The characterisation of *Ureaplasma* species isolated from the chorioamnion of women who deliver late preterm

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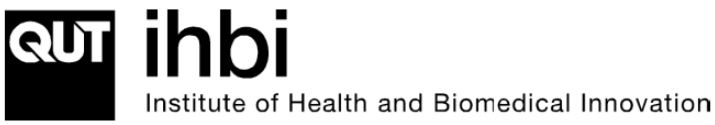

List of key words

Ureaplasma parvum; Ureaplasma urealyticum; upper genital tract infection; chorioamnion; chorioamnionitis; pregnancy; preterm birth; late preterm birth; adverse pregnancy outcomes; multiple banded antigen; real-time PCR and high resolution melt; serotyping; inflammation; *ex vivo* tissue model; host immune response

Abstract

Each year, 15 million infants are delivered preterm (< 37 weeks of gestation) and this accounts for 10% of all births worldwide. Preterm births can be further classified by the gestational age at which the infant is delivered into early preterm (< 32 weeks of gestation) or late preterm (LPT; 32 – 36 weeks). Greater than 75% of all preterm births occur LPT; however, the aetiology of LPT birth is currently unclear. Upper genital tract (UGT) infections are a major antecedent of preterm birth and the human *Ureaplasma* species are the bacteria most frequently isolated from the amniotic fluid or placentae of women who deliver preterm.

Within this thesis, the prevalence and diversity of microorganisms within placentae (n = 535) of women (n = 477) who delivered LPT or at term (≥ 37 weeks of gestation) were investigated, and the incidence of chorioamnionitis was determined. Microorganisms were detected in 9.9% of LPT and 14.1% of term chorioamnion tissues and of these, *Ureaplasma* spp. were the most prevalent clinical isolates (42/61; 68.8%). The presence of microorganisms was correlated with a history of chorioamnionitis in previous pregnancies (p = 0.025), and this may be a risk factor used by clinicians to identify women 'at-risk' of UGT infection in a current pregnancy. Significantly, the presence of *Ureaplasma* spp. within the chorioamnion (but not other microorganisms) was associated with histological chorioamnionitis (68.4% vs. 26.7%, p < 0.001). Despite the strong association between *Ureaplasma* spp. and histological chorioamnionitis, the detection of ureaplasmas within the chorioamnion was not always associated with histological chorioamnionitis and 14 (33.3%) placentae that were infected with *Ureaplasma* spp. demonstrated no evidence of histological chorioamnionitis. We identified no correlation between the number of ureaplasma CFU within the chorioamnion and the severity of disease, indicating that there may be other factors which influence the progression of disease.

To better understand why only some women with ureaplasma chorioamnion infection developed histological chorioamnionitis, the ureaplasma clinical isolates (n = 42) that were isolated from chorioamnion tissues were characterised. A novel real-time polymerase chain reaction (PCR) and high resolution melt (HRM) assay was designed and optimised to serotype the *U. parvum* clinical isolates. While *U. parvum* was the most frequently isolated species (85.7%) and the *U. parvum* serovars 1, 3 and 6 were the most frequently isolated serovars (23.8%, 21.4% and 28.6%, respectively); there was no association with ureaplasma species or serovars and the development of adverse pregnancy or neonatal outcomes. The clinical ureaplasma isolates were also tested by western blot and PCR to investigate changes/alterations in a major surface-exposed lipoprotein, the

multiple banded antigen (MBA), which is a predicted virulence factor of *Ureaplasma* spp. UGT infection. Previously, MBA size variation has been identified within a pregnant sheep model of UGT infection and differences in the size of the MBA was associated with differences in the severity of histological chorioamnionitis, but as yet no studies have investigated ureaplasma MBA size variation in human pregnancies. Within our study, some clinical isolates demonstrated MBA protein bands that were the same size as *Ureaplasma* spp. American Type Culture Collection (ATCC) strains (i.e. no MBA variation was occurring), but in numerous clinical isolates, different sized MBA protein bands were observed. Clinical ureaplasma isolates that demonstrated MBA size variation were associated with a decreased incidence of histological chorioamnionitis ($p = 0.023$). By contrast, when ureaplasmas isolated from the chorioamnion demonstrated no MBA size variation then these placentae demonstrated severe histological chorioamnionitis. Furthermore, MBA size variation correlated with the concentration of specific cytokines within cord blood. When *Ureaplasma* spp. were detected within the chorioamnion, this was associated with significantly increased levels of cord blood granulocyte colony-stimulating factor (G-CSF) (489.35 ± 183.8 pg/mL, $p = 0.04$), when compared to uninfected placentae. Furthermore, when MBA size variation was also present, there were differences in the levels of cord blood cytokines. In placentae which were infected with ureaplasmas, but no MBA variation was detected, there were significantly higher levels of G-CSF and interleukin (IL)-8; while ureaplasma-infected placentae in which MBA size variation was detected had significantly lower levels of G-CSF and IL-8 ($p = 0.008$ and $p = 0.04$, respectively). These results are consistent with *Ureaplasma* spp. modulation of the host immune response.

The specific host immune responses to *U. parvum* within an *ex vivo* placental tissue model of chorioamnion infection was further investigated. This pilot study utilised chorioamnion tissue from four human placentae that were processed within one hour of delivery. From each placenta, six sections of chorioamnion tissue were cut and suspended within Ussing chambers ($n = 6$ chambers per placentae). The chorioamnion tissue within each Ussing chamber acted as a barrier (both physical and immunological, mimicking its function *in vivo*) and each side of the membranes was separately perfused with sterile M199 media supplemented with urea and fetal calf serum. In each experiment, the maternal (chorion) side of the membranes was stimulated with live *U. parvum* serovar 6 clinical isolate ($n = 2$ chambers), UV-inactivated *U. parvum* serovar 6 clinical isolate ($n = 2$ chambers) or vehicle control ($n = 2$ chambers) and perfusing media was collected from both the maternal and fetal compartments of the Ussing chambers over time. Exposure of the maternal side of the chorioamnion membrane to live *U. parvum,* but not UV-inactivated *U. parvum* resulted in elevated secretion of the anti-inflammatory cytokine IL-10 (20.4 ± 2.8 pg/mL, $p = 0.046$) within the

maternal perfusates at 20 and 30 hours post-infection. Exposure of the chorioamnion to live *U. parvum* also resulted in increased MMP-9 production, ureaplasma invasion of the chorioamnion tissue, degradation of the chorioamnion membrane and detachment of the chorioamnion membranes, and these changes are consistent with the initial stages of membrane rupture (pPROM).

The data presented within this thesis has improved our understanding of *Ureaplasma* spp. infections of the placenta within human pregnancies. Here, we confirm that *Ureaplasma* spp. are an aetiological agent of adverse pregnancy outcomes and demonstrated, for the first time, that infection with *Ureaplasma* spp. alone within the chorioamnion was associated with histological chorioamnionitis. It was also identified that ureaplasma clinical isolates obtained from the chorioamnion of human pregnancies demonstrated differences in the size of their MBA protein and variation of the size of the surface-exposed MBA was associated with a decreased incidence of histological chorioamnionitis. Furthermore, we demonstrated that the presence of *Ureaplasma* spp. infection in human pregnancies was associated with host immune responses and elevated levels of cord blood G-CSF; however, when MBA protein variation was present, there was a significant reduction in of G-CSF and IL-8 within cord blood, despite high numbers of ureaplasmas being present within the chorioamnion. We also identified that *ex vivo* stimulation of chorioamnion tissues resulted in elevated levels of the immunosuppressive cytokine, IL-10. Taken together, these results identify the initial host immune responses to ureaplasma infections within the chorioamnion, and these first immune responses by the host may be integral for the establishment of chronic, asymptomatic UGT *Ureaplasma* spp. infections during pregnancy.

List of thesis-associated abstracts and presentations

Oral presentations

Sweeney E.L, Kallapur S.G, Jobe A.H, Stephenson, S.A, Knox C.L. *Ureaplasma* species are associated with chorioamnionitis in late preterm placentas

Perinatal Society of Australia and New Zealand annual conference, Sydney, Australia 2012

Sweeney E.L., Kallapur, S.G, Jobe A.H, Gisslen T, Stephenson S, Knox, C.L. *Ureaplasma* species is associated with histological chorioamnionitis in late preterm placentas

IHBI Inspires annual postgraduate student conference, Gold Coast, Australia 2012

Sweeney E.L., Kallapur S.G., Jobe A.H. Gisslen T, Stephenson S, Knox. C.L. *Ureaplasma* species and multiple banded antigen (MBA) variation detected in human chorioamnion: the effect on pregnancy outcome

International Organisation for Mycoplasmology biennial conference, Blumenau, Brazil 2014

Poster presentations

Sweeney E.L., Kallapur S.G, Jobe A.H, Stephenson, S, Gisslen T, Knox, C.L. *Ureaplasma* species in late preterm placentas: association with chorioamnionitis in different ethnicities

Australian Society for Medical Research annual conference, Cairns, Australia 2011

Sweeney E.L, Kallapur S.G, Jobe A.H, Stephenson S, Knox C.L. *Ureaplasma* species in late preterm placentas is associated with chorioamnionitis.

IHBI Inspires postgraduate student annual conference, Brisbane, Australia 2011

Sweeney E.L, Kallapur S.G., Jobe A.H., Stephenson S, Knox C.L. *Ureaplasma* spp. in the placentas of women with histological chorioamnionitis

Australian Society of Microbiology annual conference, Brisbane, Australia 2012

Sweeney, E.L, Kallapur, S.G, Jobe A.H, Stephenson S, Knox C.L. Detection of *Ureaplasma parvum* and *Ureaplasma urealyticum* in late preterm placentas with histological chorioamnionitis

International Organisation for Mycoplasmology biennial conference, Toulouse, France 2012

Robinson, J.W., Dando, S.J., Nitsos, I, Newnham, J.P, Kallapur, S.G., Jobe, A.H., Kramer, B, Sweeney, E.L., Knox, C.L *Ureaplasma parvum* Multiple Banded Antigen (MBA) Size Variation – Association With Fetal Inflammation in a Sheep Model

International Organisation for Mycoplasmology biennial conference, Toulouse, France 2012

Sweeney, E.L, Kallapur S.G, Jobe A.H, Stephenson S, Knox C.L. *Ureaplasma* species multiple banded antigen (MBA) variation is associated with the severity of chorioamnionitis in late preterm placentas

Perinatal Society of Australia and New Zealand annual conference, Adelaide, Australia 2013

Knox, C.L., Bryan, E.R., Pascoe, R, Rodgers, K, Sweeney, E.L., Dickinson, H, Polglase, G.R., Tolco, M, Moss, T.J. Mid-gestation intra-amniotic infection with *U. parvum* is resolved within spiny mice (*Acomys cahirinus*) by term delivery: but caused chronic infection of fetal lungs and placentae

International Organisation for Mycoplasmology biennial conference, Blumenau, Brazil 2014

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List of abbreviations

ANOVA	Analysis of variance
ATCC	American type culture collection
BLAST	Basic local alignment search tool
bp	Base pair
BPD	Bronchopulmonary dysplasia
CFU	Colony forming unit
CP	Cerebral palsy
C_T	Cycle threshold
CSF	Cerebrospinal fluid
DAB	3', 3'-diaminobenzidine tetrahydrochloride
GBS	Group B Streptococcus
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
H&E	Haematoxylin and eosin
HRP	Horseradish peroxidase
IL	Interleukin
IVF	*In vitro* fertilisation
kDa	kilo Dalton
LPS	Lipopolysaccharide
LPT	Late preterm
mba	Multiple banded antigen gene
MBA	Multiple banded antigen protein
NEC	Necrotising enterocolitis
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
pPROM	Preterm prelabour rupture of membranes

PRR	Pattern recognition receptor
PTB	Preterm birth
RDS	Respiratory distress syndrome
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TNF	Tumour necrosis factor
TLR	Toll-like receptor

Statement of original authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institute. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made.

QUT Verified Signature

11/6/2015

Emma Sweeney

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Chapter One:

Introduction

1.1 Description of the scientific problem investigated

One in ten infants (10% of all births) are delivered preterm (<37 weeks of gestation) each year. Despite significant research and targeted intervention strategies, rates of preterm birth (PTB) continue to rise in almost every country (March of Dimes 2012). The most significant increase in PTBs has occurred within the late preterm (LPT) period (32 – 36 weeks of gestation), and in the last decade the rates of LPT birth have increased by more than 40% and LPT infants now account for 80% of all preterm infants (Raju 2006; Goldenberg *et al.* 2008b). Infection of the upper genital tract (UGT) is one of the most common causes of preterm delivery, with 25 – 40% of all PTBs associated with intrauterine infection (DiGiulio *et al.* 2008; Goldenberg *et al.* 2008b; DiGiulio 2012). Whilst these estimated rates of PTB associated with infection appear to be high, these may actually be minimal estimates, as some microorganisms have fastidious nutritional requirements and are not always detected/cultured during routine microbiological processing.

The human *Ureaplasma* species (*Ureaplasma parvum* and *Ureaplasma urealyticum*) are the microorganisms most frequently isolated from the amniotic fluid (AF) and placentae of women who deliver preterm and this finding is consistent in studies which utilise traditional culture-based methods and/or newer molecular based methods (DiGiulio *et al.* 2008; DiGiulio 2012). Unlike other intraamniotic pathogens, which often cause acute infections resulting in PTB and fetal deaths, infections with *Ureaplasma* spp. are frequently chronic and asymptomatic. Ureaplasmas have been isolated from amniotic fluid as early as 15 weeks of gestation (Cassell *et al.* 1983; Cassell *et al.* 1993a; Gerber *et al.* 2003). Despite the absence of signs of infection, ureaplasma infections are frequently associated with adverse pregnancy and neonatal outcomes, including chorioamnionitis, funisitis, neonatal infections (including pneumonia, sepsis and meningitis) (Hillier *et al.* 1988; Cassell *et al.* 1993b; Goldenberg *et al.* 2002; Schelonka and Waites 2007; Waites *et al.* 2009; Kasper *et al.* 2010). Remarkably, these fastidious microorganisms are able to survive (and thrive) within the amniotic cavity, even in the presence of maternal/fetal immune responses. Given this, it is likely that *Ureaplasma* spp. have developed specific mechanisms that facilitate access to the amniotic cavity and the ability to chronically colonise/infect the AF during pregnancy without eradication. The overall hypotheses of this PhD program of study are:

(i) The *Ureaplasma* spp. infiltrate the placenta and chorioamnion during pregnancy;

(ii) *Ureaplasma* spp. are an aetiological agent of late preterm births and may be associated with adverse pregnancy and neonatal outcomes.

(iii) The host immune system may not always recognise ureaplasmas, as these microorganisms possess the ability to vary the size of their surface-exposed lipoprotein, the multiple banded antigen (MBA).

These hypotheses were examined in human pregnancies, and in a human chorioamnion tissue *ex vivo* model of ureaplasma infection.

1.2 Specific aims of this study:

(i) To determine the prevalence and diversity of microorganisms, particularly *Ureaplasma* spp. within the chorioamnion of late preterm placentae.

(ii) To detect, identify and serotype the most prevalent *Ureaplasma* species and serovars using a novel real-time polymerase chain reaction (PCR) and high resolution melt (HRM) assay.

(iii) To characterise the immunodominant surface lipoprotein of clinical ureaplasma isolates (the multiple banded antigen (MBA)) and to detect variations of this surface-exposed protein.

(iv) To investigate the host-microbe interactions of *Ureaplasma* spp. using an *ex vivo* model and human chorioamnion tissue.

By understanding these fundamental aspects of *Ureaplasma* spp. UGT infections, this project may lead to a better understanding of the role of host immune responses during *Ureaplasma* spp. infections, the identification of novel biomarkers which may predict or identify women at-risk of delivering preterm due to infections and may inform targeted therapeutic strategies. These are greatly needed in order to reduce the high rates of PTB and its associated neonatal morbidity and mortality.

Chapter Two:

Literature Review

2.1 Preterm birth: A global issue

Preterm birth (PTB: delivery < 37 weeks of gestation; term = 40 weeks) remains the single greatest obstetric challenge in the Western world (March of Dimes 2012). In 2012, 15 million births (10% of all births worldwide) occurred preterm, and whilst intervention strategies have reduced the neonatal mortality associated with PTB, rates of PTB continue to increase in almost every country (Bick 2012; March of Dimes 2012). Unlike many other health issues, PTB is a truly global issue with low- (LI), middle- (MI) and high-income (HI) countries being affected. While rates of PTB range from 5 – 18% in different countries (March of Dimes 2012), the highest reported rates of PTB are in India (MI), China (MI), the Democratic Republic of the Congo (LI) and the United States (HI) (March of Dimes 2012). In HI countries, there were 1.2 million (8%) PTBs in 2012 and more than 42% of these occurred within the United States alone (March of Dimes 2012).

Within Australia, rates of PTB increased from 6.8% in 1991 to 7.7% in 2012 (Lancaster *et al.* 1994; Hilder *et al.* 2014). Similarly, within the United States, rates of PTB have increased from 10.9% in 1991 to 11.7% in 2011 (Hamilton *et al.* 2012). These figures suggest that the current intervention strategies employed by clinicians are not effectively curbing the high rates of PTB, and due to the high number of surviving infants this places an increased burden on our healthcare system. Many countries are only now experiencing and reporting on the full extent and long-term outcomes of PTB survivors (March of Dimes 2012). Whilst the adverse effects of PTB are seen most frequently in infants born extremely preterm, there is increasing evidence that all preterm infants (regardless of their gestational age) are at increased risk of adverse sequelae (Figure 2.1).

2.1.1 Consequences of prematurity: why term babies are worth the wait

PTB remains one of the most significant threats to the health and wellbeing of infants, claiming the lives of more than 1 million children each year (Bick 2012; March of Dimes 2012). Forty percent of all newborn deaths (death within four weeks of delivery) are as a consequence of PTB, and PTB is also the second leading cause of death in children under the age of 5 (the leading cause of death is pneumonia) (March of Dimes 2012). Whilst mortality rates are inversely proportional to the age at which the child is delivered, there are also discrepancies in mortality rates between LI, MI and HI countries. A child delivered at 24 weeks of gestation has a 50% chance of survival in an HI country, whilst a child delivered at 34 weeks of age has only a 50% chance of survival in LI or MI countries (Figure 2.1) (March of Dimes 2012).

Those infants who survive their prematurity are often faced with a lifetime of disability or impairment, and this is predominantly as a consequence of immature organ systems. The most frequently encountered adverse outcome of preterm infants is lung injury, resulting from bronchopulmonary dysplasia (BPD; also known as chronic lung disease of prematurity) (Doyle *et al.* 1999). Higher incidences of recurrent respiratory infections/illnesses (Greenough 2012) and childhood asthma (Hack *et al.* 2005) are also commonly associated with PTB. Other organ systems are also at risk and children born preterm may experience visual and hearing deficiencies and language or behavioural problems (Moster *et al.* 2008). PTB is also associated with more severe sequelae, such as neuromotor and coordination disorders (Gorga *et al.* 1988), autism spectrum disorders (Abel *et al.* 2013) and cerebral palsy (Oskoui *et al.* 2013).

The childhood and long-term health outcomes of premature children are also inversely proportional to their gestational age at birth. Preterm infants (< 32 weeks of gestation) have higher rates of severe conditions (e.g. cerebral palsy), whilst those delivered at later gestations have less severe conditions (such as increased risk of non-communicable diseases later in life; Figure 2.1). Despite the apparent decreased severity of outcomes in later gestations, infants born late preterm (LPT) account for more than 75% of all PTBs and are therefore likely to have the greatest long-term impact on our healthcare system (Wang *et al.* 2004; Raju 2006). A review article on the prevention of preterm birth stated that "the true costs of prematurity, especially on a long-term, global level, are poorly understood and likely to be grossly underestimated" (Simmons *et al.* 2010). This is particularly true for infants born LPT, as despite their large numbers this cohort has remained largely unstudied.

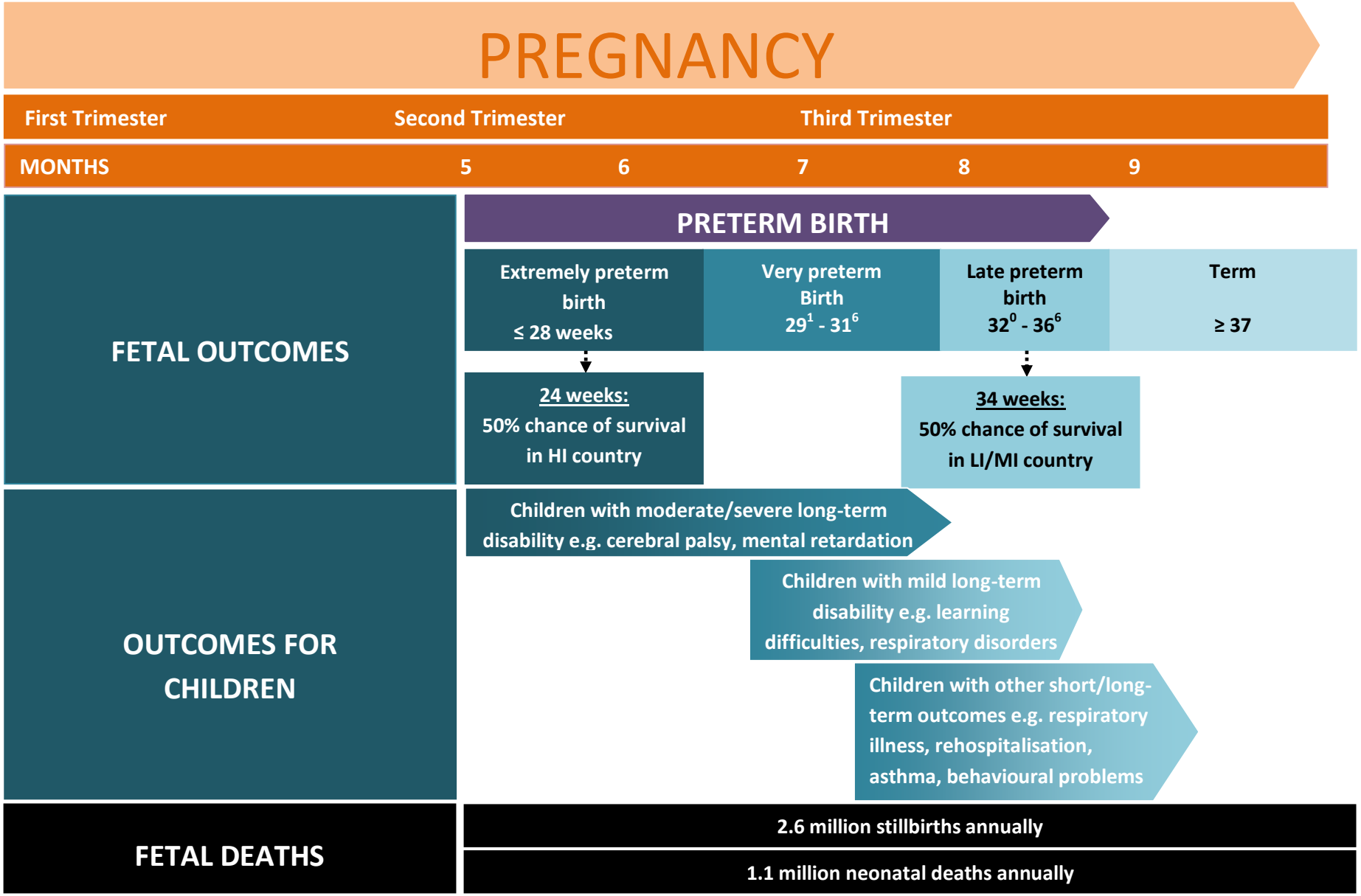


Figure 2.1. Overview of preterm birth and its associated outcomes.

HI – high income, MI – middle income, LI – low income. Image adapted from (March of Dimes 2012)

2.2 Late preterm birth: A seldom studied population at risk

Worldwide (Australia and the United States included), the most significant increase in PTB has been for neonates delivered within the LPT period (32^0 – 36^6 weeks of gestation), increasing by more than 40% between 1981 and 2005 (Loftin *et al.* 2010). LPT infants were traditionally referred to as "near term" as they were delivered just a few weeks early and were thought to be comparable to infants delivered at term (≥ 37 weeks). There is compelling evidence that LPT infants are physiologically and developmentally immature, and are at greater risk of severe outcomes (Table 2.1) than term-born infants. In the United States, the total cost of treating children born very preterm (at 25 weeks of gestation) was found to be $38.3 million dollars, while the cost of treating LPT infants was $39.3 million dollars (Gilbert *et al.* 2003). Despite the high costs of treating very premature children, the cost of treating LPT infants is higher due to the greater numbers born in this period and the increased number of surviving children. In Australia, an alarming 79.9% of preterm infants are born in the LPT period and in the US this statistic is even higher at 84.5% (Hamilton *et al.* 2012; Li *et al.* 2013). This represents a very large number of children who are at risk of chronic health problems.

LPT infants are at increased risk of temperature instability (Wang *et al.* 2004; Laptook and Jackson 2006), hypoglycaemia (Wang *et al.* 2004; Adamkin 2011), respiratory distress (Escobar *et al.* 2006a; Escobar *et al.* 2006b; Ramachandrappa and Jain 2009; Colin *et al.* 2010; Harijan and Boyle 2012), apnea (Raju 2006) and jaundice (Boyle and Boyle 2013) within the first month of life. Of those LPT infants delivered before 34 weeks of gestation, more than half will require admission to the neonatal intensive care unit (NICU) (Mally *et al.* 2010), highlighting the risk of being born "just a few weeks" preterm. The adverse outcomes for LPT infants include a higher rate of rehospitalisation, with these infants being readmitted twice as frequently as term-born infants (Escobar *et al.* 2006a; Ramachandrappa and Jain 2009; Harijan and Boyle 2012). A study by Wang and colleagues identified that the cost of caring for LPT infants during their initial admission at birth was three times higher than the cost for term infants (Wang *et al.* 2004). These infants are also at greater risk of ongoing morbidities including acute bronchitis, otitis media and pneumonia, which were associated with 2-fold higher healthcare costs for the first three years of life, when compared to the costs of treating term-born infants (Berard *et al.* 2012). More recently, a study by Gunay *et al.* (2013) examined the blood pressure and renal function of LPT children (age range: 4 – 13 yrs; n = 65) and compared these to sex- and age-matched term-born children. While the renal function of LPT children was similar to that of the term-born cohort, the mean blood pressure levels were significantly different between the cohorts. Day time, night time and 24-hour readings for both systolic and diastolic blood pressure

levels were significantly higher in the LPT group, compared to the term-born group. This may lead to childhood hypertension and ongoing health issues later in life .

2.3 Adverse outcomes associated with late preterm birth

Most organ systems of preterm infants remain vulnerable, even at late gestational ages and this predisposes infants to a range of short- and long-term adverse outcomes (Table 2.1). The final six weeks of gestation are crucial for fetal brain development, making this organ particularly vulnerable to insult following LPT birth. The brain of a LPT infant weighs one third less and the cerebral volume is approximately half the volume of a term-born infants' brain. LPT myelination and interneuronal connectivity remains incomplete, and any insult (including preterm birth) during this stage of development can lead to poor long-term outcomes for the neonate (Ramachandrappa and Jain 2009). The brainstem, which controls the suck-swallow rhythm associated with feeding is also less mature and this may result in feeding difficulties and "failure of the offspring to thrive" (Darnall *et al.* 2006; Mally *et al.* 2010). This exacerbates other sequelae of LPT infants, including dehydration, hypoglycaemia and jaundice (Boyle and Boyle 2013). Other studies have shown that preschool children who were born LPT (n = 44) performed poorly on 'verbal inhibitory control' and 'short-term verbal memory tests', compared to term-born infants of the same age (Brumbaugh *et al.* 2013) and these LPT infants had lower 'general conceptual abilities', lower 'verbal and non-verbal scores' and poorer 'visual and dexterity outcomes and adaptability', compared to term-born children ($p < 0.01$) (Baron *et al.* 2013). Up to one third of 7 year old children delivered LPT were reported by their teachers to have difficulties in basic motor skills (29%), speaking (21%), writing (32%) and mathematics (19%) (Huddy *et al.* 2001).

LPT birth has also been associated with more severe neurological conditions such as cerebral palsy, and rates of cerebral palsy are up to three times higher for children delivered late preterm, compared to their term-born counterparts (Adams-Chapman 2006; Petrini *et al.* 2009; Mally *et al.* 2010), particularly where intraamniotic infection and/or inflammation are also reported at birth (Goldenberg *et al.* 2008b). A study of 900 000 children, including almost 33 000 children born LPT reported higher incidences of cerebral palsy (relative risk: 2.7 - 14.1), mental retardation (relative risk: 1.6 – 2.1), behavioural and emotional disturbances (relative risk: 0.8 – 1.0) as well as other severe disabilities including blindness or decreased vision, hearing loss and epilepsy (relative risk: 1.5 – 2.3) for children born LPT (Moster *et al.* 2008).

The fetal lung is also particularly vulnerable following LPT birth. A study by Colin and colleagues likened the function/development of the LPT lung as more similar to that of a very preterm infant than that of a term infant (Colin *et al.* 2010). LPT infants' lungs are at the saccular stage of development, which is characterised by an increase in the number of bronchi, alveoli and at this time, surfactant is produced. Associated with these changes is the rapid increase in lung volume and lung development can be altered if infants are delivered LPT. Respiratory distress (RDS) may occur as a consequence of interrupted alveolar development and is associated with poor gas exchange due to lack of surfactant production. Infants born LPT are 8 times more likely to be diagnosed with RDS, and 9 times more likely to require continuous positive airway pressure (CPAP) ventilation due to their immature lungs, compared to term-born infants (Wang *et al.* 2004) (Ramachandrappa and Jain 2009).

This is consistent with other studies which identified LPT infants as having significant respiratory morbidities as evidenced by high rates of assisted ventilation, the use of oxygen at 36 weeks (gestational age of the infant following LPT birth) (Escobar *et al.* 2006b) and with reduced respiratory compliance, when compared to matched term-born control infants (McEvoy *et al.* 2013). A follow-up study identified a significant link between late PTB and recurrent wheezing up to three years of age (Escobar *et al.* 2010). Another study of 7295 children monitored the outcomes of infants from birth to 18 months of age (age at enrolment: 34 - 36 weeks) and showed a significant correlation between birth in the LPT period and a diagnosis of asthma within the first 18 months of age (adjusted odds ratio: 1·68) (Goyal *et al.* 2011). These studies confirm the significant impact of late PTB and identify and that the burden associated with late PTB may be underestimated. Both the short- and long-term adverse outcomes for these infants born LPT place further strain on our healthcare system and further research is required to understand the pathogenesis of LPT birth and to identify risk factors and causes of LPT birth.

Table 2.1. A comparison of neonatal and long-term outcomes for extremely preterm, very preterm, late preterm and term infants. Outcomes of children born late preterm are more closely related to those of children born very preterm, rather than those delivered at term.

	Extremely Preterm	Very Preterm	Late Preterm	Term	n =	Reference
Gestational age at birth (week^{day})	≤ 27^{6}	28^{0} - 31^{6}	**32^{0} - 36^{6}**	≥ 37^{0}	N/A	(March of Dimes 2012)
Proportion of total birth	0.4%	0.8%	**6.5%**	92.3%	301 810	(Li *et al.* 2011)
Proportion of preterm birth	5.2%	10.4%	**84.4%**	N/A	301 810	(Li *et al.* 2011)
Mortality rate (per 1000 live births)	392.7	27.2	**3.5**	0.4	301 810	(Li *et al.* 2011)
INITIAL STAY AND REHOSPITALISATION						
Mean weight (grams)	752.6 ± 350.3	1420.2 ± 454.2	**2643.1 ± 580.3**	3607.5 ± 478.4	1 390 742	(Raisanen *et al.* 2013)
Initial length of stay (days)	78.2 ± 0.70	35.92 ± 1.21	**7.28 ± 0.29**	2.0 ± 0.78	458 366	(Gilbert *et al.* 2003)
Rehospitalisation	28.75%	19.5%	**13.74%**	2.0%	263 883 33 276	(Underwood *et al.* 2007) (Escobar *et al.* 2005)
Average cost of rehospitalisation	$ 15.12 million	$ 16.66 million	**$ 64.9 million**	Insufficient data	263 883	(Underwood *et al.* 2007)
NEUROLOGICAL DISORDERS						
Cerebral palsy	9.1%	6%	**2.2%**	0.1%	903 402	(Moster *et al.* 2008)
Mental retardation	4.4%	1.8%	**1.7%**	0.4%	903 402 141 321	(Moster *et al.* 2008) (Petrini *et al.* 2009)
Autism spectrum disorder	0.6%	0.4%	**0.08%**	0.05%	903 402	(Moster *et al.* 2008)
Psychological and behavioural disorders	2.5%	0.7%	**0.6%**	0.2%	903 402	(Moster *et al.* 2008)
RESPIRATORY DISORDERS						
Respiratory distress	74.3%	44.2%	**28.9%**	4.2%	458 366 7 474	(Gilbert *et al.* 2003) (Wang *et al.* 2004)
Bronchopulmonary Dysplasia	Up to 40%	Up to 24.8%	**28.9%**	4.2%	4 185	(Gortner *et al.* 2011)
OTHER						
Visual impairment, blindness, hearing loss and epilepsy	10.6%	8.2%	**6.6%**	1.7%	903 402	(Moster *et al.* 2008)
TOTAL COST						
Average cost per gestational age group	$ 38.3 million	$ 35.95 million	**$ 39.26 million**	$ 69.95 million	458 366	(Gilbert *et al.* 2003)

2.4 *In utero* infections: the (often) silent threat to a healthy pregnancy

In utero infection during pregnancy is the most common (potentially) preventable antecedent of PTB (Goldenberg *et al.* 2000b; Goldenberg *et al.* 2008b). A seminal study conducted by Hillier and colleagues tested the placentae of women following delivery and identified that 61% of women (n = 38) who delivered preterm had bacteria present within the chorioamnion (membranes which surround the fetus during pregnancy) and of these, 47% were infected/colonised with *Ureaplasma* spp. (Hillier *et al.* 1988). It has been consistently shown that the human *Ureaplasma* spp. are the microorganisms most frequently isolated from the amniotic fluid and placentae of women who deliver preterm (Gray *et al.* 1992; Cassell *et al.* 1993b; Knox *et al.* 1997; Goncalves *et al.* 2002; Gerber *et al.* 2003; Aaltonen *et al.* 2007; Hecht *et al.* 2008; Onderdonk *et al.* 2008; DiGiulio *et al.* 2010; Kasper *et al.* 2010). DiGiulio compared the results from 37 studies that reported on the prevalence and diversity of bacteria within amniotic fluid (15 – 37 weeks) and found that this clinical fluid was most frequently infected with *Ureaplasma* spp. (24%), *Fusobacterium* spp. (20%) or *Streptococcus* spp. (16%), and less frequently with *Mycoplasma* spp. (4%), *Bacteroides* spp. (4%) and *Gardnerella* spp. (8%). It was also reported that *Ureaplasma* spp. was the most frequently isolated microorganism in cases of preterm premature rupture of membranes (pPROM); and the second most frequently isolated organism from women who delivered preterm without rupture of the membranes (DiGiulio 2012). *Ureaplasma* spp. infection has been identified in the amniotic sac as early as 15 weeks of gestation (Gerber *et al.* 2003) and whilst it has been reported that ureaplasmas can be isolated from discoloured amniotic fluid without any other clinical signs of infection (Cassell *et al.* 1983), they are most frequently isolated from women with clear amniotic fluid (as ureaplasmas are so small that they do not produce turbidity in liquids – including *in vitro* cultures in broth) and macroscopically normal placentae (though there may be histological evidence of inflammation/chorioamnionitis). This means that not only are ureaplasma infections of the amniotic cavity asymptomatic, but also that ureaplasma infection is not immediately suspected as a cause of preterm birth or adverse pregnancy outcomes.

Furthermore, it has been identified that only 35% of preterm births are medically indicated; whilst the remaining 65% of preterm births occur as a result of spontaneous premature labour and pPROM (Goldenberg *et al.* 2008b), most frequently as a result of intrauterine infection (approximately 25 – 40% of all preterm births are as a result of intrauterine infection) (Goldenberg *et al.* 2000b; Goldenberg *et al.* 2008b; DiGiulio 2012). Taken together, this indicates that intrauterine infection is

prevalent and often extremely difficult to identify/diagnose, as women appear to be apparently healthy until preterm labour ensues.

Kenyon *et al.* published two of the largest randomised clinical trials investigating antibiotic treatment of women with pPROM (Kenyon *et al.* 2001a) and spontaneous preterm labour (Kenyon *et al.* 2001b) (both are classical symptoms of intrauterine infection). The ORACLE I trial studied the efficacy of erythromycin or amoxicillin and clavulanic acid (augmentin) treatment versus placebo controls in 4826 women with pPROM. The administration of antimicrobials was effective, reducing adverse neonatal outcomes (oxygen dependency and surfactant therapy of the neonate, cerebral abnormalities and positive blood cultures); however, the use of augmentin was associated with necrotising enterocolitis (NEC) in neonates ($p = 0.0005$). Therefore, augmentin was contraindicated for use in pPROM (Kenyon *et al.* 2001a). The ORACLE II clinical trial investigated the effectiveness of erythromycin or augmentin treatment in a cohort of 6295 women with spontaneous preterm labour. In contrast to the ORACLE I trial, the ORACLE II clinical trial showed no significant improvements for neonates outcomes (parameters mentioned above) in either of the erythromycin (useful in the treatment of ureaplasmas) or augmentin (not useful in the treatment of ureaplasmas) treatment groups, or in the placebo (no antimicrobial treatment) control group; however, there was a reduced incidence of maternal infection in those women who were administered antimicrobials. They therefore concluded that women in spontaneous preterm labour should not be routinely administered antimicrobials unless there was clinical evidence of infection (Kenyon *et al.* 2001b). However, the problem largely remains that identification of intrauterine infection is extremely difficult, due to the predominantly asymptomatic nature of the infection.

A 7-year follow-up of the children born to mothers administered antimicrobials vs. placebo during the ORACLE II randomised clinical trial found there were no significant differences in the children's rate of death, the medical conditions they developed, their behavioural patterns or the educational attainment levels of the children. However, children born to mothers who received erythromycin had greater functional impairment (mild, moderate or severe impairment of vision, speech, dexterity, emotion, cognition or pain) compared to those children born to mothers who did not receive erythromycin (658/1554, compared to 574/1498, odds ratio: 1·18). Even more significant was the risk of developing cerebral palsy (in both erythromycin/augmentin treatment groups); children born to women treated with either antimicrobial were more likely to develop cerebral palsy compared to children who did not receive antimicrobials (erythromycin: 53/1611 compared to

27/1562, odds ratio: 1·93; augmentin: 50/1587 compared to 30/1586, odds ratio: 1·69) (Kenyon *et al.* 2008).

More recently, Acosta *et al.* (2014) identified that multiple-dose maternal intravenous azithromycin eradicated *U. parvum* with a 95% success rate in a non-human primate model. However, other studies investigating the effectiveness of erythromycin treatment during pregnancy have reported conflicting results. Dando *et al.* (2010) showed that maternal intramuscular administration of erythromycin failed to eradicate intrauterine ureaplasma infection in an ovine model. The failure of erythromycin treatment may be due to poor trans-placental transfer; as a previous study reported that the trans-placental transfer rate of erythromycin was as low as 3%, so these antimicrobials may not be reaching the placentae or amniotic fluid in therapeutic concentrations (Heikkinen *et al.* 2000). Dando *et al.* (2014) subsequently sequenced the domain V of the 23S rRNA gene of ureaplasmas isolates from their 2010 study and demonstrated significant genetic differences between ureaplasmas isolated from the chorioamnion and amniotic fluid of pregnant sheep. While ureaplasmas isolated from amniotic fluid showed 100% 23S rRNA sequence homology to the original strain injected, isolates from the chorioamnion demonstrated significant genetic variation (only 64 – 82% sequence homology) when compared to the original strain injected. Furthermore, the ureaplasmas isolated from the chorioamnion of sheep demonstrated the presence of macrolide resistance genes and the presence of these genes (*erm*(B) and *msr*(D)) were associated with variable minimum inhibitory concentrations (MICs), regardless of whether the isolates were exposed to erythromycin *in vivo*. Therefore, these results suggest that the microbial niche (i.e. amniotic fluid or chorioamnion tissue) may act as a selective pressure that allows the growth of some ureaplasma variant strains *in vivo*. This may also explain why it is so difficult to treat intraamniotic *Ureaplasma* spp. infections.

Other studies have also reported mutations within the 23S rRNA gene and erythromycin resistance patterns; however, macrolide resistance in these populations was low (Xiao *et al.* 2011a). A follow-up study in sheep by Kemp *et al.* (2014) attempted to resolve intraamniotic ureaplasma infections with erythromycin treatment and combinations of single or repeat intraamniotic and maternal intramuscular injections but failed to eradicate ureaplasmas from the amniotic cavity of pregnant sheep. While erythromycin treatment reduced the number of viable ureaplasmas present, the authors demonstrated the presence of several erythromycin-resistant strains following *in vivo* treatment (Kemp *et al.* 2014). Redelinghuys *et al.* (2014) have also demonstrated increasing antimicrobial resistance in human clinical isolates. Self-collected vaginal swabs were obtained from

pregnant women (n = 96) attending an antenatal clinic in South Africa. Specimens were screened and 76% (73/96) contained *Ureaplasma* spp. and the majority of isolates were *U. parvum*. Antimicrobial susceptibility assays were performed and > 80% of isolates were resistant to erythromycin (a class A antimicrobial therapy suitable for treating pregnant women), and surprisingly 73% of ureaplasma isolates were also found to be resistant to tetracycline (a class D antimicrobial therapy – contraindicated during pregnancy). Other studies have also indicated that tetracycline resistance of *Ureaplasma* spp. is increasing (Xiao *et al.* 2011a). These studies highlight the failure of erythromycin to treat intraamniotic ureaplasma infections. These findings highlight the need for further studies to identify methods to detect asymptomatic UGT infections and adequate treatment strategies to eradicate these complex pathogens during pregnancy.

In a review of drug therapies for the prevention of preterm birth, it was stated that "if the right antibiotics (with appropriate activity against a particular microorganism) were administered to women prior to 22 weeks of gestation (and before inflammation/damage *in utero*) then the incidence of preterm birth may be reduced by 40 – 60%" (Lamont and Jaggat 2007). However, the difficulty remains that: (i) intrauterine infections in early gestational periods are often polymicrobial and require more than one antibiotic to effectively treat the different microorganisms causing the infection (Kallapur *et al.* 2013); (ii) a large proportion of women have clinically silent intrauterine infections (Goldenberg *et al.* 2000b); and (iii) routine screening/testing of pregnant women for UGT infection is not part of current obstetric practice (Australian Government Department of Health and Ageing 2012).

2.4.1 Routes of *in utero* infections

The female upper genital tract (UGT) was traditionally considered to be a sterile site, as it was thought that infants developed within a sterile site and were first exposed to bacteria during birth (Tissier 1900; Wilson 2005). However, increasing evidence suggests that the UGT is not sterile and that there are specific mechanisms by which microorganisms gain access to the UGT. Goldenberg *et al.* (2000) proposed four major mechanisms: retrograde spread from the peritoneal cavity (*via* the Fallopian tubes), haematogenous dissemination *via* the placenta and maternal blood supply, accidental contamination at the time of invasive procedures such as amniocentesis or chorionic villus sampling, and ascending invasive infections from the LGT to the UGT (Fig. 2.2). Of these, the most widely accepted mechanism is that microorganisms originating from the LGT (e.g. *Ureaplasma* spp.) ascend through the cervix into the choriodecidual space and cross the chorioamnion membrane to reach the amniotic fluid and the fetus (Kim *et al.* 2009). A study by Hansen *et al.*

(2014) identified that despite the presence and intense antimicrobial activity of the cervical mucus plug (a gel-like structure which fills the cervix), *Ureaplasma* spp. were still isolated within the cervical mucus plug, indicating that its presence does not block the movement of *Ureaplasma* spp. to the UGT. A previous study identified that migration of radio-labelled particles from the vagina to the UGT can occur within 2 minutes (Zervomanolakis *et al.* 2007) and further studies conducted by Kundsin *et al.* (1984) demonstrated the recovery of *Ureaplasma* spp. increased as the duration of rupture of membranes increased, providing further evidence that bacteria can ascend from the LGT and are likely to be the primary source of UGT infections. Knox *et al.* (2003) also demonstrated that *Ureaplasma* spp. were adherent to human spermatozoa and were not always removed by assisted reproductive technology (ART) washing procedures (29/73, 39.7% remained ureaplasma-positive). By attaching to the surface of sperm, ureaplasmas may gain access to the UGT (Nunez-Calonge *et al.* 1998; Knox *et al.* 2003) and it is possible that ureaplasmas and other microorganisms may asymptomatically colonise the endometrium at (or prior to) the time of conception.

A recent study found that 10% of all endometrial samples contained *Ureaplasma* spp. (Cicinelli *et al.* 2012). This is in accordance with previous studies that report the isolation of ureaplasmas (and other bacteria) from the endometrium of healthy, asymptomatic women (Idriss *et al.* 1978; Cassell *et al.* 1993b). Pathogenic microorganisms have been identified within the endometrium of women without any evidence of pelvic infection and with negative cervical cultures (Lucisano *et al.* 1992), and there is a low correlation between microorganisms isolated from the cervix and those found within the endometrium, with 67% of endometrial specimens having distinct microbial populations, when compared to the corresponding cervical specimen (Cicinelli *et al.* 2012). It has been suggested previously that endometrial infection/colonisation may be present at the time of implantation, and that this may explain abnormal placentation (Jones *et al.* 1998), pre-eclampsia (Viniker 1999), adverse pregnancy outcomes (Gibbs *et al.* 1992) and spontaneous and recurrent abortions (Naessens *et al.* 1987). These studies provide significant evidence that not only is the UGT not sterile, but that it contains a unique population of microorganisms that may be suited to this anatomical niche. Further studies are required to determine the diversity of microorganisms present within the UGT and to follow the outcomes of women who fall pregnant whilst bacteria are present in the UGT, in order to determine the effects of these microorganisms during gestation.

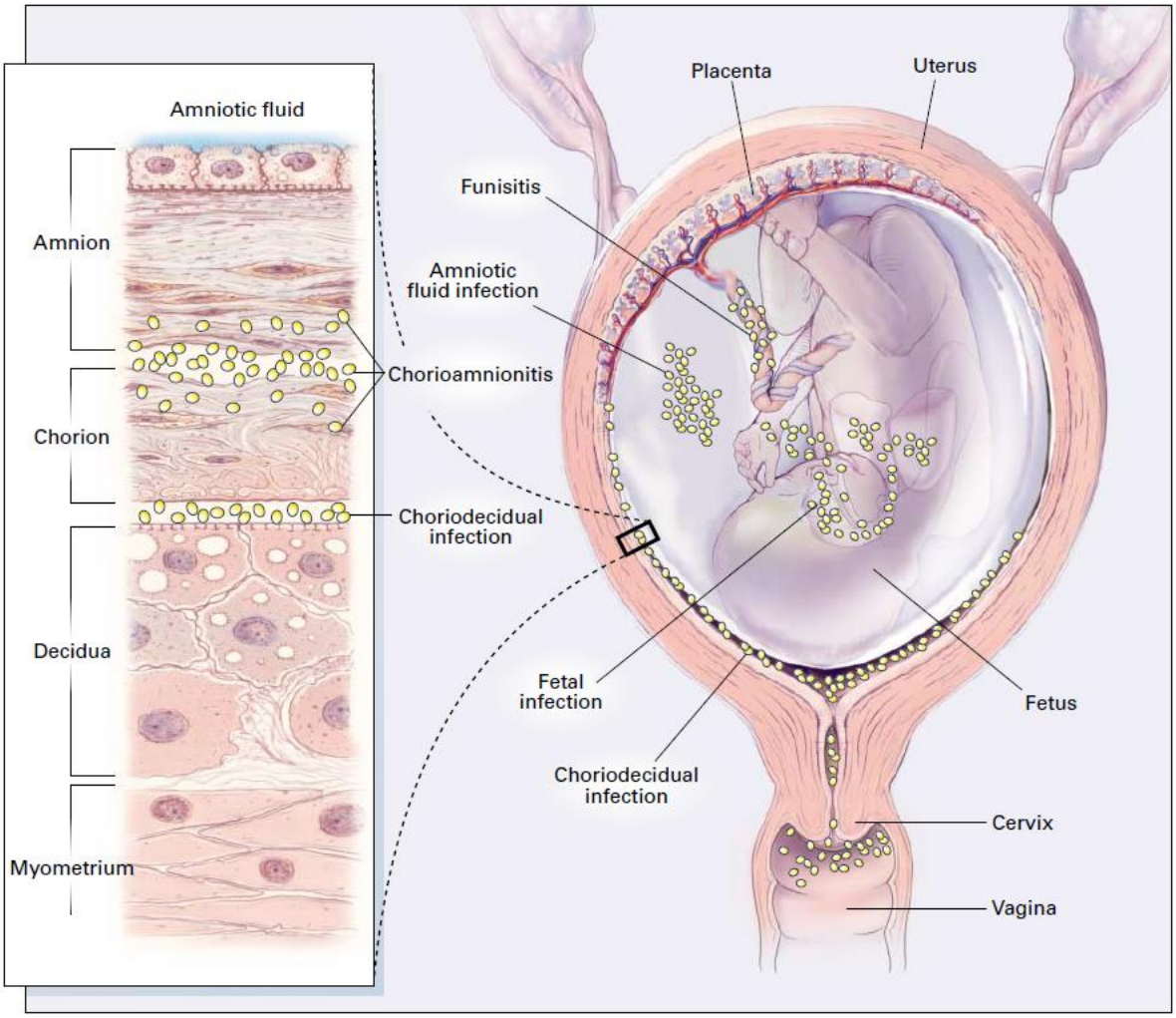


Figure 2.2. Ascending invasive infections are predicted to be the most common route of *in utero* infection. Bacteria may ascend from the LGT; invade the chorioamnion (maternal/fetal) membranes, gaining access to the amniotic fluid and fetus during pregnancy. Image source: Goldenberg *et al.* (2000).

2.4.2 Microbial mechanisms leading to preterm birth

Infection-associated PTB is most commonly thought to occur from microbial invasion of the UGT, specifically the choriodecidual space (maternal/fetal interface). Activation of the host immune system at the time of invasion is recognised by pattern-recognition receptors (PRRs), such as the Toll-like receptors (TLRs). Activation of host TLRs result in the production of cytokines, such as interleukin (IL)-1β, IL-6, IL-8, IL-10 and tumour necrosis factor (TNF)-α. These cytokines and microbial toxins initiate prostaglandin synthesis, which in turn initiates contractions of the myometrial and cervical muscles, triggering uterine contractions and opening of the cervix in preparation for labour (Goldenberg *et al.* 2000b; Challis *et al.* 2009). Increased levels of cytokines at the site of microbial invasion/infection also result in the recruitment of neutrophils and the expression of matrix

metalloproteinases, which attack and weaken the chorioamnion membranes, ultimately leading to rupture of membranes (Goldenberg *et al.* 2000b). The combination of these events results in premature labour and preterm delivery of the baby, unless intervention strategies are quickly put in place.

2.5 *Ureaplasma* species: Taxonomy and classification

Ureaplasma species are among the smallest self-replicating microorganisms. These were first isolated from urethral exudates in males with primary and recurrent non-gonococcal urethritis (Shepard 1954). Due to their close resemblance to *Mycoplasma* spp. and small colony size (size range 5 – 20 µm, compared to 200 – 500 µm of *Mycoplasma* spp.) (Shepard 1954), they were referred to as the T (tiny) strain mycoplasmas. In 1974, Shepard distinguished the T-strain mycoplasmas from other *Mycoplasma* spp. by their unique ability to hydrolyse urea as their major (95%) energy source, and so he proposed that these organisms be reclassified into their own genus: *Ureaplasma* (Shepard 1974). The urease enzyme of ureaplasmas breaks down urea (present within urine, the genital tracts of men and women and also within amniotic fluid) to produce ammonia, resulting in *de novo* ATP synthesis (Smith *et al.* 1993). Production of ammonia is also the distinguishing characteristic used for the identification of ureaplasmas in routine diagnostic *in vitro* broth culture (Shepard and Lunceford 1976), the growth of ureaplasmas is confirmed by an alkaline shift and pH indicator colour change.

Ureaplasmas belong to the division *Tenericutes*, within its own class, the *Mollicutes* (Latin: mollis – soft; cutis – skin). The *Mollicutes* are unique in their cell structure, lacking a peptidoglycan cell wall and are surrounded only by a plasma membrane. *Ureaplasma* spp. are further classified in the order *Mycoplasmatales* and the family *Mycoplasmataceae,* along with the closely related *Mycoplasma* spp. (Figure 2.3) (Bergey *et al.* 1994; Krieg 2012). *U. urealyticum* was initially proposed as a single species infecting humans, containing eight antigenically distinct serovars; however, 14 serovars have now been identified based on the results of metabolic inhibition tests and epifluorescence assays (Robertson and Stemke 1982). These 14 serovars were divided into two distinct biovars, the "parvo" biovar (also known as biovar 1 or A; containing serovars 1, 3, 6 and 14) and the "T960^{T}" biovar (also known as biovar 2 or B; containing serovars 2, 4, 5, 7 - 13) (Christiansen 1981). However, the accumulation of phenotypic and genotypic differences between these biovars, including differences in genome size, restriction fragment length polymorphisms (RFLPs), DNA-DNA hybridisation, 16S rRNA and 16S-23S rRNA intergenic region differences as well as manganese inhibition patterns and clustering of antigenic types prompted the revision of this taxonomy into two distinct species: *U.*

parvum (the "parvo" biovar) and *U. urealyticum* (the "T960^{T}" biovar), which were formally proposed (Robertson *et al.* 2002). This revised taxonomy of two species and 14 serovars of *Ureaplasma* spp. was formally accepted, however, this taxonomy has not been widely adopted within the literature, and often the 14 serovars are still referred to as *U. urealyticum.*

There are currently seven species of ureaplasmas: *U. parvum, U. urealyticum, U. diversum, U. cati, U. gallorale, U. felinum* and *U. canigenitalium* (Robertson *et al.* 2002). Of these, *U. parvum* and *U. urealyticum* (often referred to as "the ureaplasmas") infect human hosts and are most closely related to the *Mycoplasma pneumoniae* taxonomic group. *Ureaplasma* spp. have unique genomes, with the highest A+T content of all bacteria (Glass *et al.* 2000). They have the second smallest genome of all free-living prokaryotes (the smallest being *M. genitalium*) with genomes ranging in size from 0.75 Mbp – 1.2 Mbp (Glass *et al.* 2000). These minimal genomes are thought to have evolved from closely related low G+C-content Gram positive bacteria by extensive degenerative evolution (Glass *et al.* 2000) and phylogenetic analysis identified *Clostridium* spp. (*Clostridium innocuum* and *Clostridium ramosum*) as the closest relatives of ureaplasmas and mycoplasmas (Woese *et al.* 1980). More recently, studies have revealed that ureaplasmas are also closely related to the *Bacillus* spp., *Streptococcus* spp. and *Lactobacillus* spp.; and of these the *Lactobacillus* spp. and *Streptococcus* spp. are the closest relatives of ureaplasmas, based on phosphoglycerate kinase sequencing (Wolf *et al.* 2004). It is also very interesting to note that several key genes found in all other bacteria are absent in *Ureaplasma* spp. The genes encoding the heat shock proteins GroEL and GroES that mediate protein folding are found in all sequenced microbial genomes, with the exception of *Ureaplasma* spp. (Glass *et al.* 2000). Perhaps the most interesting absence within the ureaplasma genome is the gene which encodes the cell division protein FtsZ (Glass *et al.* 2000). This protein forms the "Z-ring" that constricts between two dividing cells during replication and is thought to be essential in the binary fission of free-living bacteria. However, *Ureaplasma* spp., *Chlamydiae* and *Aeropyrum pernix* are the only bacteria that do not contain this protein. Given this, the genetic mechanisms by which ureaplasma cells divide currently remain unknown.

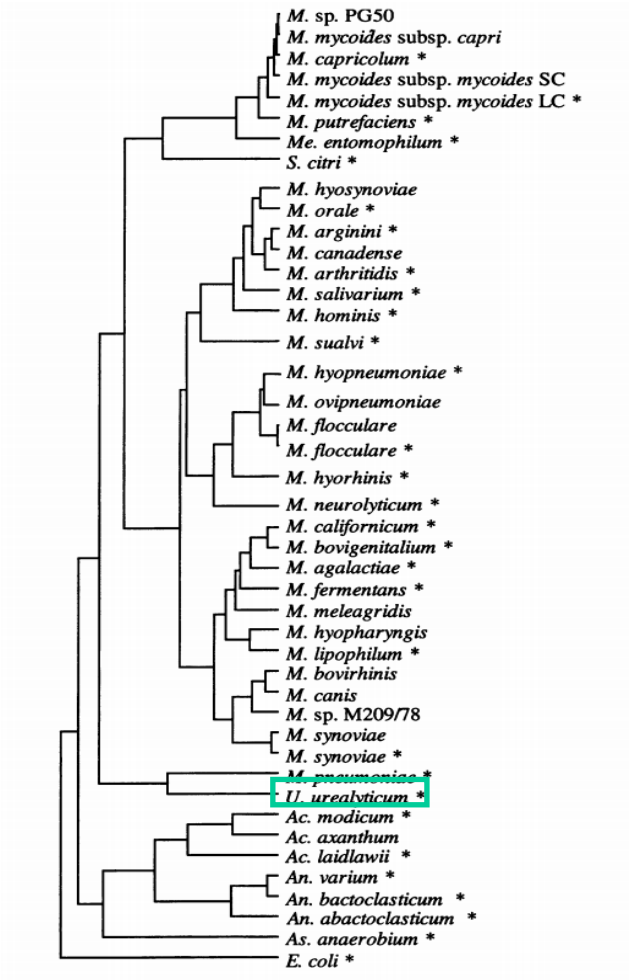


Figure 2.3. Phylogenetic relatedness of *Ureaplasma* spp., along with other *Mollicutes* including *Mycoplasma* spp., *Acholeplasma* spp., *Anaeroplasma* spp. and *Spiroplasma* spp. and *Firmicutes* including *Escherichia coli*. 16S rRNA direct solid-phase DNA sequencing was employed on well-characterised variable regions of the 16S rRNA gene. In this figure, *U. urealyticum* is representative of all 14 serovar belonging to *U. parvum* and *U. urealyticum* and is most closely related to *Mycoplasma pneumoniae*. Image from (Pettersson *et al.* 1994)

2.6 *Ureaplasma* species: Association with disease

2.6.1 LGT colonisation

2.6.1.1 LGT colonisation of females

Ureaplasma spp. are considered to be normal flora of the urogenital mucosal epithelia and are found in 40 – 80% of females of reproductive age (Cassell *et al.* 1993b). *Ureaplasma* spp. colonisation of the genital tract has been associated with a number of factors, including: age (most

frequently in 14 - 25 year age group and carriage declines with increasing age) (Tibaldi *et al.* 2009; Ruzman *et al.* 2013), ethnicity (particularly African American and Indigenous women) (McCormack *et al.* 1986; Knox *et al.* 1997; Tibaldi *et al.* 2009), level of education (Tibaldi *et al.* 2009), the number of recent sexual partners (McCormack *et al.* 1986; Knox *et al.* 1997; Nelson *et al.* 2007; Ruzman *et al.* 2013), age at first sexual intercourse (≤ 16 yrs) (Knox *et al.* 1997; Ruzman *et al.* 2013) as well as the use of non-barrier contraceptives (Knox *et al.* 1997) and IUDs (Tibaldi *et al.* 2009). Due to their high prevalence, ureaplasmas are considered to be of low virulence in the female LGT and are often referred to as asymptomatic colonisers (Volgmann *et al.* 2005). Studies have demonstrated that there were no significant differences in the isolation rates of *Ureaplasma* spp. from endocervical, vaginal or urethral swabs collected from women of reproductive age with or without symptoms of infection (and irrespective of pregnancy status) (Casari *et al.* 2010; Hunjak *et al.* 2013). By contrast, others have reported ureaplasmas as a potential source of genital tract infection, including urinary tract infections, bacterial vaginosis and vaginitis (Tibaldi *et al.* 2009; Patel and Nyirjesy 2010).

The presence of *Ureaplasma* spp. in the female LGT has also been proposed as a risk factor for adverse outcomes including chorioamnionitis, low Apgar scores at birth (infants are scored at 1 and 5 minutes after birth with respect to appearance, pulse rate, reflexes, activity and respiratory effort), admission of the infant to the NICU and preterm birth (Abele-Horn *et al.* 2000; Kwak *et al.* 2014). However, the majority of studies in pregnant women have demonstrated that *Ureaplasma* spp. colonisation of the LGT is not significantly associated with adverse pregnancy outcomes (Donders *et al.* 2009; Lee *et al.* 2009). A recent study conducted by Anderson *et al.* (2013) studied a population of 47 pregnant and 16 non-pregnant women and obtained vaginal swabs on four occasions during routine clinical visits at gestations of: < 14 wks, 14 – 28 wks, > 28 wks and 4 – 6 wks postpartum. Non-pregnant women were also screened at 12 week intervals. *Ureaplasma* spp. was prevalent in vaginal swabs from pregnant women (> 60% in all visits during pregnancy), particularly within the first and second trimesters. However, there were no significant associations between the gestational age at delivery (mean: 39 wks; range: 33 – 41 weeks), birth weight (mean: 3255 g; range: 2070 – 4640 g) or any adverse maternal or neonatal outcomes of those women colonised with *Ureaplasma* spp. (Anderson *et al.* 2013). Conversely, Breugelmans *et al.* identified *Ureaplasma* spp. as a risk factor for preterm delivery and 52/97 (53.6%) of women who had positive cervical cultures for *Ureaplasma* spp. delivered preterm ($p = 0.02$). However, they failed to comment on the fact that ureaplasmas were also isolated from the LGT of 783/1981 (41.4%) women who delivered at term (Breugelmans *et al.* 2010). Overwhelmingly, these studies indicate that *Ureaplasma* spp. are part of

the normal vaginal microflora of both pregnant and non-pregnant women and are LGT colonisation with ureaplasmas is not a significant predictor of preterm delivery.

2.6.1.2 LGT colonisation of males

Ureaplasma spp. are also considered to be asymptomatic colonisers of the male urogenital tract and can be found in up to 50% of urethral specimens (Cassell *et al.* 1993b; Volgmann *et al.* 2005; Nakashima *et al.* 2014). *Ureaplasma* spp. in the male LGT are often associated with non-gonococcal urethritis (Yoshida *et al.* 2005; Shimada *et al.* 2014), epididymitis (Zeighami *et al.* 2009) and chronic prostatitis (Skerk *et al.* 2002), though their role in these disease states are often controversial. Ureaplasmas have also been isolated in seminal fluid from both fertile and infertile men and have been associated with male infertility and adverse fertility parameters. The prevalence of *Ureaplasma* spp. in the sperm of infertile men varies from 10 – 40% (Keck *et al.* 1998) and has been correlated with: alterations in sperm motility (both increases/decreases) (Nunez-Calonge *et al.* 1998; Knox *et al.* 2003; Liu *et al.* 2014), changes in sperm pH (Wang *et al.* 2005), altered sperm membrane morphology (Nunez-Calonge *et al.* 1998), reduced sperm concentration and viscosity (Wang *et al.* 2006), decreased or abolished acrosomal reaction (impairing sperm penetration ability and increasing apoptosis of sperm) (Kohn *et al.* 1998) and membrane lipid peroxidation and oxidative stress in sperm (Fraczek *et al.* 2007). A study conducted by Shi *et al.* (2007) demonstrated cross-reactivity between antigens belonging to *Ureaplasma* spp. and human sperm membrane proteins (hSMP). They identified anti-ureaplasma antibodies in the semen of infertile men with ureaplasma infections and these antibodies also reacted with hSMP. Immunofluorescent analysis showed specific reactivity at the posterior sperm head, the area where sperm-egg binding occurs. The authors suggested that this may be a mechanism by which ureaplasma-related infertility blocks or inhibits sperm-egg binding. The authors then explored this further by pre-incubating murine spermatozoa with antibodies generated against *Ureaplasma* spp. prior to *in vitro* fertilisation with murine ova. No significant impairment of sperm motility was seen, however, there was significant inhibition of sperm-egg binding in the group pre-incubated with the cross-reactive *Ureaplasma* spp. antibodies. This inhibition of binding was abolished when the antibodies were diluted to a lower level. A similar result was seen when female mice were pre-immunised with these cross-reactive antibodies, with more than 75% of female mice becoming sterile (Shi *et al.* 2007). These results suggest that not only are spermatozoa affected by these cross-reactive antibodies, but that the presence of cross-reactive antibodies in female mice may also be a cause of infertility. Furthermore, the presence of *Ureaplasma* spp. has also been correlated with molecular changes to spermatozoa, including premature chromatic decondensation (which compromises DNA integrity), mitotic

alterations and chromatid breaks and gaps (Wang *et al.* 2010). DNA damage may affect the ability of sperm to fertilise oocytes and can have a damaging effect on embryogenesis. This may have severe implications for infertility, abortion and overall reproductive health and so further studies are required to elucidate the role of *Ureaplasma* spp. in sperm function, DNA damage and infertility.

2.7 *In utero* ureaplasma infection is associated with adverse neonatal outcomes

Ureaplasmas may be acquired by the fetus *in utero* or during delivery and this can result in severe adverse neonatal outcomes. A recent study by Hahn *et al.* (2014) determined the rate of vertical transmission of microorganisms during delivery. Of the 455 women tested, 64 (14.1%) were found to have sexually transmitted infections (STIs) cultured/detected from the LGT. For 17.2% of these women, microorganisms were transmitted vertically from the mother to the neonate. The human *Ureaplasma* spp. were found to be the most frequently identified microorganisms isolated from the LGT of mothers (n = 36; 50%) and also from the oral secretions of the neonate immediately following delivery (8/11%; 72.2%).

During development *in utero*, the fetus swallows and inhales amniotic fluid as part of normal fetal development, preparing fetal organs for their *ex utero* roles (such as swallowing and breathing). If this amniotic fluid is infected, this allows microorganisms, including ureaplasmas, to directly access the fetal lung and gut. Ureaplasmas have been isolated from the fetal lung following delivery and are associated with respiratory disorders including pneumonia (Cassell *et al.* 1993b), RDS, (Castro-Alcaraz *et al.* 2002; Kotecha *et al.* 2004; Cultrera *et al.* 2006) and bronchopulmonary dysplasia (BPD) (Benstein *et al.* 2003; Viscardi *et al.* 2006; Schelonka and Waites 2007; Sung 2010). Systemic infections were also observed in a sheep model study by Knox *et al.* (2010), in which *Ureaplasma* spp. injected intraamniotically were detected in numerous fetal body sites, including the GIT, liver, kidney, lung and cerebrospinal fluid post-delivery.

RDS is an acute dysfunction of the lung, where there is insufficient surfactant (liquid that aids gas exchange) (Hallman *et al.* 1991), while BPD is a more severe respiratory disease in which alveolar development is arrested, resulting in fewer but larger alveoli (Jobe and Bancalari 2001). Both disease states are strongly associated with inflammation, resulting from *in utero* exposure to ureaplasmas (or other microorganisms) and chorioamnionitis or funisitis. The relationship between ureaplasmas and BPD was first proposed in 1988, with several studies identifying a causative agent of BPD in low birth weight infants as *Ureaplasma* spp.(Cassell *et al.* 1988; Sanchez and Regan 1988; Wang *et al.*

1988). Since these reports, there has been an increasing body of evidence for the involvement of *Ureaplasma* spp. in BPD.

Studies utilising animal models of ureaplasma infection and the impact on fetal lung development have been used extensively. In non-human primate animal models, intraamniotic injection of *Ureaplasma* spp. resulted in fetal pneumonia, alveolar type II cell proliferation and lung injury (Novy *et al.* 2009). Pregnant baboons were exposed to *Ureaplasma* spp. *in utero* for 2 days and their fetuses subsequently demonstrated either (i) clearance of the infection and had normal lung function at birth; or (ii) were colonised persistently in the fetal lung with *Ureaplasma* spp. with inflammation and poor lung function was demonstrated at the time of delivery (Yoder *et al.* 2003). Similarly in a sheep model of intraamniotic *Ureaplasma* spp. infection, it has been shown that both acute and chronic durations of exposure to ureaplasmas have been associated with fetal lung inflammation and increased cytokine (IL-1β, IL-8 mRNA) expression, altered elastin and α-smooth muscle actin deposition, increased surfactant production and improved lung gas volumes at the time of birth (Collins *et al.* 2010).

Prematurely born babies exposed to *Ureaplasma* spp. *in utero* also have demonstrated signs of lung fibrosis, elastic fibre accumulation and increased levels of TNF-α and transforming growth factor (TGF)-β within their lungs at the time of autopsy (Viscardi *et al.* 2002; Viscardi 2010). Once ureaplasmas have access to the fetal lung, they are able to disseminate to distant anatomical sites and have been isolated in 23% of umbilical cord blood cultures in preterm neonates (Goldenberg *et al.* 2008a). *Ureaplasma* spp. are also the most frequently isolated microorganism from the cerebrospinal fluid (CSF) of neonates and are a causative agent of sepsis (Waites *et al.* 1989; Cassell *et al.* 1993b), meningitis (Garland and Murton 1987; Stahelin-Massik *et al.* 1994; Biran *et al.* 2010), brain lesions (Olomu *et al.* 2009) and intraventricular haemorrhage (Viscardi *et al.* 2008). In severe cases of infection, ureaplasmas are also a cause of fetal and neonatal death. The *Ureaplasma* spp. are associated with severe, potentially life-threatening neonatal infections and warrant further investigation. By first understanding the pathogenesis of fetal infections, more effective treatments may be developed in order to avoid severe infection or potential long-term morbidity.

Ureaplasmas are able to gain direct access to the fetal gut when the fetus swallows infected amniotic fluid. A recent study by Wolfs *et al.* (2013) utilised an ovine model of ureaplasma infection; ewes were intraamniotically injected with ureaplasmas and fetal gut was collected after surgical delivery of the fetus at 3, 7 or 14 days post-infection. Inflammatory responses were detected within

the neonatal gut of all animals exposed to ureaplasmas. Within 7 days of infection, there was significant damage to the villus epithelium, deterioration of the gut barrier and enterocyte proliferation, differentiation and maturation were significantly reduced. By 14 days of infection, severe villus atrophy was evident in animals exposed to ureaplasma infection. When animals infected with ureaplasmas were also given recombinant human IL-1 receptor agonist, these outcomes were largely prevented (Wolfs *et al.* 2013). These results are very important, and are the first to discuss the outcomes of the fetal gut following exposure to ureaplasmas. It is unknown what effect ureaplasmas may have on the human fetal gut, particularly as *in utero* infection with *Ureaplasma* spp. may be chronic (Cassell *et al.* 1993b) (much longer than the exposure times utilised in this study). Further studies focused on the fetal and infant gut would be of great interest to determine if exposure to ureaplasmas *in utero* adversely affects the fetus/infant.

2.8 Long-term sequelae of *in utero* ureaplasma infections

The developmental origin of health and disease (DOHaD) paradigm was initially proposed 25 years ago and evolved from epidemiological studies correlating rates of infant mortality and birth weight with certain types of diseases in these same infants later in life. Hales and Barker (1992) stated that "environmental factors during a phase of developmental plasticity (*in utero* development) interact with genotypic variation to change the capacity of the organism (fetus) to cope with its environment in later life". He further illustrated the severe consequences of this paradigm by identifying significant associations between chronic diseases in adulthood (e.g. heart disease, metabolic syndrome) and deficiencies and/or insults to the fetus during *in utero* development (Hales and Barker 1992; Hales and Barker 2001; Benyshek and Watson 2006). Whilst some studies have identified links between some microorganisms and long-term sequelae (Ternhag *et al.* 2008; Puntener *et al.* 2012), currently no studies have reported the long-term outcomes of infants exposed *in utero* to ureaplasmas. This is due to a lack of follow-up studies beyond the neonatal period and the difficulties in identifying a causative agent and the associated sequelae, particularly when the origin may be multifactorial.

Studies have identified that *Ureaplasma* spp. intrauterine inflammation was associated with elevated IL-6 and IL-8 levels, a potential risk factor for cerebral palsy (Yoon *et al.* 1997). Further studies (Berger *et al.* 2009) confirmed that the presence of *Ureaplasma* spp. *in utero* was associated with adverse neuromotor outcomes for two year-old children, compared to children who were not exposed to ureaplasmas *in utero*. Normann *et al.* (2009) examined the murine fetal brain after *in utero* exposure to *Ureaplasma* spp. Pups exposed to ureaplasmas showed decreased neuron density

and impaired production/maturation of interneurons, which are key to healthy cognitive function. *In utero* exposure to ureaplasmas was also correlated with abnormal myelination. This is the first report of *Ureaplasma* spp. altering brain development in an animal model of infection. A combination of animal model experiments and studies of neonates will further elucidate the role of *Ureaplasma* spp. in adverse neurodevelopmental outcomes.

Studies have also linked *Ureaplasma* spp. in the upper respiratory tract with respiratory symptoms over the first three years of life. Kundsin *et al.* (1996) recruited children (n = 88) between 2 months and 3 years of age who presented with respiratory symptoms. Throat swabs were collected for each patient and were screened for the presence of *Ureaplasma* spp. and *Mycoplasma hominis*. Overall, 29/88 (33%) of children within the study were positive for the presence of *Ureaplasma* spp. and colonisation was highest within the 0-12 month (25%) group and the 1- 2 year (43%) group. Significantly, the presence of *Ureaplasma* spp. was associated with a much higher incidence of wheezing of the infants (mean: 14 days; $p < 0.01$), compared to those infants with respiratory symptoms who were not colonised with *Ureaplasma* spp. (3 days) and the control group (0 days). The authors also noted no significant differences between infants with respiratory symptoms who were born prematurely and those born at term, which suggests that colonisation of infants occurs independently of gestational age and is associated with adverse respiratory outcomes (Kundsin *et al.* 1996).

A larger study of almost 3000 infants and their mothers identified a higher risk of wheezing in infants less than 3 years of age and the presence of *Ureaplasma* spp. (odds ratio: 2.0). The authors suggested that colonisation with ureaplasmas, acquired at or before birth, may cause reactive airway disease. Furthermore, the authors also suggested that the acquisition of certain microorganisms, such as *Ureaplasma* spp. contributes to the establishment of infant microflora and the subsequent development of allergies and wheezing (Benn *et al.* 2002). While these studies show significant associations between *Ureaplasma* spp. and the wheezing respiratory phenotype, it is unclear if *Ureaplasma* spp. also contribute to other long-term adverse respiratory diseases, such as asthma.

2.9 Ureaplasmas as a controversial pathogen

Although *Ureaplasma* spp. have been isolated most frequently from women who deliver preterm, the role of ureaplasmas in adverse pregnancy outcomes is complicated as not all pregnant women who are infected/colonised with *Ureaplasma* spp. in the UGT deliver preterm or experience adverse

pregnancy outcomes. Gerber *et al.* (2003) tested amniotic fluid from 254 asymptomatic pregnant women at 15 – 17 weeks of gestation by PCR and detected *Ureaplasma* spp. in 29/254 (11.4%) of subjects. Significantly, 24% of women with intra-amniotic *Ureaplasma* spp. infection delivered preterm with or without signs of P-PROM, compared to 4.4% of women without *Ureaplasma* spp. infection ($p < 0.0001$). However, 76% of women identified as having *Ureaplasma* spp. *in utero* delivered at term with no apparent adverse outcomes. Women who were infected with ureaplasmas and delivered preterm also had a higher rate of preterm labour in previous pregnancies (consistent with the proposal that *Ureaplasma* spp. may be present as colonisers or as an asymptomatic infection in the endometrium prior to conception), this study failed to comment on the large proportion of women who did not experience any adverse outcomes and delivered at term (Gerber *et al.* 2003).

To explain the discrepancies in these studies, researchers have suggested that there may be more "virulent" serovars of *Ureaplasma* spp.; however, there is little evidence that particular ureaplasma serovars are more virulent than others. Studies have reported that (i) *U. urealyticum* serovar 4 was associated with recurrent abortion (Quinn *et al.* 1983; Naessens *et al.* 1988) (ii) *U. parvum* serovars 3 and 14 were significantly associated with the absence of *Lactobacillus* spp. and genital tract symptoms including vaginal/cervical discharge or pain during urination (De Francesco *et al.* 2009) (iii) *U. parvum* serovars 3 and 6 were most frequently isolated from women who delivered preterm in an Australian population (Knox and Timms 1998); and (iv) *U. parvum* serovar 6 was the most adherent serovar to the surface of spermatozoa, following standard ART washing procedures (Knox *et al.* 2003). Zheng *et al.* (1992) tested ureaplasma isolates from the CSF of neonates and identified that *U. parvum* serovars 1, 3 & 6 and *U. urealyticum* serovar 8 & 10 were capable of causing systemic infections (Zheng *et al.* 1992). These results suggest that there is no single "virulent" *Ureaplasma* species or serovar, and that the serovars isolated in these studies may simply be the most prevalent microorganisms within the population studied.

It has been suggested that instead of "virulent" serovars, there may be other virulence/pathogenicity mechanisms by which microorganisms can affect the host immune response and the outcomes of those infected with *Ureaplasma* spp.

2.10 Ureaplasma virulence factors

Whilst *Ureaplasma* spp. are normal regional flora of the human LGT and are considered to be of low virulence, the *Ureaplasma* spp. have been implicated in adverse pregnancy outcomes (as described

in section 2.6) and specific virulence factors which enable ureaplasmas to contribute to disease have been investigated. There are five proposed proteins that have been investigated as virulence factors of *Ureaplasma* spp.: the multiple banded antigen (MBA), urease, immunoglobulin (Ig) A protease, phospholipase A and phospholipase C proteins (Robertson *et al.* 1984; De Silva and Quinn 1986; Teng *et al.* 1994; DeSilva and Quinn 1999; Glass *et al.* 2000).

2.10.1 The MBA

The MBA is the major antigen recognised by the host immune system in response to ureaplasma infections (Shimizu *et al.* 2008). This major antigen was first detected by Watson *et al.* (1990) by sera from patients who were infected with ureaplasmas. These patients' sera recognised a predominant 71 kDa ureaplasmal protein and subsequent monoclonal antibodies identified less intensely stained MBA bands of varying molecular weights, which formed a laddering (multiple banded) pattern (Watson *et al.* 1990).

Zheng *et al.* (1995) cloned and sequenced the gene, which encodes the *U. parvum* serovar 3 MBA protein. This gene was found to contain a single large open reading frame of 1230 bp, encoding a 409 amino acid protein. The N-terminal region of the protein consisted of a typical prokaryotic signal peptide and a membrane lipoprotein attachment site (transmembrane domain). Following the transmembrane domain, a C-terminal hydrophilic (surface-exposed) gene region was identified and was found to contain tandem repeating units, which were unique to each *Ureaplasma* spp. serovar (Zheng *et al.* 1995). Glass *et al.* (2000) demonstrated that the *mba* gene had no homology to any other known gene and was unique to *Ureaplasma* spp. (Glass *et al.* 2000). Shimizu *et al.* (2008) further confirmed that the MBA is a lipoprotein, due to its ability to be isolated in the detergent phase of Triton X-144 partitioning, and confirmed the antigenicity of the MBA, with this major antigen activating nuclear factor (NF)-kB and the production of TNF-α *via* TLRs 1, 2 and 6.

The first studies to characterise variation in the size of the MBA protein/gene used polymerase chain reaction (PCR) to demonstrate that the differences in MBA size correlated with differences in the number of tandem repeating units (Zheng *et al.* 1994). Antigenic size variation has been reported in other *Mycoplasma* spp. and is predicted to modulate the interaction between bacteria and host cells (Citti *et al.* 2010). Knox *et al.* (2010) demonstrated ureaplasma MBA size variation following 69 days of (chronic) intraamniotic ureaplasma infections within an established ovine model. A non-clonal *U. parvum* serovar 6 clinical isolate (with a single MBA antigen) was injected into the amniotic fluid and after delivery the number of MBA size variants within the amniotic fluid was assessed. When the

amniotic fluid of these pregnant ewes contained less than 5 MBA size variants, severe histological chorioamnionitis was seen within chorioamnion tissues. However, when amniotic fluid was found to contain nine or more MBA size variants, there was little or no evidence of histological chorioamnionitis within the chorioamnion of the ureaplasma-infected ewes. Based on these findings, it was suggested that the size variation of the ureaplasma MBA may be a mechanism by which *Ureaplasma* spp. can evade host immune recognition, allowing chronic infections to develop.

Robinson *et al.* (2013) further confirmed the ability of clinical ureaplasma isolates to vary in size. A non-clonal *U. parvum* serovar 3 clinical isolate was injected into the amniotic fluid of pregnant ewes (n = 32) and the fetuses were then delivered surgically after acute (3 or 7 days) or chronic (69 day) infections. Harvested amniotic fluid was tested for the presence of MBA size variants by PCR and western blot. Amniotic fluid infected with ureaplasmas for 3 or 7 days showed minimal evidence of MBA variation (when compared to the original strain injected), whilst amniotic fluid infected with ureaplasmas for 69 days showed significant MBA size variation (Robinson *et al.* 2013).

Dando *et al.* (2012) utilised *Ureaplasma* spp. isolated from the amniotic fluid of sheep infected in previous experiments (originally published in Knox *et al.* 2010). Two ureaplasma cultures were cloned and filtered to obtain two populations each derived from a single colony-forming unit (CFU). These two inocula were then injected into the amniotic fluid of cohorts of pregnant ewes. Amniotic fluid specimens were collected every two weeks of gestation and antigenic size variation was observed throughout gestation. Significantly, this is the first study to identify MBA size variations over the course of gestation. It was determined that there was an inverse correlation between the number of *Ureaplasma* spp. CFU/mL within amniotic fluid and the numbers of MBA size variants; the greater the number of MBA size variants within amniotic fluid, the fewer the numbers of ureaplasma (CFU/mL) present within the amniotic fluid. This suggests that the variation of the MBA is an important mechanism to maintain diversity within their population *in vivo* (Dando *et al.* 2012). It is proposed that the production of MBA size variants *in vivo* results in an inability of the host to accurately respond to the many antigens (MBA variants) present, and this may facilitate establishment of chronic infections. The ability of ureaplasmas to vary their surface-exposed MBA is a major virulence factor and requires further studies to fully elucidate the role of MBA variation and the host immune responses but as yet MBA variation in ureaplasmas from human infection has not been studied.

The ureaplasma MBA is also able to undergo phase variation, which involves the "switching on/off" of the MBA *in vitro*. This alternating expression of surface-exposed proteins is also proposed to be an important microbial strategy for host adaptation and evasion of the immune response, in order to maintain diversity and a propagating population (Monecke *et al.* 2003; Zimmerman *et al.* 2011; Dando *et al.* 2012). Phase variation has been demonstrated previously in other *Mycoplasmatales* (Citti *et al.* 2010), such as the variable surface antigen (*vsa*) of *Mycoplasma pulmonis* (Bhugra and Dybvig 1992; Bhugra *et al.* 1995) and the variable protein of *Mycoplasma agalactiae (vpma*) (Glew *et al.* 2000; Glew *et al.* 2002; Chopra-Dewasthaly *et al.* 2008). Monecke *et al.* (2003) and Dando *et al.* (2012) showed the MBA protein was able to be "switched off" by serially passaging ureaplasmas in broth containing polyclonal antibodies specific to ureaplasmas. The absence of the MBA was shown by western blot and variation in the length of the *mba* gene was demonstrated by PCR. "Switching off" of the MBA was shown to be reversible and this mechanism may be used by ureaplasmas to evade the host immune response *in vivo*.

Zimmerman *et al.* (2009) further investigated phase variation of the MBA by analysing the MBA locus. They identified the UU376 gene, a *Ureaplasma* spp.-specific conserved hypothetical gene, encoding a ureaplasmal protein. Expression of the MBA and UU376 was assessed by serially passaging ureaplasmas in the presence of polyclonal antibodies generated against the MBA or UU376. The alternate expression of the MBA and UU376 was associated with a DNA inversion event between the non-repetitive region and an intergenic region between the MBA and a region downstream of UU376. It was proposed that these DNA inversion events serve as a mechanism by which *Ureaplasma* spp. express their MBA protein or "switch off" the expression of this protein (Zimmerman *et al.* 2009).

These same researchers provided further evidence of DNA inversion events between the ureaplasma genes UU171, UU172 and UU144. Using polyclonal antibodies, they again used selective pressures to mediate alternate expression of these genes, and found that the open reading frames associated with these genes were not expressed alone, but were only expressed in conjunction with the N-terminal sequence of UU172. Phase variation was previously suggested to occur as a result of slipped-strand mispairing (Rocha and Blanchard 2002); however, Zimmerman *et al.* provided evidence that phase variation occurred between two inverted repeat regions – one of which is located in the ORF of UU172 and another in the intergenic region between UU171 and UU172 (Zimmerman *et al.* 2011). As yet the mechanisms by which these organisms alter their surface-exposed proteins have not been characterised nor is it known if these events occur *in vivo*. Further

investigation is required to elucidate the mechanisms, which govern surface antigen variation and the effect this has on infections within the host.

Recently, Paralanov *et al.* (2012) sequenced the genomes of 19 ureaplasma clinical isolates and compared these to American Type Culture Collection (ATCC) strains. The major finding of this study was that the *mba* gene is part of a large and complex superfamily, comprising more than 180 genes. This study confirmed that each serovar/isolate contained an *mba* gene that consisted of a conserved upstream portion, attached to downstream unique tandem repeating units. Significantly, this study also identified that the majority of ureaplasma ATCC strains and clinical isolates contained additional tandem repeating unit sequences that were not attached to the *mba* gene. These extra tandem repeating units were found in other locations within the genome and were surrounded by putative recombination sites, which suggest that these tandem repeating units may also be part of a phase-variable system. Experimental evidence is required to substantiate these claims, in order to observe the attachment of these additional tandem repeating unit sequences to the upstream conserved portion of the *mba* gene. It would be of great interest to investigate the potential phase variation of these tandem repeating units, as some may be immunogenic and contribute to the differential pathogenicity of ureaplasma clinical isolates/strains (Paralanov *et al.* 2012). These mechanisms of MBA gene expression and antigenicity are crucial in our understanding of why only a small proportion of patients with *Ureaplasma* spp. develop clinical symptoms whilst others remain asymptomatic. This is particularly pertinent to our current understanding of chronic asymptomatic ureaplasma intrauterine infections.

2.10.2 Urease

The ability of *Ureaplasma* spp. to hydrolyse urea was first identified in 1966, and the production of adenosine triphosphate (ATP) *via* this mechanism appears to be unique within ureaplasmas (Purcell 1966; Shepard 1966; Ford 1967; Shepard 1967). The urease enzyme of ureaplasmas is 30 – 180-fold more efficient than those reported for any other bacterial urease (Mobley *et al.* 1995) and the hydrolysis of urea creates an electrochemical gradient due to intracellular ammonia accumulation (with ureaplasmas having extremely high intracellular ammonia levels, measured at 21 times the extracellular concentration) (Smith *et al.* 1993). This gradient creates a chemiosmotic potential, by which *Ureaplasma* spp. are able to generate 95% of their ATP (Smith *et al.* 1993).

Urease enzyme activity is another key virulence factor of ureolytic bacteria, as the production of ammonia has been shown to be lethal following intravenous injection of bacterial ureases in a

murine animal model (Takebe *et al.* 1984). Urinary tract ureaplasma infection was found to be associated with urinary stone formation, which was attenuated when urease inhibitors were added (Takebe *et al.* 1984). Whilst the urease enzyme complex of ureaplasmas comprises a major portion of the cytoplasm, due to its location, it is unlikely to be involved in immune stimulation or inflammatory responses. However, a recent publication within our group identified that chronic (69 day) *in utero* infection with *Ureaplasma* spp. resulted in a significant increase in amniotic fluid and fetal lung pH in an ovine model of intra-amniotic ureaplasma infection. The increased pH was most likely due to the accumulation of ammonia within the amniotic fluid, which has a high concentration of urea and the urea concentration increases during gestation. This study also identified that the increased pH within the fetal lung was associated with lung damage, even in the absence of inflammatory responses (Robinson *et al.* 2013). Whilst this study provides the first evidence of increased pH *in vivo* due to ureaplasma infection, it remains unclear what the long-term effect of exposure to this alkaline environment may have on other fetal outcomes.

There are seven genes, which encode the ureaplasma urease enzyme and they are clustered together in a similar conformation to other ureolytic bacteria, such as *Proteus mirabilis* and *Escherichia coli*. Urease (*Ure) A*, *UreB* and *UreC* encode the structural subunits of the urease enzyme, whilst *UreE*, *UreF*, *UreG* and the truncated *UreD* encode for urease accessory proteins, involved in the synthesis of the nickel metallo-centre. Despite the high urease enzyme efficiency, it is interesting to note that ureaplasmas encode a urease enzyme but they do not assimilate ammonia into glutamate or glutamine (Williams and Wernegreen 2010).

2.10.3 IgA Protease

Immunoglobulin A (IgA) protease activity has been reported as one of the major contributing factors to ureaplasma pathogenesis (Kilian *et al.* 1984). A primary defence of the human immune system is the secretion of IgA antibodies at the surface of the mucosa and the cleavage of IgA may enable ureaplasmas to invade the upper genital tract (particularly during pregnancy), causing adverse outcomes. IgA protease activity was first documented in ureaplasmas in 1984 (Robertson *et al.* 1984) and was confirmed experimentally by Kilian and Freundt (1984) colleagues who showed the specific cleavage of IgA_1, resulted in intact Fab and Fc fragments. All 14 serovars of *Ureaplasma* spp. have been shown experimentally to possess IgA_1 protease activity (IgA_1 present in serum and vaginal secretions); however, they do not possess any proteolytic activity against IgA_2 (present in vaginal secretions but not in serum), IgM or IgG antibodies (Kilian *et al.* 1984). Studies conducted by

Spooner *et al.* (1992) identified that the IgA protease of ureaplasmas (a serine protease) cleaved human IgA specifically between proline and threonine residues within the hinge of the heavy chain.

Despite experimental evidence of IgA protease activity, sequencing of the entire *U. parvum* serovar 3 genome failed to identify the genes homologous to those in other urease producing bacteria that encode IgA proteases within ureaplasmas (Glass *et al.* 2000). Furthermore, genomes of the 14 ATCC strains along with *Ureaplasma* spp., along with 19 clinical isolates were compared and again there was no evidence of an IgA protease gene(s) within any of the isolates tested (Paralanov *et al.* 2012). Based on experimental evidence and the lack of computational gene evidence for an IgA protease, it has been suggested that the genes encoding this protease have evolved so significantly that it is no longer recognisable (when compared to orthologues in other prokaryotes) or that ureaplasmas possess a unique IgA protease with no sequence similarity to other bacteria. However, further evidence is required to confirm IgA protease activity and to identify the genes involved in its proteolytic activity, as this is likely to be a key virulence factor of *Ureaplasma* spp.

2.10.4 Phospholipase A and C

Phospholipases are a group of enzymes that hydrolyse phospholipids, producing fatty acids and other lipophilic substances (Istivan and Coloe 2006). Phospholipases have long been recognised as virulence factors in a range of microorganisms (Schmiel and Miller 1999), including *Legionella pneumophila* (Dowling *et al.* 1992), *Listeria monocytogenes* (Rouquette and Berche 1996), *Staphylococcus aureus* (Nygren *et al.* 1966) and *Pseudomonas aeruginosa* (Pollack 1984). The pathogenesis of phospholipases result from the production of membrane-destabilising compounds or the widespread degradation of the host cell membrane phospholipids (Istivan and Coloe 2006). There are four major types of phospholipases: A (divided into A_1 and A_2), B, C and D. Phospholipase A_1 and A_2 are responsible for the cleavage of sn-1 and sn-2 acyl chains of fatty acids, respectively. Cleavage of the sn-2 acyl chain releases arachidonic acid. Phospholipase C cleaves the phosphodiester bonds, resulting in the production of 1,2-diglyceride and phosphorylesters (Vasudevan 2011). Phospholipase activity has been identified in *Ureaplasma* spp. previously by De Silva and Quinn (1986). Endogenous phospholipase activity was demonstrated *in vitro* in ureaplasma serovars 3, 4 and 8, which were active over a wide pH range (5 – 9; the pH growth range of *Ureaplasma* spp.), and phospholipase activity of A_1 and A_2 differed from that documented in other bacteria. The unique activity of these ureaplasma phospholipases was significantly higher during the exponential growth phase compared to stationery phase growth, suggesting that these phospholipases are membrane-bound. The authors also demonstrated that *U. urealyticum* serovar 8

had a three-fold higher phospholipase A_2 activity than serovars 3 and 4. Phospholipase A_2 cleaves sn-2 acyl chain, producing free arachidonic acid, an important intermediate in inflammation and the synthesis of prostaglandins. Prostaglandins play a key role in pregnancy, with prostaglandin levels naturally increasing at the onset of childbirth (Pawelec *et al.* 2013). Prostaglandins trigger uterine contractility and high levels of prostaglandins prior to the normal onset of labour may result in premature uterine contractions and preterm delivery of the fetus (Goldenberg *et al.* 2000b). Due to the large differences in phospholipase A_2 activity in the different ureaplasma serovars tested, the authors suggested that these differences may account for virulence of particular serovars and the differences between the serovar may be of physiological importance due to their interactions with host tissues (De Silva and Quinn 1986). However, despite these previous findings, whole genome sequencing of *Ureaplasma parvum* serovar 3 showed no evidence of genes encoding the phospholipase A_1, A_2 or C enzymes (Glass *et al.* 2000). Further genome analysis of a range of *Ureaplasma* spp. ATCC serovars and clinical isolates again showed no significant evidence of phospholipase A1, A2 or C genes (Paralanov *et al.* 2012). The experiments of De Silva and Quinn were repeated in conjunction with a commercial kit to detect phospholipase C activity. However, no phospholipase activity at either the stationery or exponential phases of ureaplasma growth was detected and so it was suggested that ureaplasmas do not possess a phospholipase C enzyme. However, a gene containing significant similarities to phospholipase D enzyme was identified in all *Ureaplasma* spp. ATCC strains and in all clinical isolates tested (Paralanov *et al.* 2012). Further functional characterisation and determination of phospholipase D activity would be of great interest to further our understanding of this ureaplasma virulence mechanism.

2.11 Host immune response to ureaplasma infections

Immunologically, pregnancy is a unique period in which the maternal immune system undergoes significant changes. The presence of the fetus, an amalgam of two individuals, expresses MHC cell surface markers that would normally be recognised as 'foreign' by the host immune system (Abrahams 2008). Because of the capacity of the fetus to elicit such a strong immune response, it was historically thought that the interface between the mother and the fetus was immunologically inert, in order to prevent the eradication or rejection of the fetus; however, this hypothesis does not take into account *in utero* infections.

Infection of the UGT during pregnancy are complex, as there is not simply a single host; but multiple hosts (both the mother and the fetus) that may respond to the invading pathogen. Numerous studies have shown that the fetal-maternal interface adopts a predominantly 'protective' TH2

immune phenotype and reduces any TH1 (proinflammatory) immune responses to protect the fetus and placenta. However, the host is still capable of recognising and responding to "infectious, non-self" threats, such as bacterial infections (Koga and Mor 2010; Mor and Cardenas 2010; Mor *et al.* 2011). Importantly, while the maternal immune responses to intraamniotic infection have been well characterised (such as *E. coli* lipopolysaccharide [LPS]), the human maternal immune responses to *in utero Ureaplasma* spp. infections are not well understood.

2.11.1 Innate immune responses

The innate immune system is the immunological “first line of defence” during pregnancy, providing an immediate response to invading pathogens. The innate immune system is unique during pregnancy and there are high numbers of circulating leukocytes (70% natural killer cells, 20 – 25% macrophages and 1.7% dendritic cells) within the uterus and placenta (Abrahams *et al.* 2005). Within serum, the complement system acts to identify pathogens and trigger proteolytic cascades, resulting in the production of proinflammatory mediators, opsonisation of the pathogen and targeted cell lysis. More recently, the complement pathway has been described as the functional “bridge” between the innate and adaptive immune systems, allowing an integrated host response to pathogenic organisms (Dunkelberger and Song 2010).

Recently, Beeton *et al.* (2013) demonstrated the serovar-specific bactericidal activity of serum against ureaplasmas. *U. parvum* serovars 1, 3, 6 and 14 were incubated with human sera collected from normal healthy patients (n = 12) and from immunodeficient patients (n = 4) to determine the bactericidal activity against each particular serovar. *Ureaplasma* spp. killing was calculated as the fold decrease in ureaplasma colour changing units (CCU), when compared to the ureaplasma CCU incubated with heat-inactivated human serum from the same patient. Western blots using patient sera were carried out against all *U. parvum* serovars to determine specific immunoreactivity. The study identified that sera from healthy patients was frequently associated with bactericidal activity against *U. parvum*, when compared to the sera collected from immunodeficient patients (83% serum killing from healthy patients, compared to 25% serum killing from immunodeficient patients, respectively). Serovars 1, 6 and 14 activated classical complement pathways and an increased bactericidal activity was correlated with the presence of immunoreactive bands by western blot. Interestingly, *U. parvum* serovar 3 complement activation was harder to define, but was demonstrated to be completely C1q-dependent. Significantly, the serum-killing ability for all *U. parvum* serovars was abolished following removal of IgG1 from serum, showing that the bactericidal activity of serum is antibody-dependent. The results of this study suggest that surface-exposed

antigens may play a significant role in complement activation, and the immunodominant MBA antigen of *Ureaplasma* spp. (as described in section 2.10.1) would be an excellent target for further studies of complement activation and bactericidal activity against *Ureaplasma* spp.

Antimicrobial peptides are also produced as natural antimicrobial agents and are present during gestation. Defensins, including α-defensins 1 – 3, 5; as well as β-defensins 1 – 3 have been identified at various sites of the UGT during pregnancy (including the decidua, amnion, chorion, trophoblast and syncytiotrophoblast). Other antimicrobial peptides such as secretory leukocyte protease inhibitors and elafin have also been identified within amniotic fluid and other tissues, suggesting the production of these natural antimicrobials within the uterus play an important role in prevention of intraamniotic infections during pregnancy (King *et al.* 2007). More recently, Xiao and colleagues further elucidated the role of antimicrobial peptide production in response to *Ureaplasma* spp. (Xiao *et al.* 2014). Monocytic (THP-1) cells co-cultured with *U. parvum* and *U. urealyticum* demonstrated significantly lower gene expression of α-defensins 1 and 6, β-defensin 1 and down-regulation of human cathelicidin genes, when compared to uninfected controls. The down-regulation of α-defensins resulted in an inability of the host to inhibit different types of pathogens (bacteria, fungi and enveloped viruses), while down-regulation of β-defensins hindered the epithelial surfaces resistance to microbial colonisation. Furthermore, cathelicidns serve a critical role in mammalian innate immune defences against invasive bacterial infections. While the authors found no evidence of chromatin modification or DNA methylation alterations, they concluded that down-regulation of these antimicrobial peptides may be an important mechanism for *Ureaplasma* spp. to establish chronic infections and "avoid" this portion of the innate immune defences (Xiao *et al.* 2014).

Another major facet of the innate immune system includes the TLRs, which are key mediators of inflammation during infection (Abrahams 2005; Mor and Cardenas 2010). TLRs recognise pathogen-associated molecular patterns (PAMPS, expressed on the surface of microorganisms) and damage-associated molecular patterns (endogenous nuclear/cytosolic molecules released during cell and tissue injury). There are ten known TLRs (TLR 1 – 10), all of which are expressed within the placenta, along with their various co-receptors and accessory proteins (Mitsunari *et al.* 2006).

TLR expression changes over the course of pregnancy, both spatially and temporally, in order to protect the fetus. For example, TLRs 2 and 4 are expressed within the trophoblast and cytotrophoblast cells (the embryo), but not in syncytiotrophoblast cells (the outer epithelial cell layer). It is thought that this syncytiotrophoblast remains "TLR negative" as there is no immediate threat to the embryo unless a pathogen is able to breach this cell layer and reaches the TLR-

expressing trophoblast and cytotrophoblast (Abrahams *et al.* 2004; Kumazaki *et al.* 2004). TLR 6 is expressed by trophoblast cells in the third trimester, but not during the first trimester of pregnancy (Koga and Mor 2010).

Shimizu *et al.* (2008) first investigated the TLRs that interact/bind to *Ureaplasma* spp. by exposing murine macrophages to ureaplasmas *in vitro*. The ureaplasma surface-exposed MBA protein was specifically recognised by TLRs 1, 2 and 6, which activated NF-kB and signalled the production of cytokines TNF-α, IL-1β, IL-6 and IL-8. Triantafilou *et al.* (2013) further elucidated the role of TLR signalling *in vitro.* Human amniotic epithelial cells were exposed to *Ureaplasma parvum* serovars 3 and 14. These amniotic epithelial cells expressed minimal levels of all TLRs tested (TLR 1 – 9) prior to ureaplasma stimulation, but upon exposure to ureaplasmas, expression of TLRs 2, 6 and 9 was significantly increased and this also corresponded to significant increases in TNF-α, IL-1β, IL-6 and IL-8. Amniotic epithelial cells were further exposed to a recombinant *Ureaplasma* spp. MBA antigen and significant increases in TLRs 2 and 6 were measured. TLR 2/6 heterodimers recognise the diacylated MBA protein on the cell surface of *Ureaplasma* spp. The expression of TLRs 2, 6 and 9 was then silenced by RNA interference with the silencing of TLR 2 resulting in the most significant loss of cellular activation (as evidenced by significantly decreased IL-6 concentration). Silencing of TLRs 6 and 9 also inhibited cellular activation, but to a much lesser extent (Triantafilou *et al.* 2013).

Taken together, the findings of Shimizu *et al.* (2008) and Triantafilou *et al.* (2013) confirm that the ureaplasma MBA (the major antigen and pathogen-associated molecular pattern of *Ureaplasma* spp.) is recognised by TLR 2 and stimulates the production of TNF-α, IL-1β, IL-6 and IL-8. These cytokines have also been detected in human cells infected with *Ureaplasma* spp. (Triantafilou *et al.* 2013) and also in animal cells infected with *Ureaplasma* spp. (Shimizu *et al.* 2008). Additional studies should investigate the efficacy of these immune modulators, in combination with others, as markers of ureaplasma infection. However, cytokines are not only elevated in response to infection, but have been shown to be elevated in other pregnancy sequelae. For example, increased IL-8 levels have been detected in the amniotic cavity of women with preeclampsia, in the absence of infection (Yada *et al.* 2010), suggesting that more reliable and consistent biomarkers should be investigated. Additionally, panels of biomarkers may prove a useful addition to current clinical practices, as the use of multiple markers of UGT infection may also improve the efficacy of successful detection and identification of sequelae.

More recently, Allam *et al.* (2014) also confirmed that expression of TLRs 1, 2, 6 and the accessory protein CD14 was significantly increased following intraamniotic *U. parvum* infection in BALB/c mice. Immunohistochemistry revealed intense co-localisation of TLR 2 and CD14 in syncytiotrophoblast cells, which lined the chorionic plate and also in neutrophils at the choriodecidual junction/chorionic plate. CD14 is an important accessory protein, which is known to be a signal enhancer of TLRs (including TLR 1, 2 and 6). Animals which displayed significant placental histopathology (funisitis and chorionic vasculitis) and fetal pathology (encephalitis, pneumonitis, myocarditis or hepatic necrosis) demonstrated the most intense co-localisation of TLR 2 and CD14, suggesting that these factors together may enhance the maternal inflammatory response to *in utero* ureaplasma infections and result in severe adverse outcomes for the pregnancy and for the fetus. This is significant, as over activation of the host immune system can lead to serious acute or chronic outcomes that have deleterious effects during pregnancy. Intraamniotic ureaplasma infection and inflammation has been linked to human fetal inflammatory response syndrome (Dammann *et al.* 2003) and other serious long-term neurological conditions, such as cerebral palsy (Berger *et al.* 2009).

Interestingly, in response to human intraamniotic infection with *Ureaplasma* spp. it has been reported that the levels of cytokines may be highly elevated (Holst *et al.* 2005; Witt *et al.* 2005), moderately elevated (Menon *et al.* 2009) or there may be no correlation between infection and cytokine levels (Perni *et al.* 2004). This could be, in part, attributed to the number of CFU of ureaplasmas present *in utero*; however, while some studies have shown correlations between the numbers of ureaplasmas present and the levels of inflammatory cytokines within amniotic fluid (Kasper *et al.* 2010), this is not always consistent. In sheep model experiments, the severity of inflammation within the chorioamnion and fetal tissues was different in each animal; varying from no inflammation in ureaplasma-infected tissues, to moderate/severe inflammation or to resolution of infection and scarring. This is despite the fact that all animals received identical inocula of the same strain and dose of *Ureaplasma* spp. (Knox *et al.* 2010; Robinson *et al.* 2013). Furthermore, after identical *U. parvum* strains/doses were introduced into the urinary tract of rats and different severities of infection ensued; non- complicated and complicated UTIs and urinary stone formation (Reyes *et al.* 2009). These animal models confirm that the severity of inflammation was independent of the numbers of ureaplasmas present. Similarly, human studies have also shown discordant host cytokine responses, depending on maternal ethnicity. Different cytokine profiles were generated *in vitro* within chorioamnion tissues incubated with autologous amniotic fluid. Tissues and amniotic fluid were derived from Caucasian and African-American women and were stimulated with either *U. parvum* or *U. urealyticum* (Peltier *et al.* 2012). These differences in host cytokine responses may be

as a result of the host-microbe interactions within the animals and individuals tested, but they may also be due to the ability of ureaplasmas to vary their surface-exposed antigens, including the MBA *in vivo.*

2.11.2 Adaptive immune responses

Adaptive immune responses to *Ureaplasma* spp. have been confirmed in pregnant women by the detection of circulating anti-ureaplasma IgA, IgM and IgG (Quinn 1986; Liepmann *et al.* 1988; Dinsmoor *et al.* 1989; Cunningham *et al.* 1996). Furthermore, the placenta allows circulating immune cells and some antibodies (IgG) to be transferred to the fetus, creating a feto-maternal microchimerism (Tan *et al.* 2011). However, the fetus also responds to ureaplasma infections *in utero*. A study by Cunningham *et al.* (1996) identified that immunoreactivity to ureaplasmas alters throughout pregnancy. Pregnant women (n = 80) were recruited for this study and maternal sera was collected at ≤ 30 weeks of gestation and again at the time of delivery. Anti-ureaplasma IgG and IgA antibodies were detected by western blot. Of the women tested, 93% were found to have serum IgG, which reacted against *Ureaplasma* spp. For five of these women (8%), the number of immunoreactive bands increased between the initial screening and at birth. Four of the five women who had increases in the number of immunoreactive bands also showed corresponding increases in the number of IgA bands against *Ureaplasma* spp. The authors concluded that maternal antibody responses to ureaplasmas altered during pregnancy and this may be a hallmark of ureaplasma infections (Cunningham *et al.* 1996).

Other studies have correlated infection, anti-ureaplasma antibodies and pregnancy losses (Quinn *et al.* 1983), preterm delivery (Horowitz *et al.* 1995), postpartum fever (Lee and Kenny 1987) and low birth weight neonates and fetal death (Horowitz *et al.* 1995). Quinn *et al.* (1983) demonstrated strong links between serum anti-ureaplasma maternal antibodies and severe adverse neonatal outcomes. Anti-ureaplasma antibodies were detected in sera from mothers who experienced stillbirths (77%), neonatal deaths (69%) or had neonates with respiratory sequelae (58%). By contrast, only 6.5% of sera obtained from healthy mothers with healthy neonates had these anti-ureaplasma antibodies (De Silva and Quinn 1986).

Despite these results, the relationship between anti-ureaplasma antibodies and *Ureaplasma* spp. infection are not always clear. Anti-ureaplasma antibodies have been detected in the sera of women whose LGT specimens tested ureaplasma-negative (Liepmann *et al.* 1988), in women who delivered at term with no apparent complications and in healthy neonates (who were delivered at term)

(Horowitz *et al.* 1995). These reports demonstrate the variability in not only the innate inflammatory responses generated *in vivo* during ureaplasma infections, but also in the adaptive immune responses to ureaplasmas.

In a sheep model study by Dando *et al.* (2012), ureaplasmas were isolated from the chorioamnion of a sheep with no apparent inflammation and from the chorioamnion of a sheep with severe inflammation (published in Knox *et al.* 2010). These isolates were cloned and filtered to produce two clonal cultures (an 'avirulent' strain and a 'virulent' strain) and the strains were then inoculated into the amniotic fluid of two pregnant sheep cohorts. Amniotic fluid from each animal was tested by western blot and these demonstrated that there was no difference in the propensity for these isolates to vary their surface-exposed MBA (i.e. antigenic variation is not specific to virulent strains of *Ureaplasma* spp.) and there was no difference in the maternal anti-ureaplasmal IgG antibody production in either the 'virulent' or 'avirulent' cohorts. Maternal and cord blood collected from these animals were also tested by western blot using the MBA proteins of the corresponding inocula. Interestingly, the anti-ureaplasma IgG antibodies detected in maternal sera by western blot did not always correlate with those MBA size variants found within the amniotic fluid of the same ewe. This is a novel finding and may be attributed to antigenic variation of *Ureaplasma* spp. *in vivo*. This further suggests that when antigenic variation occurs *in vivo*, the host may not be able to effectively produce neutralising antibodies to eradicate the *Ureaplasma* spp. Additionally, variation of the surface-exposed MBA throughout pregnancy may be a mechanism, which facilitates chronic *in utero* infections. These observations are significant, if ureaplasmas can evade/avoid host immune eradication; the fetus is exposed to live *Ureaplasma* spp. for extended periods, which may predispose the infant to severe adverse outcomes.

2.12 Models for the study of ureaplasma infections

Animal models including rats, mice sheep and non-human primates have been used to the study intraamniotic infections and their sequelae. The challenge is to use a cost-effective animal model, which accurately reflects disease pathogenesis in humans so that the findings are translatable to human gestation/parturition. There will always be concerns when comparing one animal model to another and ultimately extrapolating these data to human gestations. Therefore, it has been suggested that there is no "single best animal model".

2.12.1 Murine models

Rats and mice are among the most common cost-effective animal models. Rats and mice are easy to house, they have short gestations of approximately 20 and 22 days, respectively. Mice are able to be modified genetically they have well-characterised immune systems, with a wide variety of commercially available antibodies, small interfering ribonucleic acids (siRNAs) and microarrays are available (Kemp *et al.* 2010). Whilst studies have shown that treatment of mice with *E. coli* LPS resulted in an increase of IL-1 in maternal sera and preterm delivery of pups (Fidel *et al.* 1994); these models are not directly translatable to human intraamniotic infections as the major underlying mechanisms of parturition are very different. In mice and rats, labour and delivery results from the withdrawal of progesterone and corresponding luteolysis, brought about by the production of prostaglandin $F_{2\alpha}$ (Mitchell and Taggart 2009). In contrast, human labour and delivery result from increased production of prostaglandin E_2 or antiprogestin, oxytocin and their corresponding receptors. Furthermore, the placental anatomy of both mice and rats is very different to that of humans, with mice/rats having hemotrichorial labyrinthine placentae, whilst humans have hemomonochordial villous discoid placentae (Mitchell and Taggart 2009). Further to this, the short gestational period of rats and mice, as well as the poorly developed organ systems of the fetus (particularly the lung) at the time of birth limit the usefulness of these models for studying chronic intrauterine infections and fetal outcomes. This is particularly crucial in the study of *Ureaplasma* spp. infections, as these infections are often chronic and fetal outcomes are of great importance. Whilst mice are not particularly useful for studying pregnancy outcomes, both rats and mice have been used to study *Ureaplasma* spp. infections, including urogenital tract infections (Reyes *et al.* 2009; Allam *et al.* 2011), lung infections (Viscardi *et al.* 2002) and brain inflammation (Normann *et al.* 2009) as well as infertility (Wang *et al.* 2010).

In contrast to other rodent and mouse models, the Egyptian spiny mouse (*Acomys cahirinus*) is a more suitable rodent model for studying chronic intrauterine infections and fetal outcomes following infection and preterm delivery. The spiny mouse is a relatively new animal model, but is proving to be a valuable model in the studies of fetal and placental growth. This is due to the relatively long gestation (38 – 40 days) and small litter size (1 – 5; usually 2 – 3 pups per pregnancy). Furthermore, the offspring are relatively active from the time of birth with well-developed organ systems; including the kidney, liver, brain and immune system and are highly comparable to that of the human infant at the time of birth (Dickinson *et al.* 2008). Spiny mice have been utilised as the animal model of choice in birth asphyxia treatment and fetal neurodevelopmental outcomes (Hutton *et al.* 2009; Fleiss *et al.* 2012), fetal hypoxia (Ireland *et al.* 2011), placental development (O'Connell

et al. 2011), as well as fetal responses to a range of hormones (O'Connell *et al.* 2011; Quinn *et al.* 2013).

2.12.2 Ovine models

Both acute and chronic durations of ureaplasma intraamniotic infection have been studied using the ovine model; as their gestational period is significantly longer than that of rats and mice (term in sheep = approximately 147 days). Preterm fetal sheep are also comparable in weight to human fetuses (Mitchell and Taggart 2009) and have well-developed organ systems, particularly the lung; and so this also provides the opportunity to study fetal outcomes following *in utero* infections.

Our research group has extensively utilised ovine models for both acute and chronic *Ureaplasma* spp. infections (Moss *et al.* 2005; Moss *et al.* 2008; Moss *et al.* 2009; Collins *et al.* 2010; Dando *et al.* 2010; Knox *et al.* 2010; Polglase *et al.* 2010; Dando *et al.* 2012; Robinson *et al.* 2013; Dando *et al.* 2014). These studies have provided significant evidence that *in utero Ureaplasma* spp. infections are associated with increased surfactant production and lung maturation (Moss *et al.* 2005; Moss *et al.* 2008; Polglase *et al.* 2010), histological chorioamnionitis (Knox *et al.* 2010) and infection and resultant inflammation caused by ureaplasmas within the fetal lung (Knox *et al.* 2010; Dando *et al.* 2012; Robinson *et al.* 2013), fetal gut (Wolfs *et al.* 2013) and fetal cerebrospinal fluid (Knox *et al.* 2010). However, there are several disadvantages in the use of an ovine model of intrauterine infection. Ovine parturitions are dependent on progesterone withdrawal, which occurs due to fetal adrenocorticotropic hormones and cortisol production (Mitchell and Taggart 2009). The production of cortisol stimulates the maturation of fetal lungs, signalling fetal tissues to produce estrogen and initiates labour (Mitchell and Taggart 2009). Other disadvantages of this model are that intrauterine inflammation does not trigger the induction of labour, nor does the production of inflammatory cytokines in the amniotic fluid and fetal tissues trigger preterm delivery. However, an advantage of this model is that long-term, chronic intraamniotic infections can be studied since preterm delivery is independent of inflammation. Furthermore, the placental anatomy of sheep is very different to that of humans. Sheep have a highly layered epithelial-chordial cotyledonary placenta, very different to the hemomonochodial villous, discoid placentae of humans (Enders and Carter 2004). The layered nature of the ovine placenta means that maternal antibodies and importantly antibiotics cannot effectively traverse the placenta to reach the amniotic fluid. This means that results relating to the maternal and fetal immune responses to UGT ureaplasma infections are not directly translatable to UGT infections within humans.

2.12.3 Non-human primate models

Pregnancies in non-human primates are the most similar to human pregnancies. The gestational length in non-human primates (term = 160 days) is longer than in the sheep model (term = 145 days) and is more comparable to that of human pregnancies (term = 266 days). The placental anatomy is very similar, with both humans and non-human primates having hemomonochordial villous, discoid placentae. Another major advantage of using non-human primates is that their labour and delivery are not dependent on progesterone withdrawal (as discussed above), and so this makes the non-human primate an ideal model for studying intrauterine infection and preterm birth as a consequence of intrauterine infection/inflammation.

Novy *et al.* (2009) utilised the non-human primate model (Rhesus macaques) for the study of *Ureaplasma* spp. intrauterine infection. Rhesus monkeys were infected with *U. parvum* serovar 1 clinical isolate (n = 5; originally isolated from the placenta of a women who had chorioamnionitis and delivered an infant with ureaplasma sepsis) or with sterile media (n = 3). Intraamniotic injection of 10^7 CFU of *U. parvum* resulted in the rapid proliferation of *Ureaplasma* spp. (to $14.8 \pm 6.6 \times 10^5$ CFU/mL) within the first 24 hours. Dramatic increases in cytokine concentrations of TNF-α, IL-1β, IL-6 and IL-8 were detected in amniotic fluid within the first 48 – 72 hours. There were also dramatic increases in IL-1 receptor agonist (RA) in *U. parvum* infected animals at 48 – 72 hours, which persisted until preterm delivery. Levels of prostaglandins and matrix metalloproteinases (MMPs) were both significantly increased ($p < 0.05$), with prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ and MMP-9 remaining significantly increased until delivery (however prostaglandin levels varied between animals preceding delivery). Infection with *U. parvum* also resulted in early acute chorioamnionitis or in animals with longer infection-to-delivery times, subacute inflammatory responses in both fetal membranes and decidua were evident.

Non-human primate fetuses exposed to intraamniotic *U. parvum* were also affected by infection, with 100% of fetal lungs and fetal membranes testing culture-positive for *Ureaplasma* spp.. Bronchoalveolar lavage fluid and tracheal aspirates were predominantly culture-positive for ureaplasmas and this correlated with acute histological changes in fetal lungs. In some cases, pneumonia was evident (characterised by alveolar neutrophils and macrophages accompanied by necrosis of the airways). Fetuses infected with *Ureaplasma* spp. for greater than ten days showed partial resolution of acute inflammation, however, proliferation of epithelial cells and increased collagen deposition gave the appearance of thickened alveolar walls (a key characteristic of BPD) (Jobe and Bancalari 2001). Furthermore, in some experiments the fetal blood and cerebrospinal fluid

were also positive for the presence of *Ureaplasma* spp. The Rhesus monkey model of intraamniotic ureaplasma infection produced similar outcomes to those seen in human pregnancies; the severity of inflammation was consistent with human chorioamnionitis and human fetal bronchopulmonary dysplasia in the lung. Therefore, this model may be best used in future investigations to model the efficacy of treatment of *Ureaplasma* spp. infections (Novy *et al.* 2009). Other studies have utilised baboons to study the respiratory outcomes after the fetus was exposed to intraamniotic *Ureaplasma* spp. infection. Fetal lungs demonstrated extensive fibrosis and significant proinflammatory cytokine responses (Viscardi *et al.* 2006). Taken together, these studies confirm that non-human primate models of intraamniotic ureaplasma infections produce pregnancy and neonatal complications that are similar to those reported in humans' pregnancies. However, there are strict ethical considerations in the use of human primates, and it has become increasingly difficult to obtain ethical clearance for these studies. This animal model is also very expensive to maintain and so fewer animals are tested in each cohort, reducing the scientific power gained from these experiments. This means that this animal model is not used routinely for the study of *Ureaplasma* spp., infections and is not available within Australia.

An alternative is research that utilises human-derived cells and tissues. The ultimate goal of most research is to accurately model and reflect the mechanisms and consequences of intraamniotic infection, which is not always possible within a single animal model. This can be simplified by the use of human cells and tissues.

2.12.4 Human cell lines

Cell lines derived from humans are frequently used for *in vitro* studies of cell-microbe interactions. A number of female reproductive tract cell lines and tissues have been used (either alone or in co-culture systems) to model both healthy/normal interactions as well as disease states. Hormonal regulation of apoptosis by estrogen and progesterone has been studied using Ishikawa endometrial adenocarcinoma cell lines (Song *et al.* 2002), whilst another endometrial adenocarcinoma cell line, Hec1B, has been utilised for the study of invasive *Neisseria gonorrhoeae* infections (Shaw and Falkow 1988). Human placental (trophoblast) carcinoma cells (BeWo and JEG-3) have been used to model *Toxoplasma gondii* (Castro *et al.* 2013), hepatitis B virus (HBV) (Bai *et al.* 2013) and human immunodeficiency virus (HIV) 1 (Phillips and Tan 1992; David *et al.* 1995) infections to better understand invasion by these pathogens during pregnancy. *Ureaplasma* spp. have also been studied using cell culture systems, to investigate ureaplasma attachment to human cervical adenocarcinoma (HeLa) cells (Smith *et al.* 1994; Monecke *et al.* 2003). Human-derived cell lines offer a uniform

cellular population that originates from a well-defined tissue type, however, as with any model there are some disadvantages associated with the use of cell lines. Most cell lines used in research have undergone significant mutations to become immortalised, which can alter their cell biology and cellular processes. This means that some of the responses seen *in vitro* may not truly reflect the cellular responses seen *in vivo*. To circumvent these problems, primary cells obtained from human tissues have also been studied. Padmini *et al.* (2011) isolated placental endothelial cells from women with or without preeclampsia and these were exposed to *U. parvum ex vivo* to further understand stress responses to microbial stimuli in 'healthy' and 'non-healthy' pregnant women. Primary placental endothelial cells from both cohorts responded similarly to stimulation with *U. parvum*: changes in cellular iron, magnesium and calcium content were demonstrated, as well as increased apoptotic changes of the cells. While the responses seen in primary cell culture more closely mimics the responses seen *in vivo*, these experiments tested only a single cell type, which also limited study of the cell-to-cell signalling to a single cell type. This is different to the diverse cellular populations present *in vivo* and so therefore the immune responses seen may still not truly reflect what occurs during pregnancy.

Human primary tissues may also be used to study the interactions of bacteria and human tissues. Again, whilst there are strict ethical and clinical considerations, which regulate the use of human-derived samples, from a practical viewpoint, fetal tissues (such as placentae and umbilical cord) are easily obtained following delivery as these tissues are normally discarded as medical waste and are not often utilised for any other purpose. Primary tissue explants of the chorioamnion (also commonly referred to as the fetal membranes) have been used as the membranes are the major physical and immunological barrier of the amniotic sac, protecting the fetus against infection and insults during gestation. The chorioamnion contains a variety of cell types and the tissue integrity and 3-dimesional structure of tissue explants can be maintained *ex vivo* and utilised to study the host tissue immune response after exposure to microorganisms.

2.12.5 *Ex vivo* models

Abrahams *et al.* (2013) exposed chorioamnion explants to heat-killed *M. hominis ex vivo* and demonstrated increased mRNA expression of TLR 4, TLR 6 and TLR 8 and similarly *U. parvum* increased the mRNA expression of TLR 8. However, chorioamnion explants exposed to *U. urealyticum* induced no significant changes in mRNA expression of any TLRs. Menon *et al.* (2009) measured the cytokine and prostaglandin responses after chorioamnion tissue was exposed to heat-killed microorganisms; *E. coli* stimulated the production of TNF-α and PGE_2, whilst exposure to *U.*

parvum resulted in significantly elevated TNF-α and IL-10 concentrations. The greatest increases in IL-10 levels were detected after chorioamnion tissue was exposed to *U. parvum*; however, there were no detectable changes in prostaglandin levels when chorioamnion tissue was exposed to *U. parvum*.

To further understand the complexities of intraamniotic infection, this group also examined cytokine responses in tissues collected from women of different ethnicities. Chorioamnion explants were collected from Caucasian and African-American women and then incubated with heat-killed pathogens in the presence or absence of autologous amniotic fluid. *U. parvum*, *M. hominis* and *U. urealyticum* stimulated TNF-α and IL-10 production by the explants. Interestingly, the addition of autologous amniotic fluid affected the immune responses of both ethnicities in a race- and pathogen-dependent manner. Chorioamnion from African-American women exposed to *U. parvum* elaborated significantly higher TNF-α levels, compared to levels produced by tissues from Caucasian women. By contrast, tissues from Caucasian women when exposed to *U. parvum* secreted higher IL-10 levels ($p = 0.031$) than the levels detected in tissues of African-American women ($p = 0.630$). The authors concluded that the host response to infection of the chorioamnion is complex and cannot be generalised, as evidenced by the significantly different racial differences and altered responses to the individual pathogens (Peltier *et al.* 2012).

Whilst the results of these studies are intriguing, there are some experimental parameters that should be discussed. The use of heat-killed microorganisms may affect the results, due to a number of factors. Killing of the organisms means that the pathogen is no longer able to replicate and cannot produce biofilms, which may be a key to the pathogen's successful invasion and establishment of an infection during pregnancy. Furthermore, the action of heat-killing may result in cell lysis and destruction of their surface receptors, such as the MBA lipoprotein of *Ureaplasma* spp. The lack of cell surface receptors may significantly augment TLR activation and subsequent immune responses. Therefore, the results of these experiments should be interpreted with caution as they may not truly reflect the immune responses to live *Ureaplasma* spp. (or other microorganisms) infections. Other limitations of these explant-based studies result from a lack of tissue structure and difficulties in distinguishing the specific cellular responses of the amnion and the chorion cells which comprise the chorioamnion.

It is known that there are differences in cellular immune response of the amnion and chorion, so to further understand these differences, Keelan *et al.* (2009) developed and validated an *ex vivo*

chorioamnion tissue model to investigate an NF-kB pharmacological inhibitor in response to *E. coli* LPS. This model utilised an Ussing chamber, traditionally used to study permeability and secretory functions of the gut mucosa (Heyman *et al.* 1988; Foitzik *et al.* 1997; Hotz *et al.* 1998). The model produced by Keelan *et al.* contained two distinct compartments, separated by a barrier, the chorioamnion tissue itself. Each compartment was separately perfused with cell culture media that bathed the amnion and the chorion tissue. The 'maternal' (chorion) compartment was exposed to LPS over a 20 hour period. Aliquots of perfusing media were collected at different time-points and these samples were subsequently tested for cytokines, chemokines and prostaglandin levels in each chamber over the course of each experiment. The *ex vivo* Ussing chamber system used was found to be an accurate and valid method for studying immune responses to LPS, which approximated the conditions seen during pregnancy. Exposure of the decidual ('maternal') portion of the membranes to LPS resulted in increased accumulation of proinflammatory cytokines and chemokines, followed by an inflammatory response within the amniotic ('fetal') portion of the membranes. Most notably, increases in the concentrations of proinflammatory cytokines/chemokines macrophage-derived chemokine (MDC), TNF-α and PGE_2 were seen, along with increases in the anti-inflammatory cytokine transforming growth factor (TGF)-β were increased in both the 'maternal' and 'fetal' compartments following LPS exposure. Co-treatment with the NF-kB inhibitor resulted in an inability of nuclear translocation of NF-kB p65 and a subsequent inhibition of cytokine and chemokine production within the 'maternal' compartment. Similarly, cytokine and chemokine production was reduced within the fetal compartment, but to a much lesser extent. Whilst these results were promising, there was also a significant increase in the rate of apoptosis within the chorion, which the authors highlighted as a major concern regarding its effect on the placenta, if the treatment were to be administered during pregnancy (Keelan *et al.* 2009). However, these studies highlight the usefulness of human tissues, and in some cases human primary cells to better understand the variability observed in humans with intraamniotic *Ureaplasma* spp. infections. This *ex vivo* model may be further utilised to advance our current understanding of human placental host-microbe interactions occurring during ureaplasma infections.

2.13 Summary

This literature review has highlighted the need for further research, in order to better characterise the role of *Ureaplasma* spp. UGT infections during gestation. The results chapters (Chapters 4, 5 and 6), which follow this review of literature report the findings of studies which investigate: the prevalence of *Ureaplasma* spp. chorioamnion infection in human pregnancies and the effect on

maternal and fetal outcomes; ureaplasma MBA variation in clinical isolates obtained from human chorioamnion tissue and the cord blood immune responses; and the optimisation and use of an *ex vivo* chorioamnion tissue model to investigate the host immune responses at the maternal and fetal interface, in response to *Ureaplasma* spp. exposure. This PhD project will address major knowledge gaps that have been identified within the area of research concerning *Ureaplasma* spp. infections during pregnancy and is the first major study to focus on ureaplasma infections within the chorioamnion (rather than the amniotic fluid) to investigate the role of ureaplasma infections on the mother and fetus. Within this thesis, the host immune response to *Ureaplasma* spp. infections in human cord blood and also within an *ex vivo* chorioamnion model of infection will be investigated to further our knowledge and understanding of host immune responses to infections caused by ureaplasmas.

Chapter Three:

Materials and Methods

3.1 Experimental Design

This thesis presents the results of a large prospective microbiological study of placentae obtained from 477 women within the United States of America (USA); and a second, smaller pilot study which examined the *ex vivo* immune responses of placentae from four women in Brisbane, Australia.

For the microbiological component of this research, organisms were identified within placental tissue by traditional culture and molecular microbiological techniques (results discussed in Chapter Four) and the most prevalent microorganisms, the *Ureaplasma* spp., were characterised (results discussed in Chapter Five). A single clinical *Ureaplasma parvum* isolate cultured from the placenta of a woman enrolled in the study was used as an infectious agonist to challenge the maternal surface of human chorioamnion tissue immune response *ex vivo*. This pilot study is the final aim of this PhD study (results discussed in Chapter Six). All methods and materials pertaining to each of these research chapters are presented in this chapter.

3.2 Microbiology study population and specimen collection

From July 2010 to July 2013, women giving birth at the Good Samaritan Hospital (Ohio; United States of America) were recruited and enrolled in this study. Women who smoked during pregnancy and those with intrauterine growth restriction were excluded from this study. This study was approved by the ethics committees of the Good Samaritan Hospital (Ohio; USA) and Cincinnati Children's Hospital Medical Centre (CCHMC; Ohio, USA) and was reviewed by the human research ethics committee (HREC) of Queensland University of Technology (QUT; 2009001885) and deemed exempt from the need for university HREC review. Written informed consent was obtained from all women enrolled in this study. Placentae were collected and de-identified, with the demographic and outcome data from each pregnancy entered into the research electronic capture (REDCaP) database.

Upon delivery, placentae were placed into sterile containers and transported to a procedure room for sampling under strict aseptic conditions within 24 hours of delivery. The external placental surface was decontaminated using 70% alcohol and areas in which the amnion had detached from the placenta were avoided for sampling, to minimise contamination of the membranes by vaginal microflora. Using sterile surgical implements, an incision was made into the amnion (fetal) membrane, until the interface of the chorion and amnion membranes was reached and identified. The amnion membrane was then lifted and chorioamnion specimens were excised from the placenta and placed into sterile cryogenic vials and then snap frozen. The exposed membrane interface was

then swabbed and swabs were placed into BBL-port-a-cul media (for isolation of bacteria and yeast; Becton Dickenson and Company, Maryland, USA) and into universal viral transport media (for isolation of ureaplasmas/mycoplasmas; Becton Dickenson and Company). All samples were stored at -80 °C and shipped on dry ice to the Institute of Health and Biomedical Innovation (IHBI), Queensland University of Technology (QUT), Queensland, Australia. Shipments were sent on a regular basis with 30 – 40 patient samples sets per shipment.

3.3 Primary isolation media for culture of microorganisms

Columbia horse blood agar and chocolate agar pre-prepared plates were purchased from Thermo Fisher Scientific (Thebarton, Adelaide; South Australia). Schaedler anaerobe agar, MacConkey no. 1 agar, deMan Rogosa Sharpe agar, Sabauraud's dextrose agar and Brewer's thioglycollate medium were purchased from Thermo Fisher as dehydrated powders, were prepared according to the manufacturer's instructions then poured into sterile Petri dishes. Thioglycollate broth was aliquotted into McCartney bottles and then autoclaved at 121 °C for 15 minutes.

10B broth and A8 agar was used for cultivation of *Ureaplasma* spp. and these were prepared according to previously published protocols (Shepard and Lunceford 1976). The basal medium of both the broth and agar were autoclaved at 121 °C for 15 minutes. Sterile medium supplements were then added after the medium had cooled to < 56 °C. The complete medium was then dispensed into sterile single-use culture tubes or Petri dishes (Techno Plas, St Marys; South Australia).

3.4 *Ureaplasma* spp. strains

U. parvum and *U. urealyticum* strains were purchased from the American Type Culture Collection (ATCC, Virginia, USA), except for *U. urealyticum* serovar 8, which is a reference/progenitor strain of the ATCC strains (kindly provided by H. Watson; University of Sydney, Australia). All strains were cultured in 10B broth (Shepard and Lunceford 1976) and stored at -80 °C. *Ureaplasma* spp. strains have been summarised in Table 3.1.

3.5 Bacterial strains

Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus agalactiae, Listeria monocytogenes, Haemophilus influenzae, Klebsiella pneumoniae and *Pseudomonas aeruginosa* were obtained from the QUT culture collection (see Table 3.1). These microorganisms were inoculated onto Columbia horse blood agar (Thermo Fisher Scientific) overnight, then three to five well-isolated colonies were aseptically collected and used to inoculate heat-inactivated horse serum supplemented with 20 % v/v glycerol prior to storage. DNA was extracted from each of these microorganisms and these served as controls for 16S ribosomal (r) RNA and multiple banded antigen (*mba*) PCR assays.

Table 3.1. Microorganisms utilised within the PhD program of study.

Organism	Source
***Ureaplasma parvum* serovar 1**	ATCC 27813
***Ureaplasma parvum* serovar 3**	ATCC 27815
***Ureaplasma parvum* serovar 6**	ATCC 27818
***Ureaplasma parvum* serovar 14**	ATCC 33697
***Ureaplasma urealyticum* serovar 2**	ATCC 27814
***Ureaplasma urealyticum* serovar 4**	ATCC 27816
***Ureaplasma urealyticum* serovar 5**	ATCC 27817
***Ureaplasma urealyticum* serovar 7**	ATCC 27819
***Ureaplasma urealyticum* serovar 8**	Reference strain
***Ureaplasma urealyticum* serovar 9**	ATCC 33175
***Ureaplasma urealyticum* serovar 10**	ATCC 33699
***Ureaplasma urealyticum* serovar 11**	ATCC 33695
***Ureaplasma urealyticum* serovar 12**	ATCC 33696
***Ureaplasma urealyticum* serovar 13**	ATCC 33698
Staphylococcus aureus	QUT culture collection
Staphylococcus epidermidis	QUT culture collection
Streptococcus pyogenes	QUT culture collection
Streptococcus agalactiae	QUT culture collection
***Streptococcus pneumoniae* type II**	QUT culture collection
Listeria monocytogenes	QUT culture collection
Haemophilus influenzae	QUT culture collection
Klebsiella pneumoniae	QUT culture collection
Pseudomonas aeruginosa	QUT culture collection

3.6 Propagation and quantification of *Ureaplasma* spp.

3.6.1 ATCC and Reference strain propagation

Ureaplasma spp. stocks stored at -80 °C were thawed on ice prior to use. One volume of the ureaplasma ATCC/reference stock was added to 9 volumes of 10B broth and these were then incubated at 37 °C; under O_2 until log-phase growth was achieved (indicated by the phenol red pH colour change).

3.6.2 Quantification of *Ureaplasma spp.*

To determine the number of *Ureaplasma* spp. colony forming units (CFU) within a given sample, serial dilutions (doubling or ten-fold) were carried out in 10B broth. A total of four dilutions for each sample were carried out and from each broth dilution, six 5 µL drops were then subcultured onto A8 agar plates. A8 agar were then incubated at 37 °C, under 5% CO_2 for a minimum of three days before examining microscopically using a stereomicroscope (Leica Microsystems; North Ryde, New South Wales). The number of ureaplasma colonies per drop of 10B broth were then counted, averaged and then calculations were performed to determine the total number of ureaplasma colony forming units (CFU) per swab or per gram of tissue.

10B broth dilutions were also incubated at 37 °C, under O_2 and checked daily for signs of log-phase ureaplasma growth.

3.7 Culture of microorganisms from clinical samples

All tissue and swab specimens were thawed on ice prior to culture.

3.7.1 Ureaplasma swab culture protocol

Swabs stored in universal viral transport media were inoculated into 10B broth and then serially diluted in four two-fold dilutions. The number of *Ureaplasma* spp. CFU was determined as detailed above (section 3.6.2). For specimens that were blood-stained, the inoculated broths were incubated

for 24 hours and subcultured daily for up to 5 days to ensure any ureaplasma growth was detected. Broths and plates were incubated at 37 °C, O_2 for up to 14 days.

3.7.2 Bacterial swab culture protocol

Swabs stored in BBL port-a-cul media were inoculated into thioglycollate broth and onto a range of microbiological media, including: Horse blood agar, Chocolate Agar with Isovitalex, Schaedler anaerobe agar, Sabauraud's Dextrose agar, deMan Rogosa Sharpe agar and MacConkey no. 1 agar, using a 16-streak technique. Media were incubated either aerobically, under 5% CO_2 or anaerobically in jars (Oxoid anaerogen gas pack, 2.5L; Oxoid) as appropriate. Agar plates were checked daily for signs of growth for up to 5 days. Thioglycollate broths were checked daily for signs of growth for up to 14 days. Any positive thioglycollate broths (as evidenced by turbidity) were subcultured onto media (as above) for the identification of microorganisms growing within the broth.

The number of bacterial CFU was measured semi-quantitatively after 24 hours by counting the number of colonies on the plate and multiplying by a factor of 10^2 (for a 10 µL inoculation loop) to give the number of CFU/mL of each organism present (see table 3.2; quantitative analysis was only possible for organisms that grew on primary isolation media after the direct subculture of the specimens and not for those isolates that were first enriched and then subcultured from thioglycollate broths). Each colony type was Gram-stained and a stock of the organism was stored at -80 °C for subsequent DNA extraction, 16S rRNA PCR and sequencing to identify the genus/species of each organism present.

Table 3.2. Semi-quantitative method for determining the number of bacterial colony forming units (CFU/mL) from swabs and tissue specimens.

Total amount of growth	Number of colonies	Approximate CFU/mL
0+ (primary inoculum only)	1	10^3
1+	1 – 10	10^4
2+	10 – 100	10^5
3+	100 – 1000	10^6
4+	> 1000	> 10^6

3.7.3 Chorioamnion tissue culture protocol

Tissue specimens were weighed and homogenised in sterile cryogenic vials containing 1 mL of sterile phosphate-buffered saline (PBS) and sterile glass beads using the Mini Beadbeater-16 cell disruptor (Daintree Scientific; Tasmania, Australia). Tissues were homogenised in one minute cycles for up to four minutes (average: three minutes) and placed on ice between each homogenisation step. This was performed to ensure that the samples did not overheat and affect the viability of any microorganisms present.

Tissue homogenates were inoculated into 10B broth and doubling or ten-fold serial dilutions were carried out. The *Ureaplasma* spp. were quantified as previously described (see section 3.6.2). For specimens that were blood-stained, the inoculated 10B broths were incubated for 24 hours and then subcultured daily for up to 5 days to distinguish the ureaplasma growth.

Tissue homogenates were also inoculated into thioglycollate broth and onto a range of bacterial media (as described above) and incubated either aerobically, under 5% $C0_2$ or anaerobically in jars. Agar plates were checked daily for up to 5 days and thioglycollate broths were checked daily for signs of growth for up to 14 days.

Each morphologically distinct colony type isolated was Gram-stained and a stock of each microorganism was stored at -80 °C for subsequent DNA extraction, 16S rRNA PCR and sequencing to identify the genus/species of each clinical isolate.

3.8 DNA extraction

DNA was extracted from all specimens using the QIAamp mini DNA extraction kit (Qiagen) according to the manufacturer's instructions. For DNA extractions a Proteinase K solution (100 µg/mL; Sigma-Aldrich) was used instead of the Proteinase K provided with the DNA extraction kit. All DNA samples were eluted in sterile DNAse/RNAse-free dH_20. Extracted DNA was stored at -20 °C until required.

3.8.1 *Ureaplasma* spp. ATCC and clinical isolate cultures

For the extraction of DNA from *Ureaplasma* spp., each *Ureaplasma* spp. ATCC and clinical isolate was culture in 10 mL of 10B broth until exponential growth was achieved. Broths were then centrifuged

at 5000 x g for 30 minutes at 4 °C. The supernatant was aspirated and DNA was extracted from the pellet using the manufacturer's instructions for 'tissues'.

Prior to extraction, the specimen was incubated with a Proteinase K solution (100 µg/mL; Sigma Aldrich) at 56 °C for a minimum of 1 hour. DNA was then eluted in 400 µL of sterile DNAse/RNAse-free dH_20 (Gibco) in two elutions and then stored at -20 °C until testing.

3.8.2 Clinical isolates and ATCC strains of other cultured microorganisms

For the extraction of DNA from microorganisms cultured from tissue and swabs, three to five well-isolated bacterial colonies (of the same colonial morphology) were aseptically transferred from the agar plate to a tube containing 500 µL of sterile PBS. The bacterial suspension was then centrifuged at 5000 x g for 5 minutes at room temperature. The supernatant was aseptically removed and the pellet retained for DNA extraction using the manufacturer's instructions for 'tissues'.

3.8.3 Placental tissue

Tissue homogenates (approximately 500 µL) from each patient were incubated at 56 °C with tissue lysis buffer (provided with the commercial DNA extraction kit) and 100 µL of 100 µg/mL Proteinase K solution overnight, or until the tissue had completely degraded. DNA was extracted as per the manufacturer's protocol for 'tissues'.

3.9 Conventional polymerase chain reaction (PCR) assays for the identification of microorganisms and *Ureaplasma* spp.

All tissue homogenates and cultured microorganisms were tested by conventional PCR assays using the PTC-2000 Thermal Cycler (BioRad; Gladesville, New South Wales).

3.9.1 16S rRNA polymerase chain reaction (PCR) assays

PCR primers targeting a variable region of the 16S rRNA gene were designed by using the Primer3 primer design program in conjunction with Geneious gene alignment software (Biomatters Ltd). Each 16S rRNA PCR reaction, in a total volume of 20 µL, contained 4 µL of extracted DNA, 100 µM dNTPs (Roche), 1 X PCR buffer (Tris HCl, KCl, $(NH_4)_2SO_4$, pH 8.7; Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 0.5

µM of both forward and reverse primers (Bact_RNA1F and Bact_RNA1R – see Table 3.3; Sigma-Aldrich), 2.5 U Platinum *taq* polymerase (Invitrogen) and sterile DNAse/RNAse free dH_20 (Gibco). PCR cycling consisted of initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of 94 °C for 30 seconds, primer annealing at 60 °C for 30 seconds and extension at 72 °C for 30 seconds.

DNA extracted from pure cultures of Gram positive and Gram negative organisms served as the template for positive control PCR reactions (see Table 3.1). Negative controls included replicates of master mix only and water substituted for template in order to identify contamination.

3.9.2 *mba* polymerase chain reaction (PCR) assays

Ureaplasma spp., within tissue homogenates and cultured clinical isolates, were detected and speciated using a conventional PCR assay targeting the *mba* gene. Optimised PCR assays were carried out in a total volume of 20 µL and consisted of 4 µL of extracted DNA, 100 µM dNTP mix (Roche), 1 X PCR buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.5 µM of both forward and reverse primers (UMS-125 and UMA-226 – see Table 3.3; Sigma-Aldrich), 2.5 U of Platinum *taq* DNA polymerase (Invitrogen) and sterile DNAse/RNAse-free dH_20 (Gibco). PCR cycling involved initial denaturation at 95 °C for 5 mins; followed by 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 55 °C for 30 seconds and extension at 72 °C for 30 seconds.

Positive PCR control reactions included DNA extracted from pure cultures of ATCC *U. parvum* and *U. urealyticum* serovars (See Table 3.1). *U. parvum* isolates produced a PCR product of ~404 bp, while *U. urealyticum* serovars produced a PCR product of ~448 bp. Negative controls included replicates of master mix only and reaction mixtures in which DNAse/RNAse-free dH_20 was substituted for template.

Table 3.3. PCR primers used for the 16S rRNA and *mba* conventional PCR assays

Primer Name	Sequence[1]	Tm	Source
Bact_RNA1 F	5' CYGGYAGYCCACGCCGYAAA 3'	60 °C	novel
Bact_RNA1 R	5' ACAYCYCACGACACGAGCYG 3'	60 °C	novel
UMS-125 (F)	GTATTTGCAATCTTTATATGTTTTCG	55 °C	(Teng *et al.* 1994)
UMA-226 (R)	CAGCTGATGTAAGTGCAGCATTAAATTC	55 °C	(Teng *et al.* 1994)

[1] Y – combination of C/T included into the primer sequence

3.10 Agarose gel electrophoresis

PCR amplicons were separated using a 2% agarose and Tris Borate-EDTA (TBE) gel containing ethidium bromide (final concentration 1 µg/mL). Products were electrophoresed at 100 V for 60 minutes and then visualised using the Grab-It Gel Dock (Ultraviolet Products, Ltd., Cambridge, United Kingdom).

3.11 Purification of PCR products

PCR products were purified using the PureLink PCR purification kit (Invitrogen) according to the manufacturer's instructions, with a final elution in sterile DNAse/RNAse-free dH_20 (Gibco).

3.12 Sequencing of PCR amplicons

Purified PCR amplicons were then labelled with the BigDye Terminator (BDT) v3.1 cycle sequencing kit (Thermo Fisher Scientific; Scoresby, VIC) and both forward and reverse direction sequencing reactions were carried out for each PCR amplicon. In order to incorporate the BDT dye within the PCR amplicon, a BDT-labelling PCR assay was performed in a 20 µL reaction volume, which contained 10 µL of purified PCR amplicon, 1 X BDT sequencing buffer, 1 µL of BDT ready reaction mix, 0.3 µM of the forward or reverse primer (16S rRNA or *mba* PCR primers – see Table 3.3; Sigma-Aldrich) and DNAse/RNAse-free dH_20 (Gibco). PCR cycling included initial denaturation at 96 °C for 1 minute; followed by 35 cycles of denaturation at 96 °C for 10 seconds, primer annealing at 50 °C for 5 seconds and extension at 60 °C for 4 minutes. BDT-labelled amplicons were purified using EDTA (125 mM, pH 8.0), sodium acetate (3 M) and 100% ethanol. BDT-labelled amplicons were centrifuged at 5000 x g for 20 minutes and the supernatant removed. Amplicons were then washed with 80% ethanol, centrifuged, and the tubes dried at 50 °C for 1 hour. Products were then sequenced at the Molecular Genetics Research Facility, QUT using the Ion personal genome machine (PGM) molecular sequencer (Life Technologies).

Once generated, the forward and reverse sequence data were imported, manipulated and analysed using the Geneious bioinformatics software (Biomatters Ltd.). Forward and reverse sequences were trimmed and the sequence identity of each PCR amplicon was obtained using the basic local alignment search tool (BLAST; National Centre for Biotechnology Information [NCBI]).

3.13 Western blotting of *Ureaplasma* spp. multiple banded antigen (MBA) proteins

All ureaplasma clinical isolates and ATCC strains were cultured in 10 mL of 10B broth until the late log-phase and then centrifuged at 5000 x g for 20 minutes at 4 °C. The supernatant was discarded and the pellet was resuspended in 100 µL of sterile PBS. The protein concentration was determined using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and proteins were stored at -20 °C prior to use.

Protein from each clinical isolate (30 µg) was added to SDS-PAGE loading buffer (Tris HCl, pH 6.8, 50% glycerol, 8% w/v SDS, bromophenol blue, 1 M dithiothreitol [DTT]) and incubated at 95 °C for 5 minutes then electrophoresed in a 10% SDS-PAGE gel at 150 V for 1 hour. Proteins were then transferred from the SDS-PAGE gel to a nitrocellulose membrane (Pall Corporation; Cheltenham, Victoria) in transfer solution (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, dH_20) at 90 V for 1 hour. After protein transfer, the membrane was blocked to prevent non-specific binding using a skim milk solution (5% skim milk, 150 mM NaCl, 50 mM Tris) for 1 hour, prior to an overnight incubation with the primary antibody at 4 °C (serovar-specific anti-ureaplasma rabbit antisera kindly provided by Emeritus Dr Patricia Quinn (The Hospital for Sick Children, Toronto). The primary antibodies used were raised against whole ureaplasmas and we have shown previously that the protein band(s) demonstrated by western blot correspond directly to the amplicons generated by *mba* PCR assays (Knox *et al.* 2010). Concentrations for each primary and secondary antisera are summarised in Table 3.4. After incubation, the membranes were washed and incubated with a goat anti-rabbit IgG secondary antibody conjugated with horse radish peroxidase (Sigma Aldrich) for 1 hour with gentle agitation. Membranes were again washed and the MBA protein visualised using 3', 3'-diaminobenzidine tetrahydrochloride (DAB) with cobalt chloride enhancer (Sigma Aldrich). Images were then captured (CanoScan 8600F). For each western blot, proteins obtained from ATCC and reference *Ureaplasma* spp. serovars served as positive controls. Sterile 10B medium was treated in the same manner as the ATCC ureaplasmas and clinical isolate cultures and this served as a negative control, to confirm the antibody did not react with components within the 10B medium.

Table 3.4. Primary and secondary antibody concentrations used for western blot analysis of the *Ureaplasma* spp. MBA protein

Ureaplasma serovars	Primary antibody concentration [a]	Secondary antibody concentration [b]
U. parvum		
1	1/100	1/1000
3	1/10000	1/5000
6	1/2500	1/5000
14	1/20	1/1000
U. urealyticum		
2	1/20	1/1000
4	1/500	1/5000
5	1/20	1/1000
7	1/20	1/1000
8	1/20	1/1000
9	1/20	1/1000
10	1/20	1/1000
11	1/20	1/1000
12	1/20	1/1000
13	1/20	1/1000

[a] Serovar-specific anti-ureaplasma rabbit antisera kindly provided by Emeritus Dr Patricia Quinn, The Hospital for Sick Children, Toronto

[b] Goat anti-rabbit IgG conjugated to horse radish peroxidase was commercially sourced from Sigma-Aldrich

3.14 Polymerase chain reaction (PCR) targeting the downstream repetitive region of the *mba*

The downstream repetitive region of *U. parvum* clinical isolates was targeted using previously published PCR assays (Knox *et al.* 2010; Robinson *et al.* 2013). These primers amplified *U. parvum* serovars 1 and 6; or serovars 3 and 14, by binding to regions surrounding the repetitive region of the *mba* gene and revealed *mba* size variants that resulted due to variation in the number of tandem repeating units.

These PCR reactions were carried out in a 50 µL volume and consisted of: 8 µL DNA template, 100 µM dNTPs (Roche Diagnostics), 1 x PCR buffer (Tris HCl, KCl, $(NH_4)_2S0_4$, pH 8.7 – Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.5 µM of forward (MPDF3; see Table 3.5) and reverse primers (3DR3 or 6DR4; Sigma-Aldrich – see Table 3.5), 2.5 U Platinum *taq* DNA polymerase (Invitrogen) and DNAse/RNAse-free dH_20 (Gibco). PCR cycling involved initial denaturation at 94 °C for 9 minutes; followed by 40 cycles of denaturation at 94 °C for 45 seconds, primer annealing at 54 °C for 75 seconds, extension at

72 °C for 2 minutes; with a final extension step at 72 °C for 15 minutes. Positive PCR controls included DNA extracted from ATCC strain serovars (Serovars 1, 3, 6 and 14). Negative controls included master mix only with DNAse/RNAse-free dH_20 substituted for template.

PCR amplicons were analysed by agarose gel electrophoresis (section 3.10). The *mba* amplicon profiles of *Ureaplasma* spp. clinical isolates were then compared to the *mba* amplicons produced by ATCC strains. *Ureaplasma* spp. clinical isolates, which produced *mba* amplicons of the same size as ATCC strain controls were designated as having 'no *mba* variation'; whilst clinical isolates which produced amplicons of a different size to that of ATCC strains were considered to be '*mba* variants'. In some cases multiple *mba* variants were amplified from the same clinical isolate. These isolates were further characterised as having a "single" or "multiple" unique *mba* size variants. These *mba* amplicon profiles were also correlated to the outcome of western blot analysis, to ensure that true MBA variation was occurring, at both the genetic and protein level.

Table 3.5. PCR primers targeting the downstream repetitive region of the *mba* gene

Primer Name	Sequence	Serovars amplified	Tm	Source
MPDF3	5' TAATCAAGACTTCAGGTTTG 3'	All *U. parvum* serovars	54 °C	(Knox *et al.* 2010)
3DR3	5' TCGCTTTTTTCATTACGAGTC 3'	Serovars 3 and 14	54 °C	(Knox *et al.* 2010)
6DR4	5' TAATGTAAATAAAGCACTTATTC 3'	Serovars 1 and 6	54 °C	(Knox *et al.* 2010)

3.15 Real-time polymerase chain reaction (PCR) for the speciation and serotyping of *U. parvum* clinical isolates

3.15.1 PCR Primer Design

Whole genome sequences for the 14 serovars belonging to *U. parvum* and *U. urealyticum* were downloaded from the GenBank sequence database (NCBI; http://www.ncbi.nlm.nih.gov/) and interrogated using the Geneious bioinformatics software (Biomatters Ltd). From each ureaplasma whole genome sequence, the *mba* gene and its surrounding intergenic (non-coding) regions were located. These sequences for the 14 ureaplasma serovars were then aligned and regions which contained high levels of homology (either within *U. parvum, U. urealyticum* or high homology to both *Ureaplasma* species) were identified. Single nucleotide polymorphisms (SNPs) that were unique to each individual ureaplasma serovar were also identified.

These regions were further scrutinised using the basic local alignment (BLAST) search tool (NCBI) to identify any homology with other bacterial species or with human chromosomal DNA. Primers were then designed to amplify regions (no greater than 400 base pairs);) with specific homology to only *U. parvum* species, areas which also contained SNPs for each of the *U. parvum* serovars (1, 3, 6 and 14).

Multiple primer sets were designed, purchased from Sigma-Aldrich and tested for their efficacy in real-time PCR assays for their ability to amplify only *U. parvum* serovars. Primers were further tested for their ability to differentiate *U. parvum* serovars 1, 3, 6 and 14 by high resolution melt analysis.

A number of PCR primer sets were designed to specifically amplify *U. parvum* serovars at different regions of the *mba*.

Table 3.6. Novel PCR primers targeting areas of the multiple banded antigen (*mba*) gene, designed for use in real-time PCR and high resolution melt assays

Name	Primer Sequence	Tm	Source
UpuF	5' CTAATAATGTTATTGATAATGCAG 3'	55 °C	novel
UpmbaR	5' GTTTTCAATTTCGTAAACTGC 3'	53 °C	novel
UpuF2	5' TTATAATAAAAAATATCTAATAATG 3'	50 °C	novel
UpmbaR2	5' CCAGCTCCAACTAAGGTAAC 3'	58 °C	novel
UpuF3	5' TTATATAATTAAAAGTGCAAGTGC 3'	55 °C	novel
UpmbaR3	5' TTGTTCATTAGGTTTTGGTTCACGA 3'	61 °C	novel
UpuF4	5' GTGCTAAATAAAAAGTATTTGC 3'	53 °C	novel
UpmbaR4	5' CCTGAAGTCTTGATTAATCCAC 3'	58 °C	novel
UpmbaR5	5' GTTTCAAAGTTCACTTTTTCTG 3'	55 °C	novel

Once primers were received, each PCR assay was optimised and validated in order to assess the ability of each PCR assay to selectively amplify *U. parvum* serovars 1, 3, 6 and 14.

3.16 Design of *U*. parvum real-time polymerase chain reactions

Conventional PCR assays were used to optimise PCR parameters for each primer set. Optimisation included identifying the ideal annealing temperature, annealing time, $MgCl_2$ concentration and primer concentrations. Once optimised parameters were obtained for each primer set, the ability of primers to specifically amplify *U. parvum* serovars was assessed. Primers which selectively amplified

U. parvum serovars 1, 3, 6 and 14, but not *U. urealyticum* or any other bacterial strain tested were utilised for further analysis.

3.17 Validation of *U. parvum* real-time PCR assays

The optimised real-time PCR assays were further assessed for their ability to differentiate *U. parvum* serovars 1, 3, 6 and 14 by real-time PCR and high resolution melt (HRM) analysis.

PCR assays were performed in a total volume of 20 µL and contained 5 µL of DNA (all samples were standardised to 5 µg), 100 µM dNTPs, 1 X PCR buffer, 1 X SYBR green dye, 2.5 U *taq* DNA polymerase and DNAse/RNAse-free dH_20 (Gibco). Assays also included optimised concentrations of forward and reverse PCR primers and $MgCl_2$. All real-time PCR assays were carried out in the Rotor-Gene 6000 real-time PCR cycler (Qiagen). Cycling conditions included 95 °C for 3 minutes to activate Platinum *taq* DNA polymerase; followed by 40 cycles of 95 °C for 15 seconds, optimised annealing temperatures for between 20 - 30 seconds, and extension at 72 °C for 20 seconds. Rotor-Gene Q Series Software (Version 1.7, Build 87; Qiagen) was programmed to acquire fluorescent signals with each extension step of cycling.

Real-time PCR cycling was followed by a standard melting program, which included continuous fluorescent monitoring between 65 °C - 85 °C, with temperature increasing at a rate of 1 °C/s. Standard melting profiles were assessed for the presence of a single fluorescent 'peak', which was consistent with the presence of only a single PCR amplicon. This was further assessed by electrophoresing real-time PCR products on agarose gels and imaging for the presence of a single PCR band/amplicon.

Following standard melting, a high resolution melt (HRM) assay was performed. This included continuous fluorescent monitoring between 70 °C - 85 °C, with temperatures increasing at a rate of 0.02 °C/s. Upon completion of all programs, data was viewed and manipulated using the Rotor-Gene Q Series Software.

3.18 Differentiation of *U. parvum* serovars using high resolution melt (HRM)

The Rotor-Gene Q series software enables HRM data to be viewed and manipulated as both normalised melting curves, or as difference plots. Normalised melting curves plot the negative

derivative of fluorescence over temperature and illustrate the amplicon melting temperatures and patterns (which may differ, depending on the presence of SNPs). Difference plots display a user-defined high resolution melting curve as the 'baseline' (x-axis), by which all other melting curves are plotted against. To differentiate *U. parvum* serovars 1, 3, 6 and 14, a user-defined 'baseline' was selected and all remaining serovars were compared to the baseline. Melt curves were considered to be the 'same' as the user-defined control (serovar) if the highest fluorescent peak was within ± 5 units (U). By contrast, melt curves were classified as 'different' from the user-defined control (serovar) if the highest fluorescent peak was greater than ± 5 U (Stephens 2008).

PCR primers which produced *U. parvum* serovar amplicons, which were unable to be differentiated using the criteria above were excluded from further analysis. Combinations of individual PCR primers were also assessed for their ability to differentiate *U. parvum* serovars 1, 3, 6 and 14.

3.19 Analysis of *U. parvum* clinical isolates by real-time PCR and HRM

Clinical specimens from several sources were used to assess the efficacy of the designed *U. parvum* real-time PCR and HRM assay. These consisted of 31 *U. parvum* clinical isolates obtained from the chorioamnion of women who delivered LPT or at term (see Chapter Four). Other *U. parvum* clinical isolates were obtained from previous studies, in which endocervical swabs, sperm and washed sperm samples were cultured for the presence of *Ureaplasma* spp. *U. parvum* isolates (characterised previously in (Knox *et al.* 2003) were cultured in 10B broth (as per section 3.6.1) and DNA extracted (as per section 3.8.1). The remaining clinical isolates used to assess the *U. parvum* real-time PCR and HRM assays were obtained from pure (uncultured) amniotic fluid specimens taken from sheep that were injected with *U. parvum* serovar 6. The presence and amount of *U. parvum* serovar 6 within the amniotic fluid has been previously published (Dando *et al.* 2012). From these amniotic fluid samples, 200 µL of uncultured sample was used for DNA extraction and subsequent PCR.

DNA from these clinical isolates was standardised to 5 µg/µL and used in optimised real-time PCR and HRM assays.

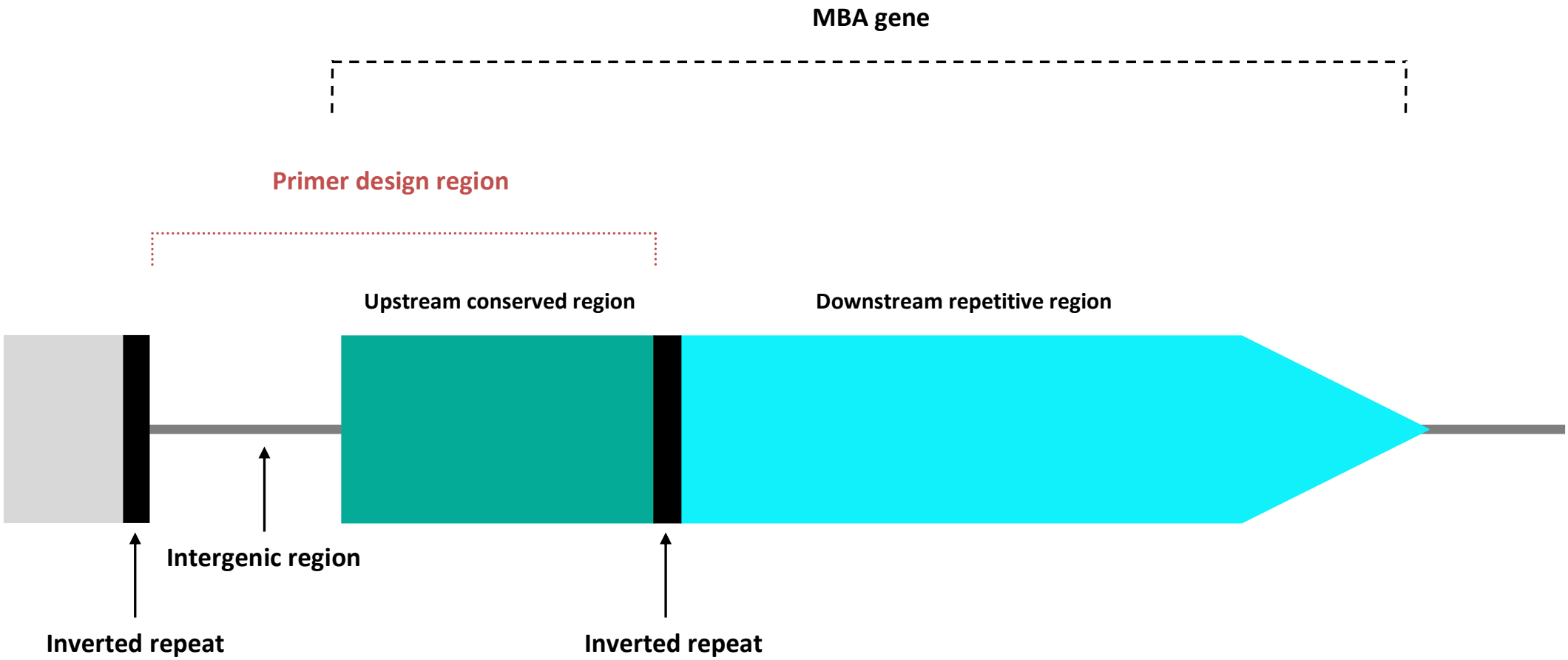


Fig 3.1. Diagram of the *mba* gene and its surroundings, including areas targeted for primer design. The *mba* gene consists of an upstream, highly conserved region (depicted in green) and a highly variable downstream region that is composed of tandem repeating units (depicted in blue). The *mba* locus also contains two inverted repeat regions, which are proposed sites of DNA inversion events. The upstream conserved regions of the *mba* gene, including some intergenic (non-coding; depicted in dark grey) areas were targeted for primer design.

3.20 *U. parvum* inoculum preparation for the *ex vivo* model

The ureaplasma strain selected for use in these experiments was isolated from the chorioamnion of a woman who delivered late preterm (at 34 weeks of completed gestation; Term = 40 weeks). Histological examination of the placenta demonstrated severe chorioamnionitis (Maternal grade 2; fetal grade 2; plate positive; using Redline *et al.* (2003) criteria). This clinical isolate, No. 429 was characterised as *U. parvum* serovar 6 – the ureaplasma serovar most frequently isolated in placentae delivered late preterm in our study population (discussed in Chapter Five).

The ureaplasma inoculum was prepared as previously described (Moss *et al.* 2008). Briefly, a low passage culture of clinical isolate 429 was thawed on ice, serially diluted in 10B broth and incubated until exponential growth was achieved. The ureaplasma cultures were then pooled and centrifuged at 14 000 x g for 1 hour at 4 °C (Beckman and Coulter, Gladesville NSW). The supernatant was then removed and the pellet was carefully resuspended in cold 10B broth (held on ice). The concentrated ureaplasmas were vortexed to create an homogenous suspension, before being aliquotted into sterile cryogenic vials (all held on ice) and stored immediately at -80 °C until required. One aliquot of concentrated ureaplasmas then was thawed and the number of ureaplasma CFU/mL within the prepared inoculum was determined as described above (section 3.6.2).

Sterile 10B broth (1 L) was also centrifuged and concentrated and prepared as above and this preparation served as the vehicle control inoculum.

Prior to use, the prepared ureaplasma and 10B broth inocula were thawed on ice. The ureaplasmas were diluted in sterile PBS to achieve an inoculum of 2×10^7 CFU. The prepared 10B broth control inoculum was similarly diluted in sterile PBS and both were retained on ice until use.

3.21 UV-inactivation of *U. parvum* inoculum

An aliquot of the ureaplasma inocula was also exposed to ultraviolet light (UV), in order to kill the ureaplasmas (preventing replication of the microorganism), but preserving the surface-exposed lipoproteins of the organism.

The ureaplasma inoculum was aliquotted into 100 µL volumes in a 96-well plate. The plate was attached to a retort stand at a distance of 5 – 10 cms from the UV lamp within a class II biohazard cabinet. The inoculum was then exposed to constant ultraviolet light for 20 minutes. The loss of

viability was then confirmed by subculturing the inoculum into 10B broth. No ureaplasma growth was detected (data not shown).

3.22 Optimisation of Ussing chamber model

Prior to performing the *ex vivo* experiments using human chorioamnion tissues; we first optimised the experimental conditions and parameters. We confirmed that the ureaplasmas were able to survive and propagate within the cell culture media (M199) when supplemented with urea and serum. We also determined an optimal concentration of *U. parvum* (CFU) to elicit an immune response. Finally, the oxygen tension, that best replicated the *in vivo* placental microenvironment and allowed the growth of ureaplasmas, was also optimised.

3.23 Optimisation of *U. parvum* growth in M199 cell culture media

The growth of *U. parvum* serovar 6 clinical isolate 429 in M199 media, supplemented with animal serum and urea at concentrations reported for term human amniotic fluid, was assessed. This growth was compared to the growth of ureaplasmas in 10B broth, the ideal *in vitro* growth medium for these bacteria (Shepard and Lunceford 1976).

3.24 Immune study population and specimen collection

From June 2014 to September 2014, women giving birth at the Royal Brisbane and Women's Hospital (RBWH - Herston; Queensland, Australia) were recruited for this study. Women who delivered *via* Cesarean section (with uncomplicated pregnancies) were enrolled in this study and informed written consent was obtained for the collection of placentae following delivery. The work was approved by the ethics committees of the RBWH and The University of Queensland (UQ) (HREC/12/QRBW/391) and was reviewed by the HREC of QUT and deemed exempt from the need for university HREC review. Following delivery, theatre staff placed the placenta into a sterile container, which was then transported to the University of Queensland Centre for Clinical Research (UQ-CCR; Herston, Queensland). All placentae were de-identified.

3.24.1 Placental dissection

Within one hour of delivery, each placenta (Fig 3.2A) was dissected under aseptic conditions. The placenta was placed onto a sterile tray where the chorioamnion membranes were carefully cut from the edge of the placenta (Fig 3.2 B and C) using sterile scissors. Areas in which the amnion had detached from the chorion were excluded. Once the chorioamnion was detached from the placenta (Fig. 3.2 D), the membranes were placed into sterile PBS and washed to remove any adherent blood clots. The membranes were then spread over a sterile Petri dish and six circular sections of the membrane (measuring approx. 6.5 cm diameter) were cut using a sterile membrane cutter (Fig 3.2 E). The sections were then placed into pre-warmed M199 media (Invitrogen) (Fig. 3.2 F). Remaining tissue was then cut to size (measuring approx. 1 x 3 cm^2) and placed into a tissue cassette and placed in paraformaldehyde for histology and into sterile cryogenic vials and stored at -80 °C.

3.24.2 Ussing chamber preparation

The chorioamnion tissue sections were then sandwiched between two discs of sterile semi-rigid mesh and secured within an Ussing chamber (Fig 3.2 G - I). Using sterile syringes, the maternal and fetal compartments were slowly filled with pre-warmed M199 media (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 100 U/mL penicillin (CSL Biosciences; Parkville, Victoria). The maternal membrane perfused media was also supplemented with fluorescein isothiocyanate (FITC)-dextran (4000 kDa, final concentration: 10 µM; Sigma-Aldrich) to assess membrane integrity/permeability over time. Different M199 media types were used for each maternal and fetal compartment (M199 with phenol red for maternal compartments; M199 without the addition of phenol red for fetal compartments) in order to immediately identify any leaks between the compartments within each Ussing chamber.

The entry/exit ports of the Ussing chambers (Fig 3.3) were connected to maternal and fetal media reservoirs *via* sterile silicone tubing (BioRad; Gladesville, New South Wales). This allowed the media to exit the maternal or fetal compartment, into the respective media reservoir thereby creating a closed loop for each compartment (Fig 3.2). Each compartment of the Ussing chamber held approximately 4 mL of media, with the tubing holding approx. 6 mL. Tubing from each 'maternal' and 'fetal' compartment was then connected to a pump (EconoGradient Pump; BioRad) with a set flow-rate of 1.5 mL/min and chambers were maintained within an incubator (Memmert INCO Trigas incubator; Schwabach, Germany) at 37 °C, 5% CO_2 and 8% O_2 for 30 hours. The Ussing chambers

were allowed to equilibrate within the incubator for 30 minutes prior to the commencement of each experiment.

Immediately following the equilibration period, the experiment was started (t = 0 hrs) and media was collected from each of the maternal and fetal reservoirs to act as a baseline for all further measurements. Immediately following this baseline reading, duplicate chambers were injected with each experimental condition (total of 6 Ussing chambers per placenta) and were exposed to: (i) 2 x 10^7 CFU of live *U. parvum* serovar 6 clinical isolate 429; (ii) 2 x 10^7 CFU of UV-inactivated *U. parvum* serovar 6 clinical isolate 429; or (iii) 10B media vehicle control.

The maternal and fetal perfusates were sampled *via* a three-way stopcock (decontaminated at each sampling using 70% ethanol) at 2, 4, 8, 20 and 30 hours post-infection; transferred to sterile cryogenic vials and stored at – 80 °C for future analysis.

At the completion of the 30 hour experiment, the remaining media was drained from each maternal and fetal chamber (and reservoir) and centrifuged at 5000 x g for 20 minutes. The resulting pellet was then resuspended in 100 µL of sterile PBS and used for Western blot in order to assess MBA variation occurring within the ureaplasma population present within the media.

Chorioamnion tissue from each chamber was also dissected and placed into paraformaldehyde for histology and the remainder was placed into sterile cryogenic vials and stored at -80 °C.

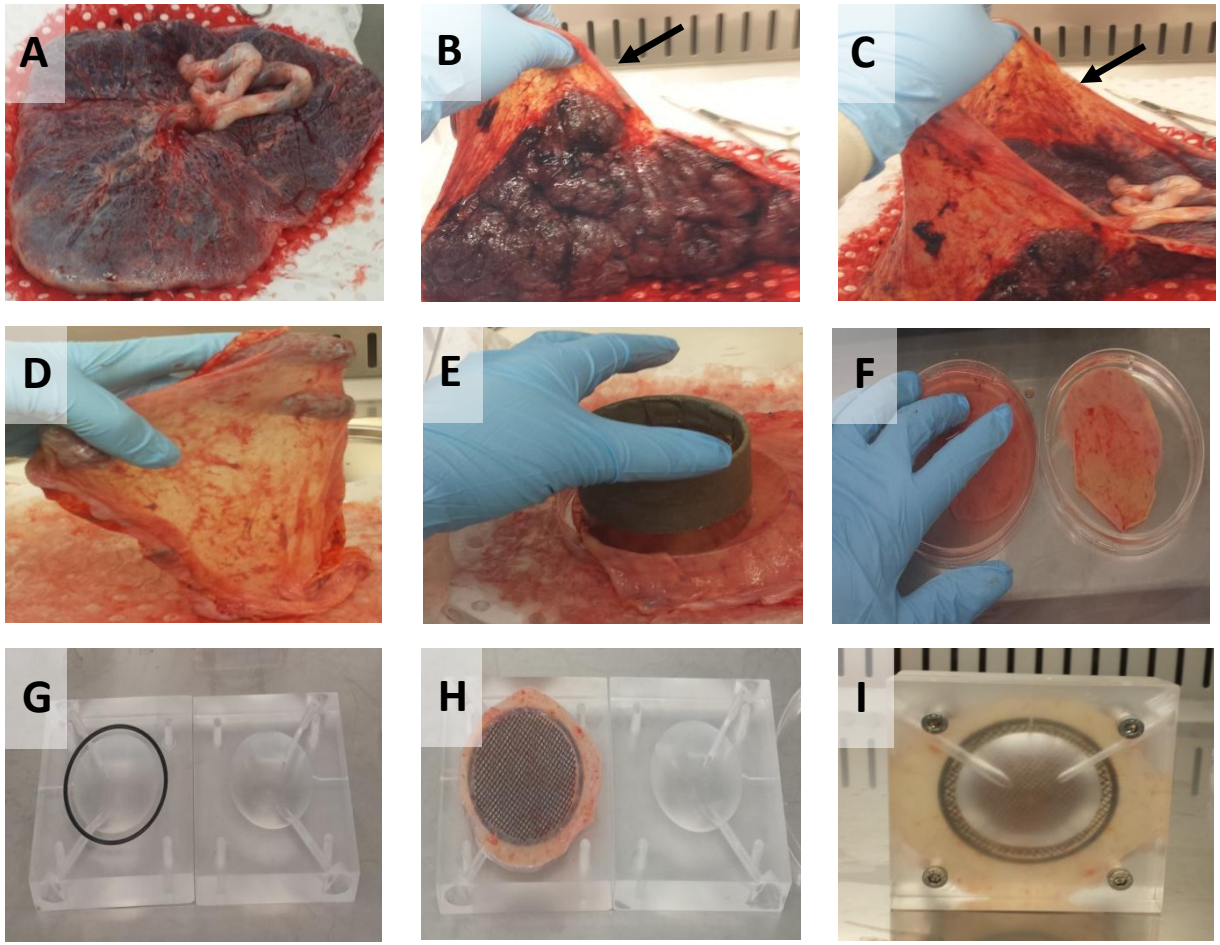


Figure 3.2. Photos illustrating placental tissue dissection (panels A – F) and Ussing chamber assembly (panels G-I) for *ex vivo* immune response experiments.

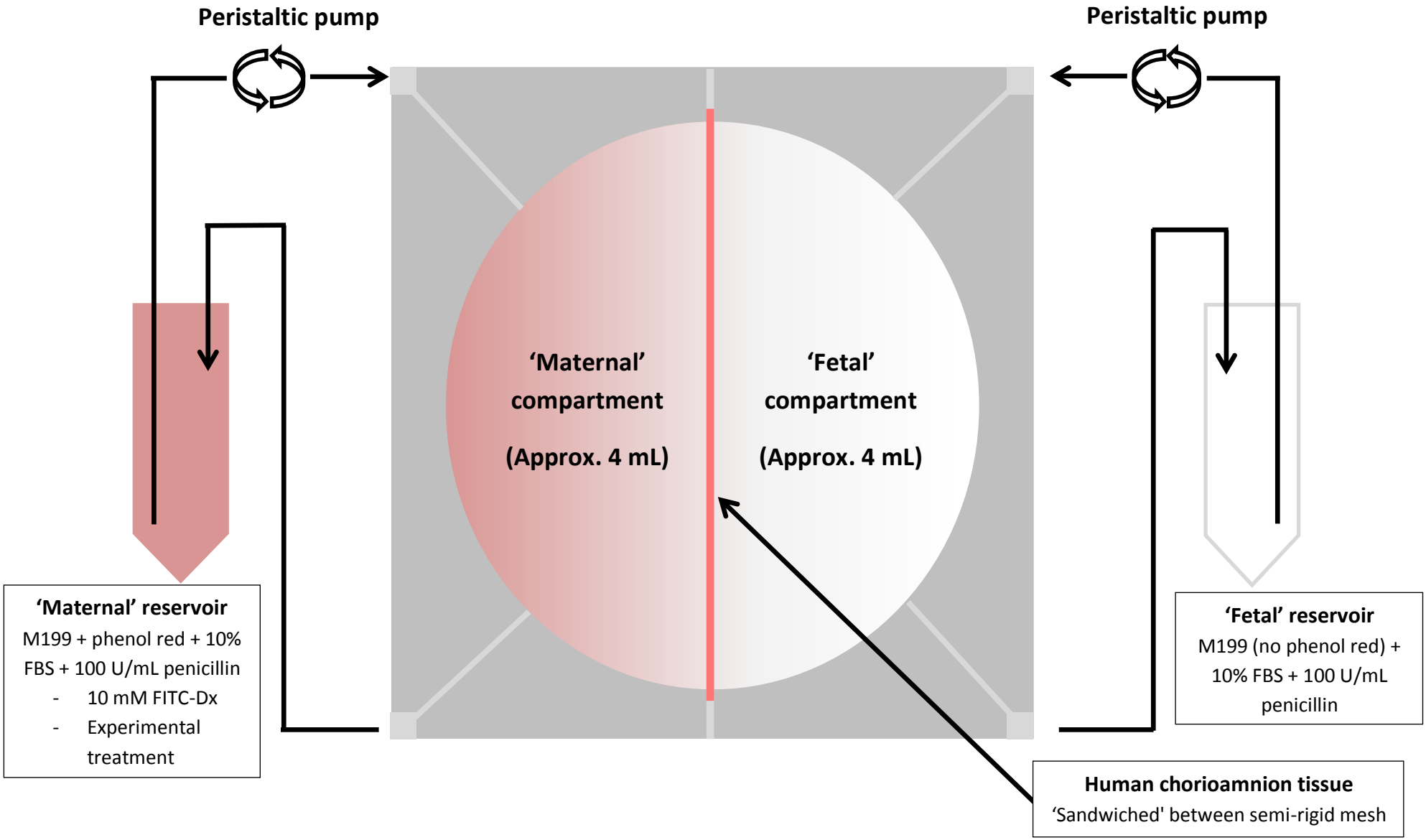


Figure 3.3. Ussing chamber design used for *ex vivo* chorioamnion infection with *Ureaplasma* spp. Chorioamnion tissue represents a physical barrier between the two compartments and both compartments were separately perfused with sterile supplemented M199 media. The experimental treatments were then added to the maternal compartment of each Ussing chamber (n = 6)

3.25 Analysis of maternal and fetal perfusates

3.25.1 FITC-dextran analysis

Alterations in the integrity or permeability of the chorioamnion tissue were monitored using FITC-dextran, which was added to the maternal compartment. A measure of the transfer from the maternal to fetal compartment was assessed using a fluorescent plate reader (SpectraMax Paradigm plate reader, Molecular Devices). 100 µL of each maternal and fetal perfusate was aliquotted into the well of a 96-well plates and the levels of FITC-dextran were measured at excitation/emission of 495/520 nm. FITC-dextran levels in each perfusate were then represented as the percentage transfer between the maternal and fetal compartment over the course of each 30 hour time-point experiment.

3.25.2 BioPlex Assays for the detection of cytokines in maternal and fetal perfusates

Maternal and fetal perfusates from replicate Ussing chambers within each experiment (n = 4 experiments) were pooled for each of the time points (0 hr, 8hr, 20 hr and 30 hr) and analysed in duplicate using the BioPlex Pro Human proinflammatory cytokine immunoassay for interleukin (IL)-2, IL4, IL-6, IL-8, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α and interferon (IFN)-γ (BioRad; Gladesville, New South Wales), according to the manufacturer's instructions.

3.25.3 MMP-9 activity and gelatin zymography

Pooled maternal and fetal perfusates from each individual Ussing chamber were also used to determine the presence of matrix metalloproteinase (MMP)-9 within each Ussing chamber. 50 µL of pooled perfusate was mixed with protein loading dye and loaded into 10% gelatin zymography gels. Gels were electrophoresed at 120 V for 1 hour and gels were then incubated in 0.5% Triton X solution (renaturing buffer) for 1.5 hours. Gels were then gently washed with water before the addition of incubation buffer (50 mM Tris, 10 mM $CaCl_2$, 50 mM NaCl) at 37 °C for 24 – 48 hours. After incubation, each gel was then stained with Coomassie blue for 1 hour and then destained with dH_20 for at least 1 hour before gel images were captured (CanoScan 8600F). MMP activity was

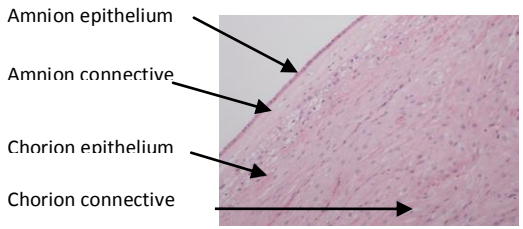
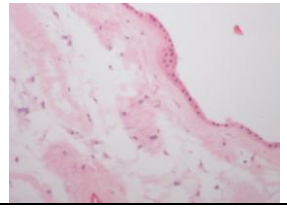
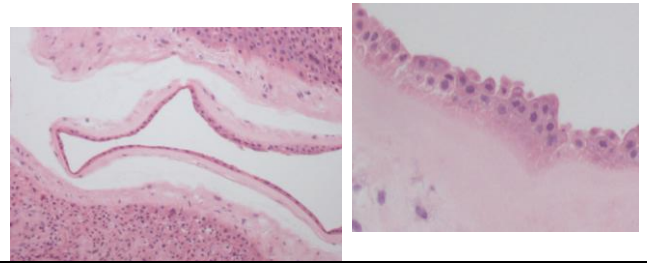
represented by a clear band of 92 kDa, with a blue stained background (MMP-9 positive control kindly provided by Dr Eliza Whiteside). Controls included an additional gel loaded with samples and positive controls treated in the same manner, but with the addition of 10 mM EDTA to the incubation buffer. EDTA prevents MMP activity and these gels showed no significant MMP activity and the absence of a clear band of 92 kDa following staining.

3.26 Analysis of chorioamnion tissues following *ex vivo* experiments

Tissue sections stored in paraformaldehyde were processed and embedded in paraffin. Tissue blocks were then cut into 5 μm sections, placed onto glass slides, dewaxed and then subsequently stained with Haemotoxylin and Eosin (QIMR Berghoefer Medical Research Institute Histology Department). Stained sections were viewed using a light microscope (Olympus CX21) and a total of ten representative images captured for each chorioamnion tissue from each placental experiment.

As no previous studies have attempted to grade chorioamnion tissues within an *ex vivo* model, grading criteria were specifically devised for this study (Table 3.7) and provided to two impartial scientists (blinded to outcome) for pathology grading. Multiple images of chorioamnion tissue from each of the three treatment groups from each placental experiment (n = 4) were randomised and provided to the scientists, along with the grading criteria. Each image was given a score (1 – 3) and the total mean value for each treatment group were then calculated.

Table 3.7. Pathology grading and tissue severity guidelines for *ex vivo* chorioamnion tissues. Tissues were graded (score: 1 - 3) according to key features of the chorioamnion. Grading was performed by two independent researchers

SCORE	Example	Key features
1		- **No significant pathology** - Intact amnion epithelial layer and connective tissue - Intact chorion epithelial layer and connective tissue
2		- **Mild/moderate** - Partial separation between the amnion connective tissue and the chorion epithelial layer
3		- **Severe** - The amnion and chorion layers are detached - Amnion epithelial is thickened

Chapter Four:

Chorioamnion infection and histological chorioamnionitis in late preterm and term placentae

Introduction

Preterm birth (PTB; delivery < 37 weeks) is a major obstetric challenge, accounting for 50% of neonatal mortality and up to 70% of the long-term neonatal and infant morbidity (March of Dimes 2012). Approximately 10% of all births occur preterm and despite improved intervention strategies, alarmingly the rates of PTB continue to increase in almost every country (March of Dimes 2012). PTBs can be grouped according to the gestational age at the time of delivery: infants who are born prior to 28 weeks of pregnancy are considered to be extremely preterm; very preterm infants are delivered at less than 32 weeks of completed gestation; while late preterm (LPT) infants are delivered between 32 – 36 weeks of gestation (Bick 2012). LPT infants are particularly important, as these infants account for 79 - 84% of all PTBs and their incidence has increased by more than 40% in the last 25 years (Raju 2006; Goldenberg *et al.* 2008b; Hamilton *et al.* 2012; Li *et al.* 2013). Importantly, while it has been determined that these infants are at increased risk of adverse sequelae, such as cerebral palsy (Soleimani *et al.* 2014), learning and visual deficits (Morse *et al.* 2009; Soleimani *et al.* 2014) and increased risk of chronic diseases in adulthood (Sullivan *et al.* 2012), the aetiology of LPT birth is currently unknown.

Infection within the female upper genital tract (UGT) is the most frequent (and potentially preventable) cause of PTB (Hillier *et al.* 1988; Goldenberg *et al.* 2000b; DiGiulio *et al.* 2008; DiGiulio *et al.* 2010; DiGiulio 2012). Of those women who experience PTB, up to 40% of have evidence of intraamniotic infections (March of Dimes 2012). While there is currently overwhelming evidence implicating intraamniotic (UGT) infection with early PTBs (< 32 weeks) (DiGiulio 2012), there is significantly less information on the prevalence of infection in later gestations. Researchers have stated that "spontaneous births at less than 30 weeks, an association with infection is the rule... while late spontaneous preterm births do not usually occur in association with infection" (Goldenberg *et al.* 2002), however, there is little scientific evidence to confirm this and the lack of association between birth in the LPT period and intraamniotic infection has not been adequately explained. Given this, it is necessary to further investigate the prevalence of infection in later gestations (in particular, the LPT and term periods).

Numerous microorganisms have been isolated from the UGT of women who deliver very preterm (Figure 4.1) and infections early in gestation are often polymicrobial, making it difficult to determine if there is an association between individual microorganisms and adverse outcomes (Hillier *et al.* 1988; DiGiulio *et al.* 2008; Onderdonk *et al.* 2008; DiGiulio *et al.* 2010). However, of the microorganisms associated with UGT infections, the human *Ureaplasma* spp. are among those most

frequently isolated bacteria from the amniotic fluid (Figure 4.1A) and placentae (Figure 4.1B) of women who deliver at less than 32 weeks of gestation.

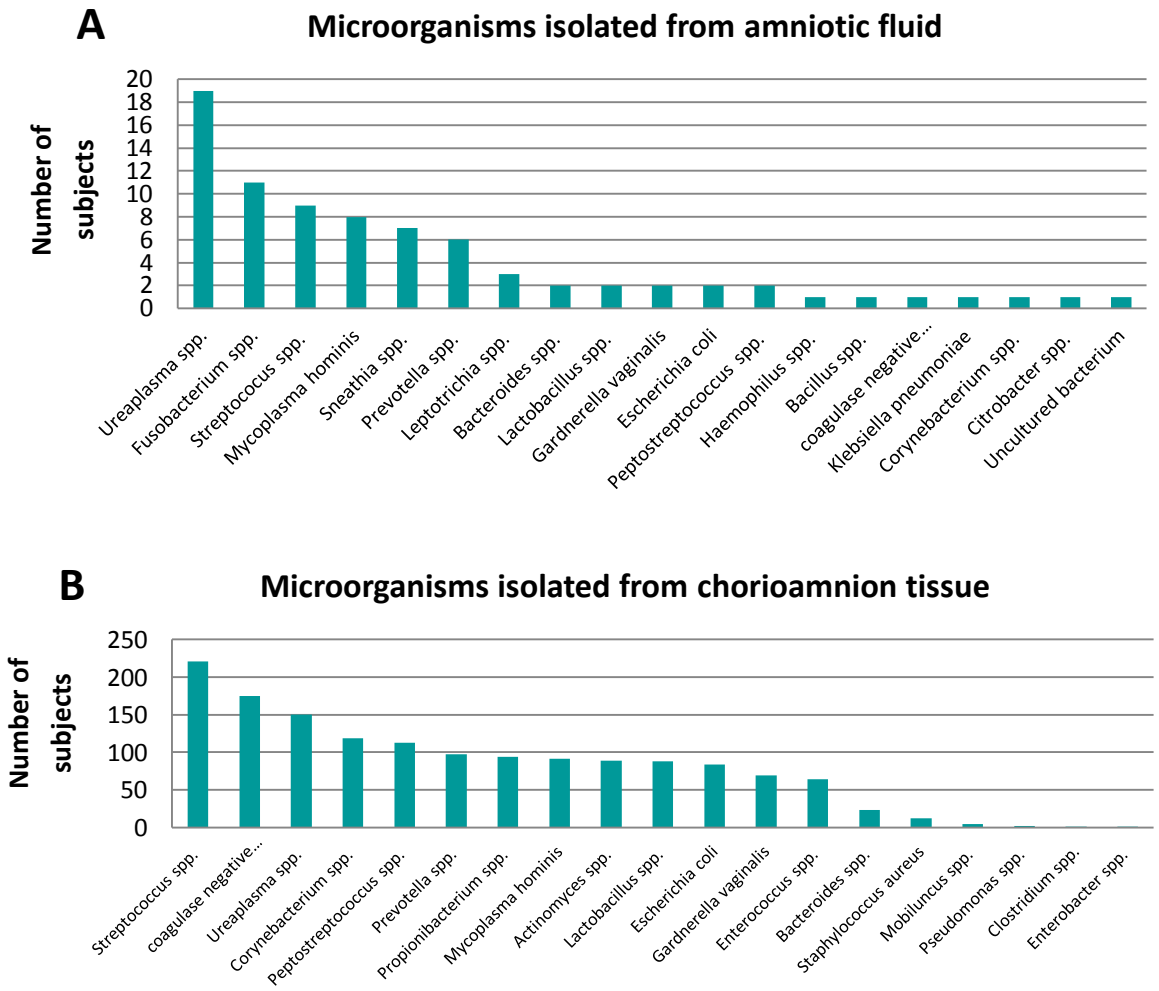


Figure 4.1. Prevalence and diversity of microorganisms isolated from (A) amniotic fluid and (B) chorioamnion tissue of women experiencing preterm birth (< 32 weeks). Only studies which included the classification and total number of positive patient samples (by culture or PCR) have been included. Data compiled from (Hillier *et al.* 1988; Jalava *et al.* 1996; DiGiulio *et al.* 2008; Onderdonk *et al.* 2008; Han *et al.* 2009; DiGiulio *et al.* 2010; Namba *et al.* 2010; Marconi *et al.* 2011)

The presence of infections within the UGT have been widely associated with adverse pregnancy outcomes, including spontaneous preterm premature rupture of membranes (pPROM) (Jacobsson *et al.* 2009; DiGiulio *et al.* 2010), PTB (Hillier *et al.* 1988; Gibbs *et al.* 1992; Goldenberg *et al.* 2002; Goncalves *et al.* 2002) and spontaneous abortion or miscarriage (Naessens *et al.* 1988). Additionally, these infections may also affect the neonate and have been associated with both short- and long-term adverse outcomes, including respiratory distress syndrome (RDS) (Gomez *et al.* 1998; Kramer

2008) or bronchopulmonary dysplasia (BPD) (Viscardi and Hasday 2009; Sung *et al.* 2010; Viscardi 2010), sepsis (Goncalves *et al.* 2002), meningitis (Garland and Murton 1987) and adverse CNS outcomes, such as intraventricular haemorrhage and cerebral palsy (Viscardi *et al.* 2008; Berger *et al.* 2009; Oskoui *et al.* 2013). While infection has been associated with adverse pregnancy outcomes, it is important to note that not all women with intraamniotic infections will deliver preterm. A study by Gerber *et al.* (2003) identified that the *Ureaplasma* spp. was present in 29/257 (11.4%) amniotic fluid specimens collected at 15 – 17 weeks of gestation. However, only 21% of these women developed pPROM and 24% experienced PTB, while >70% of women with diagnosed ureaplasma infections delivered at term with no apparent adverse outcomes. Others have reported similar findings with up to 20% of women in these studies identified as infected with *Ureaplasma* spp. within the UGT at the time of term delivery (Hillier *et al.* 1988; Gray *et al.* 1992). It is currently not clear why some women with intraamniotic infections deliver preterm, while others do not; however, a consistent finding within the literature is the association between UGT infection and an inflammatory response, such as histological chorioamnionitis (Czikk *et al.* 2011).

Chorioamnionitis is defined as inflammation within the chorioamnion membranes (which surround the developing fetus during pregnancy) and is present in up to 70% of pregnancies affected by infection (Eschenbach 1993). Diagnosis of chorioamnionitis may be associated with the presence of clinical signs or symptoms including: maternal temperature or tachycardia, uterine tenderness and pPROM; however, most cases of chorioamnionitis are clinically silent (Czikk *et al.* 2011; Martinelli *et al.* 2012). In suspected cases of chorioamnionitis, diagnoses can be made by performing culture and/or PCR of amniotic fluid (collection of this specimen is an invasive procedure) to identify microorganisms (DiGiulio *et al.* 2010; Romero *et al.* 2014), or by retrospective histological analysis of placentae for inflammation and necrosis (Redline *et al.* 2003). The prevalence of histological chorioamnionitis is inversely correlated with gestational age, with 40 – 70% of placentae from early PTBs showing evidence of chorioamnionitis; but chorioamnionitis is detected in only 2 - 4% of term placentae (Czikk *et al.* 2011). However, a study by Gordon *et al.* (2011) demonstrated an interesting bimodal trend in the prevalence of histological chorioamnionitis (Figure 4.2). This study retrospectively analysed placentae from 952 pregnancies and demonstrated histological chorioamnionitis in 22.6% of all women, with an increased prevalence of chorioamnionitis at ≤ 29 weeks of gestation (up to 17%), and also later in gestation (≥ 36 weeks, up to 30%; Figure 4.2). While histological chorioamnionitis has been correlated with intraamniotic infections, there is some debate as to the cause of the host immune response, especially if there are polymicrobial infections. While studies have demonstrated an association between *Ureaplasma* spp. and the development of

chorioamnionitis (Hillier *et al.* 1988; Goldenberg *et al.* 2008a; Hecht *et al.* 2008; Kasper *et al.* 2010; Namba *et al.* 2010), further studies are required to determine if these microorganisms can cause chorioamnionitis independently of other microorganisms.

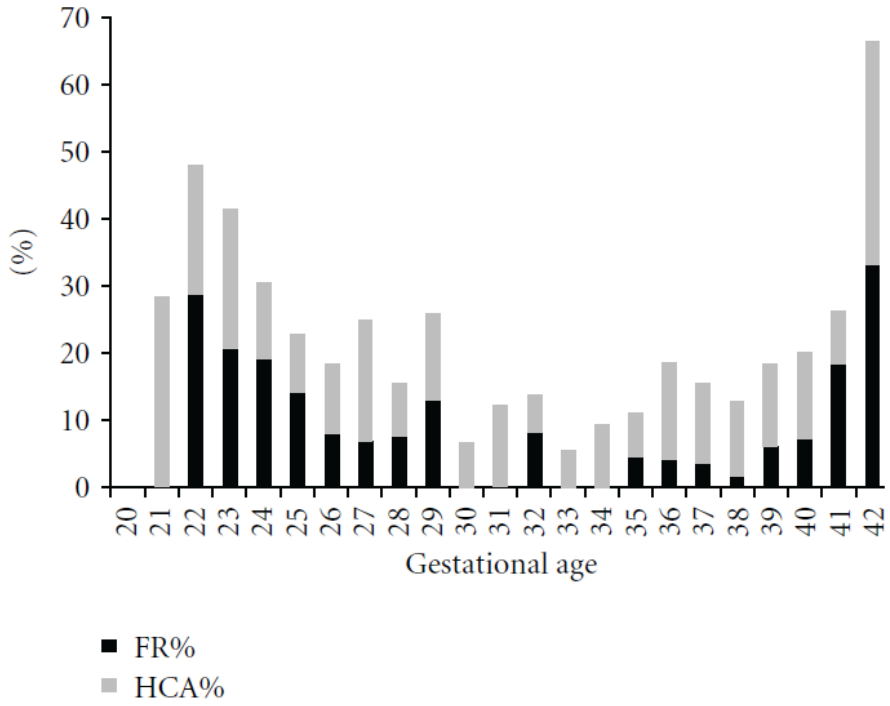


Figure 4.2 Prevalence (% of total pregnancies, n = 952) of histological chorioamnionitis (HCA; grey shading) and fetal inflammatory response (FR; black shading) at which each infant/placenta is delivered. Image adapted from Gordon *et al.* (2011).

For this current study, we hypothesised that *Ureaplasma* spp. and other microorganisms would be isolated from the chorioamnion of women who delivered LPT or at term, and that the presence of these microorganisms may be associated with adverse outcomes, such as histological chorioamnionitis. The aims of this study were to (i) identify the prevalence and diversity of microorganisms from LPT and term chorioamnion tissue; (ii) to identify if infection was associated with adverse pregnancy or neonatal outcomes; and (iii) to identify if particular microorganisms were more frequently associated with adverse outcomes.

Materials and methods

All methods pertaining to this chapter are summarised in Chapter three. Briefly, placentae were collected and processed aseptically post-delivery and tissue specimens were snap frozen as per Chapter Three, Section 3.2. Placental samples were shipped to QUT on a regular basis for microbiological testing. This included culture (Chapter three, section 3.7) of placental swabs and chorioamnion tissues to identify any microorganisms present. DNA was extracted from chorioamnion tissues and cultured clinical isolates (Chapter Three, section 3.8) and these specimens were tested by 16S rRNA (Chapter three, section 3.9) to identify any microbial species present. 16S rRNA amplicons were then detected by agarose gel electrophoresis (Chapter three, section 3.10) and these amplicons were subsequently purified and then sequenced (Chapter three, section 3.11) to identify the genera of the organisms present.

Statistical analysis

Data within the chapter are presented as the mean value, plus the standard error of the mean (SEM).

All data within this study were imported and manipulated within the statistical package for social sciences (SPSS) software program. Relationships between patient outcomes (maternal demographic data, maternal pregnancy outcomes or neonatal outcomes) were analysed against the variables of late preterm/term birth; the presence/absence of microorganisms (regardless of the type of organisms present); presence/absence of *Ureaplasma* spp. and the presence/absence of microorganisms other than ureaplasmas. Analyses within this chapter included both binary logistic regression analysis and analysis of variance (ANOVA) to determine statistical significance between cohorts and the types of tests used within this study are noted throughout the chapter.

Statistical significance was accepted as $p < 0.05$.

Results

Study population

A total of 517 women were recruited during this study period. Of those, 477 women and their 535 offspring were included in the final microbiological study and data for these women and their offspring are presented in this chapter.

The majority of women in this study were Caucasian (66.4%) or African American (25.8%), with 26 women of mixed ethnicity and 8 women of Asian descent (Table 4.1). Of the 535 infants included in the study, 421 were singleton births and 114 infants were multiple births (54 sets of twins and two sets of triplets). The rate of fetal malformations and other syndromes such as isoimmunisation in this study population was very low (13/535; 2.4%). Since the prevalence of these factors was very low, these were not considered to be factors which contributed to LPT birth and so these infants were included in our analyses.

Late preterm vs. term pregnancy characteristics

Maternal demographic

There were no significant differences in maternal age, gravida, marital status, ethnicity, level of medical insurance (Table 4.1) or mode of delivery between women who delivered LPT or at term (Table 4.2). Almost all women included in this study (both cohorts > 97%) attended at least one prenatal care visit during their current pregnancy. However, there was a significant difference in the mean level of parity of women who delivered at term. These women had a lower parity (mean: 1.7 ± 0.1) than those women who delivered LPT (mean: 2.1 ± 0.6; Table 4.1).

Table 4.1. Demographic data of mothers (n = 477) who delivered late preterm and at term

	Late Preterm (n = 385)	Term (n = 92)	Significance
Maternal age in years (mean, range)	27.4 ± 0.3 (15 – 43)	27.7 ± 0.5 (18 – 39)	NS[1]
Gravida[2]	2.5 ± 0.1 (1 – 11)	2.2 ± 0.5 (1 – 8)	NS
Parity[3]	2.1 ± 0.1 (1 – 10)	1.7 ± 0.1 (1 – 6)	0.015
Marital Status			
- **Single**	190/385 (49.4%)	32/92 (34.8%)	NS
- **Married**	191/385 (49.6%)	60/92 (65.2%)	NS
- **Unknown**	4/385 (1.0%)	0/92 (0.00%)	NS
Ethnicity			
- **Caucasian**	247/385 (64.2%)	69/92 (75.0%)	NS
- **African-American**	111/385 (28.8%)	15/92 (16.3%)	NS
- **Mixed**	19/385 (4.9%)	7/92 (7.6%)	NS
- **Asian**	7/385 (1.8%)	1/92 (1.1%)	NS
- **Undisclosed**	1/385 (0.3%)	0/92 (0.0%)	NS
Medical Insurance			
- **Private**	204/385 (53.0%)	91/92 (66.3%)	NS
- **Medicaid**	162/385 (42.1%)	24/92 (26.1%)	NS
- **Self pay/uninsured**	16/385 (4.1%)	4/92 (4.3%)	NS
- **Undisclosed**	3/385 (0.8%)	3/92 (3.3%)	NS
Evidence of one prenatal care visit	379/385 (98.4%)	90/92 (97.8%)	NS

[1]NS - Not statistically significant

[2]Gravida - number of clinical pregnancies

[3]Parity - number of viable offspring resulting from all pregnancies

Data was analysed by logistic regression analysis and compared the maternal demographic data of women whose pregnancies were delivered late preterm to those delivered at term.

Adverse pregnancy outcomes

There were no significant differences in the prevalence of signs/symptoms of infection in women who delivered LPT or at term, nor was there any difference in the rate of prior chorioamnionitis within these two cohorts. Women who delivered LPT had a higher incidence of hypertension (29.6%), compared to those women who delivered at term (7.6%), however this difference was not statistically significant. Unsurprisingly, the presence of pregnancy-induced hypertension was significantly higher in women who delivered LPT (30.4%), compared to those women who delivered at term (4.3%; p = 0.007) (Table 4.2).

There was also a higher prevalence of cervical incompetence (66.7%) and pPROM (45.4%) in women who delivered LPT than for women who delivered at term (0.0% and 0.0%, respectively). Due to the nature of logistic regression analysis, it was not possible to derive p-values for these outcomes, due to complete separation of data within the LPT and term cohorts. Women who delivered LPT were treated with antibiotics significantly more frequently during their labour (62.9%), compared to those women who delivered at term (22.8%; p = 0.049). Despite the high number of women who delivered LPT, there were no differences in the rate of vaginal or Cesarean section deliveries in these two cohorts (Table 4.2).

Table 4.2. Maternal pregnancy outcomes for mothers who delivered late preterm and at term

	Late preterm (n = 385)	Term (n = 92)	Significance
At least one sign/symptom of infection[1]	27/385 (7.0%)	7/92 (7.6%)	NS[2]
Previous history of chorioamnionitis documented	17/385 (4.4%)	3/92 (3.3%)	NS
Hypertension	114/385 (29.6%)	7/92 (7.6%)	NS
Pregnancy induced hypertension	117/385 (30.4%)	4/92 (4.3%)	0.007
Cervical incompetence	257/385 (66.7%)	0/92 (0.0%)	#
Antibiotics administered during labour[3]	242/385 (62.9%)	21/92 (22.8%)	0.049
Preterm premature rupture of membranes (pPROM)	175/385 (45.4%)	0/92 (0.0%)	#
Mode of delivery			
- **Vaginal**	265/385 (68.8%)	69/92 (75.0%)	NS
- **Cesarean**	120/385 (31.2%)	23/92 (25.0%)	NS

[1]Signs and symptoms of infection included: maternal temperature > 38 °C, uterine or abdominal tenderness, foul-smelling vaginal discharge, maternal tachycardia (> 120 bpm) or fetal tachycardia (> 160 bpm)

[2]NS - Not statistically significant

[3] While the antibiotic dose and type were not recorded, antibiotics were administered to women more than 3 hours prior to delivery.

- Logistic regression analysis was unable to derive p-values for these outcomes/conditions due to complete separation of data within the two cohorts.

Data was analysed by logistic regression analysis and compared maternal pregnancy outcomes of women who delivered late preterm or at term

Fetal characteristics

The mean gestational age of LPT infants was 34.9 ± 0.1 weeks, compared to 39.4 ± 0.1 for those infants delivered at term. There were no differences in the mean Apgar scores of LPT and term infants at either 1 or 5 minutes post-delivery.

There were, however, some interesting differences between these two cohorts. LPT infants had a lower birth weight, compared to term-delivered neonates (2535.9 ± 52.4 grams vs. 3489.0 ± 44.3 grams, respectively; $p = 0.001$), but there was no difference in the mean placental weight for LPT and term pregnancies (422.2 ± 6.3 vs. 469.0 ± 7.9 grams, respectively). There were no differences in the incidence of histological chorioamnionitis in LPT and term cohorts, nor were there any differences in the mean maternal and fetal stages of inflammation (Table 4.3). Following delivery, LPT infants displayed more signs of respiratory distress during their first 24 hours of life (23.7%), they were administered oxygen for prolonged periods (> 6 hours; 12.4%), and were treated with continuous positive airway pressure (CPAP) respiratory support more often (13.5%) than those infants who were delivered at term (3.3%, 0.0% and 0.0%, respectively) (Table 4.3). LPT infants were also diagnosed with respiratory distress syndrome more frequently than those infants delivered at term (12.0% vs. 0.0%, respectively) (see Table 4.3). As a consequence of these adverse outcomes, the overall length of stay for LPT infants was significantly higher than those infants who were delivered at term (7.1 days vs. 2.2 days, respectively; $p < 0.001$; Table 4.3).

Table 4.3. A comparison of outcomes for neonates (n = 535) delivered late preterm and at term

	Late preterm (n = 443)	Term (n = 92)	Significance
Gestational age at delivery (mean, range)	34.9 ± 0.1 (32 – 36)	39.4 ± 0.1 (37 – 41)	NS[1]
Apgar[2] score – 1 minute (mean, range)	7.8 ± 0.1 (1 – 10)	8.1 ± 0.2 (2 – 9)	NS
Apgar[2] score – 5 minutes (mean, range)	8.8 ± 0.03 (2 – 10)	8.9 ± 0.03 (8 – 10)	NS
Birth weight (mean, range)	2535.9 ± 52.4 (1060 - 4530)	3489.0 ± 44.3 (2680 - 4350)	0.001
Placental weight (mean, range)	422.2 ± 6.3	469.0 ± 7.9	NS
Chorioamnionitis in current pregnancy[3]	69/443 (21.7%)	25/92 (27.2%)	NS
- **Maternal stage inflammation**	1.4 ± 0.03 (1 – 3)	1.5 ± 0.1 (1 – 3)	NS
- **Fetal stage inflammation**	2.3 ± 0.04 (1 – 3)	2 ± 0.1 (1 – 3)	NS
Male : Female	225 : 217[4]	51 : 41	NS
Continuous positive airway pressure (CPAP)	60/443 (13.5%)	0/92 (0.0%)	#
Features of RDS[5] < 24 hours after birth	105/443 (23.7%)	3/92 (3.3%)	0.100
Required oxygen support for > 6 hrs	55/443 (12.4%)	0/92 (0.0%)	#
Diagnosed RDS[5]	53/443 (12.0%)	0/92 (0.0%)	#
Length of Stay	7.1 ± 0.3 (1 - 43)	2.2 ± 0.1 (1 - 7)	< 0.001

[1]NS - Not statistically significant

[2] Apgar – a measure of neonatal health scored at 1 min and 5 minutes post-delivery; measures appearance, pulse, reflex, activity and respiration of the newborn.

[3] Chorioamnionitis was determined by US pathologists (blinded to outcome) and was graded for severity of inflammation according to (Redline *et al.* 2003)

[4] The sex of one infant was not disclosed

[5]RDS - respiratory distress syndrome

Logistic regression analysis was unable to derive p-values for these outcomes/conditions due to complete separation of data within the two cohorts.

Data analysed by logistic regression analysis and compared the outcomes of neonates delivered late preterm or at term.

Chorioamnion infection

Chorioamnion infection, as detected by PCR and/or culture of microorganisms from clinical specimens, was identified in 10.6% (57/535) placentae (Table 4.4, Supplementary Table 8.1). In some placentae, polymicrobial chorioamnion infections were identified; multiple microbial species were isolated from 4/57 (7.0%) placentae (Supplementary Table 8.1).

Of the bacteria detected within chorioamnion tissue, the most prevalent were *Ureaplasma parvum* (36/59; 59.0%), *Ureaplasma urealyticum* (6/61; 9.8%), *Streptococcus agalactiae* (Group B beta haemolytic Streptococcus (GBS); 6/61; 9.8%), *Bacteroides fragilis* (2/61; 3.3%), *Bifidobacterium* spp. (2/61; 3.3%), *Gardnerella vaginalis* (1/61; 1.6%), *Propionibacterium* spp. (1/61; 1.6%) and *Escherichia coli* (1/61; 1.6%) (Table 4.4 and Figure 4.3). No *Lactobacillus* spp. were identified by culture or PCR methods, nor were any fungi or yeasts detected within any chorioamnion tissues sampled. Non-cultivable bacteria were detected by molecular methods in six (9.8%) placentae.

When comparing the prevalence of infection, there were no significant differences in the presence of microorganisms within the chorioamnion of women who delivered LPT or at term (9.9% and 14.1%, respectively). Since there was no difference in the prevalence of infection between these groups, we also compared the microbial load (number of CFU per swab or per gram of tissue) in placentae delivered LPT and at term. The microbial load was determined by counting the number of colonies isolated on primary isolation media (this was not able to be determined for those microorganisms which did not grow on primary isolation media, or those cultured only after enrichment within thioglycollate broths). There was no significant difference in the mean microbial load within LPT or term placentae (9.73×10^7 CFU vs. 5.85×10^7 CFU, respectively; $p = 0.159$), nor was there any difference in the prevalence of histological chorioamnionitis in LPT or term placentae (59.1% and 38.5%, respectively).

Regardless of when placentae were delivered, *U. parvum* and *U. urealyticum* were isolated most frequently within placentae (71.2%; (Figure 4.3). Ureaplasmas were also detected in higher numbers (CFU) than any other microorganism within this study (Table 4.4).

Table 4.4. The diversity and prevalence of microorganisms isolated from placentae delivered late preterm (n = 46) and at term (n = 9)

MICROORGANISMS DETECTED WITHIN PLACENTAE				
	Late preterm (32 – 36 weeks)		Term (> 37 weeks)	
Microorganism	**Frequency[1] (%)**	**Bacterial load (mean)[2]**	**Frequency[1] (%)**	**Bacterial load (mean)[2]**
Ureaplasma parvum	27 (58.7%)	4.86×10^8	9 (60.0%)	1.16×10^8
Ureaplasma urealyticum	6 (13.0%)	2.92×10^8	0 (0.0%)	-
Uncultured bacterium	3 (6.5%)	-	3 (20.0%)	-
Streptococcus agalactiae (GBS)	3 (6.5%)	1×10^3	3 (20.0%)	1×10^6
Bacteroides fragilis	2 (4.3%)	1×10^3	0 (0.0%)	-
Bifidobacterium spp.	2 (4.3%)	1×10^3	0 (0.0%)	-
Gardnerella vaginalis	1 (2.2%)	1×10^3	0 (0.0%)	-
Escherichia coli	1 (2.2%)	1×10^3	0 (0.0%)	-
Propionibacterium spp.	1 (2.2%)	1×10^3	0 (0.0%)	-
TOTAL NUMBER OF ORGANISMS ISOLATED	**46**		**15**	
FREQUENCY OF INFECTION[3]	**44/443 (9.9%)**	**-**	**13/92 (14.1%)**	**-**
Infection and chorioamnionitis	**26/44 (59.1%)**	**-**	**5/13 (38.5%)**	**-**

[1] Frequency- the total number of microorganisms isolated by standard culture, growth in enrichment broth or by PCR. No other statistically significant differences were seen in the incidence of infection

[2] Quantitative analysis was not always possible, as some microorganisms were isolated in enrichment broth or by PCR only. Quantitative analyses shown where possible

[3] Some placentae (n = 4) contained polymicrobial infections. This number represents the total number of placentae which were found to have infection, regardless of the number of organisms isolated from each placentae

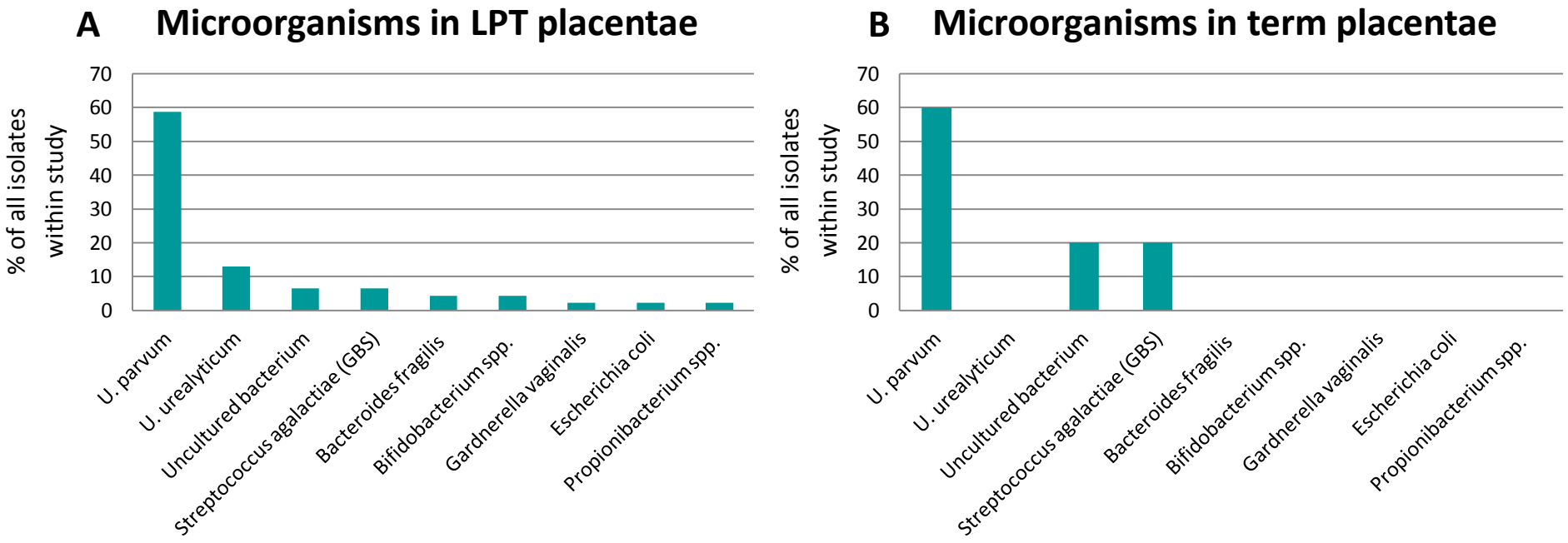


Figure 4.3. The diversity and prevalence of microorganisms detected in late preterm (A) and term (B) placentae

Association of chorioamnion infection with adverse pregnancy and neonatal outcomes

To determine if there was an association between infection and adverse outcomes, pregnancies were grouped based on the presence or absence of chorioamnion infection and the pregnancy outcomes and fetal outcomes for these women were compared.

Maternal demographic data

There were no statistically significant differences in the maternal demographic data of women whose chorioamnion was infected with microorganisms, when compared to those women whose chorioamnion had no detectable microorganisms (by culture and/or PCR; Table 4.5).

Table 4.5. Demographic data for women (n = 474) whose pregnancies were complicated by chorioamnion infection compared to those who had no chorioamnion infection

	Chorioamnion infection (n = 47[1])	**No chorioamnion detected (n = 427[1])**	**Significance**
Maternal age (mean, range)	25.0 ± 0.7 (17 – 38)	27.8 ± 0.3 (15 – 43)	NS[2]
Gravida[3] (mean, range)	2.2 ± 0.2 (1 – 5)	2.5 ± 0.1 (1 – 11)	NS
Parity[4] (mean, range)	1.9 ± 0.1 (1 – 4)	2.1 ± 0.1 (1 – 10)	NS
Marital Status			
- **Single**	28/47 (59.6%)	232/427 (54.3%)	NS
- **Married**	19/47 (40.4%)	190/427 (44.5%)	NS
- **Undisclosed**	0/47 (0.0%)	5/427 (1.2%)	NS
Health insurance			
- **Private**	16/47 (34.0%)	247/427 (57.9%)	NS
- **Medicaid**	25/47 (53.2%)	160/427 (37.5%)	NS
- **Self pay/uninsured**	3/47 (6.4%)	16/427 (3.7%)	NS
- **Undisclosed**	3/47 (6.4%)	4/427 (0.9%)	NS
Ethnicity			
- **Caucasian**	26/47 (55.3%)	289/427 (67.8%)	NS
- **African-American**	20/47 (42.6%)	104/427 (24.3%)	NS
- **Mixed**	1/47 (2.1%)	25/427 (5.8%)	NS
- **Asian**	0/47 (0.0%)	8/427 (1.9%)	NS
- **Undisclosed**	0/47 (0.0%)	1/427 (0.2%)	NS
Evidence of one prenatal care visit	46/47 (97.9%)	419/427 (98.1%)	NS

[1] Some women (n = 3) delivered multiple placentae and one placenta was found to be infected, while the other was shown to be uninfected by culture and 16S rRNA PCR. Data from these women with contrasting placental microbiology results were excluded from this analysis

[2] NS – Not statistically significant

[3] Gravida - the number of clinical pregnancies

[4] Parity - the total number of viable offspring resulting from all clinical pregnancies

Adverse pregnancy outcomes

There were no differences in the prevalence of hypertension or pregnancy-induced hypertension in those women (18.9% vs. 17.0%) with or without (26.7% and 26.9%, respectively) chorioamnion infection.

Interestingly, there was no significant difference in the clinical histories or signs/symptoms of infection in women with or without chorioamnion infection; however, women in both cohorts were treated with antibiotics at approximately the same rate (53.2% and 55.7%, respectively). Significantly, those women in whom chorioamnion infection was identified also had a much higher incidence of chorioamnionitis in previous pregnancies (10.6%), when compared to women in whom no infection was identified (3.5%; p = 0.025) (Table 4.6).

Women with UGT infection were more likely to deliver vaginally than women in whom no infection was identified (89.4% vs. 67.6%; p = 0.012); however, there was no significant difference in the prevalence of pPROM or cervical incompetence in women with or without chorioamnion infection (Table 4.6).

Table 4.6. Outcomes for women (n = 474) whose pregnancies were complicated by chorioamnion infection compared to outcomes for women who had no chorioamnion infection

	Chorioamnion infection (n = 47[1])	**No chorioamnion infection detected (n = 427[1])**	**Significance**
At least one sign/symptom of infection [2]	5/47 (10.6%)	29/427 (6.8%)	NS[3]
Previous history of chorioamnionitis documented	5/47 (10.6%)	15/427 (3.5%)	0.025
Hypertension	7/47 (18.9%)	114/427 (26.7%)	NS
Pregnancy induced hypertension	8/47 (17.0%)	115/427 (26.9%)	NS
Cervical incompetence	26/47 (55.3%)	230/427 (53.9%)	NS
Antibiotics administered during labour	25/47 (53.2%)	238/427 (55.7%)	NS
Preterm premature rupture of membranes (pPROM)	17/47 (36.2%)	158/427 (37.0%)	NS
Mode of delivery			
- **Vaginal**	42/47 (89.4%)	290/427 (67.9%)	0.012
- **Cesarean**	5/47 (10.6%)	135/427 (31.6%)	NS
- **Undisclosed**	0/47 (0.0%)	2/427 (0.5%)	NS

[1] Some women (n = 3) delivered multiple placentae and one placenta was found to be infected, while the other was shown to be uninfected by culture and 16S rRNA PCR. Data from these women with contrasting placental microbiology results were excluded from this analysis

[2] Signs and symptoms of infection included: maternal temperature > 38 °C, uterine or abdominal tenderness, foul-smelling vaginal discharge, maternal tachycardia (> 120 bpm) or fetal tachycardia (> 160 bpm)

[3] NS – Not statistically significant

Adverse neonatal outcomes

Neonates were classified into two groups, those whose chorioamnion was found to be infected and neonates whose placentae were non-infected.

While there were no differences in the mean maternal or fetal stages of inflammation between these cohorts, the presence of histological chorioamnionitis was associated with chorioamnion infection ($p < 0.001$). For these neonates, there were no differences in the gestational age at delivery, the Apgar scores at 1 or 5 minutes, the birth weights and the weight of placentae (Table 4.7). Those infants who were exposed to chorioamnion infections required prolonged oxygen support (> 6 hours) more frequently (21.0%) than those infants who were not exposed to chorioamnion infection (9.0%; $p = 0.009$) (Table 4.7). The infants exposed to chorioamnion infection were also diagnosed with respiratory distress syndrome more often (17.5%) than infants who were not exposed to chorioamnion infection (9.0%), albeit this was not statistically significant. Despite the association between chorioamnion infection and adverse neonatal sequelae, there was no significant difference in the mean length of stay post-delivery (Table 4.7).

Table 4.7. Outcomes for neonates (n = 535 placentae) after pregnancies complicated by chorioamnion infection compared to pregnancies without evidence of chorioamnion infection

	Chorioamnion infection (n = 57)	**No chorioamnion infection detected (n = 478)**	**Significance**
Gestational age at delivery (mean, range)	35.7 ± 0.3 (32 – 41)	35.6 ± 0.1 (32 - 41)	NS[2]
Apgar score[1] – 1 minute (mean, range)	7.6 ± 0.3 (1 – 9)	7.8 ± 0.1 (1 - 10)	NS
Apgar score[1] – 5 minutes (mean, range)	8.7 ± 0.1 (5 – 9)	8.9 ± 0.03 (2 - 10)	NS
Birth weight (mean, range)	2642.4 ± 84.6 (1380 – 3925)	2658.0± 29.8 (1060 - 4530)	NS
Placental weight (mean, range)	441.3 ± 16.2 (199 – 710.7)	428.7 ± 5.8 (132 - 1099)	NS
Chorioamnionitis[3] in current pregnancy	31/57 (54.4%)	90/478 (18.8%)	< 0.001
- **Maternal Stage**	1.6 ± 0.1 (1 – 3)	1.2 ± 0.02 (1 – 3)	NS
- **Fetal Stage**	2.4 ± 0.1 (1 – 3)	2.2 ± 0.04 (1 – 3)	NS
Male : Female	24 : 33	252 : 225[4]	NS
Continuous positive airway pressure (CPAP)	12/57 (21.0%)	48/478 (10.0%)	NS
Features of RDS[5] < 24 hours after birth	15/57 (26.3%)	93/478 (19.5%)	NS
Required oxygen support for > 6 hrs	12/57 (21.0%)	43/478 (9.00%)	0.009
Diagnosed RDS[5]	10/57 (17.5%)	43/478 (9.00%)	NS
Length of Stay	7.0 ± 1.1 (1 – 37)	6.1 ± 0.3 (1 - 43)	NS

[1] Apgar - a measure of neonatal health upon delivery and measures of appearance, pulse, reflex, activity and respiration.

[2] NS – Not statistically significant

[3] Chorioamnionitis was determined by US pathologists according to (Redline *et al.* 2003)

[4] The sex of one infant was not disclosed

[5] RDS - respiratory distress syndrome

Ureaplasma spp. and other microorganisms and their association with adverse pregnancy and neonatal outcomes

To compare the outcomes of women infected with specific microorganisms, pregnancies with chorioamnion infection were further divided into two groups: those in which the placentae were infected with *Ureaplasma* spp., the most prevalent microorganisms detected within the chorioamnion, and those with chorioamnion infection caused by other microorganisms. It was not possible to further divide those women with 'other microorganisms' into individual groups due to the small sample sizes of women affected by each individual microorganism isolated.

Maternal demographic data

Women with chorioamnion infection caused by *Ureaplasma* spp. had a lower maternal age (24.2 ± 0.8 yrs) than women infected with other microorganisms (28.2 ± 1.4 yrs) and women who had no chorioamnion infection (27.8 ± 0.3 yrs; $p = 0.002$).

There was no other significant differences in maternal demographic data for women in whom other microorganisms were identified, when compared to those women in whom no microorganisms were detected (Table 4.8).

Table 4.8. Demographic data for women (n = 470) with chorioamnion infection caused by *Ureaplasma* spp., or other microorganisms compared to pregnancies that were not affected by chorioamnion infection

	***Ureaplasma* spp. detected (n = 31[1])**	**Significance**	**Other microorganisms detected (n = 12[1])**	**Significance**	**No microorganisms detected (n = 427[1])**
Maternal age (mean, range)	24.2 ± 0.8 (17 - 32)	0.002	28.2 ± 1.4 (21 – 38)	NS[2]	27.8 ± 0.3 (15 – 43)
Gravida[3] (mean, range)	2.12 ± 0.2 (1 - 5)	NS	2.3 ± 0.3 (1 – 4)	NS	2.5 ± 0.1 (1 – 11)
Parity[4] (mean, range)	1.7 ± 0.2 (1 - 4)	NS	2.3 ± 0.3 (1 – 3)	NS	2.1 ± 0.1 (1 – 10)
Marital Status					
- **Single**	22/31 (70.9%)	NS	9/12 (75.0%)	NS	232/427 (54.3%)
- **Married**	9/31 (29.0%)	NS	3/12 (25.0%)	NS	190/427 (44.5%)
- **Undisclosed**	0/31 (0.0%)	NS	0/12 (0.0%)	NS	5/427 (1.2%)
Health insurance					
- **Private**	10/31 (32.2%)	NS	7/12 (58.3%)	NS	247/427 (57.9%)
- **Medicaid**	17/31 (54.8%)	NS	4/12 (33.3%)	NS	160/427 (37.5%)
- **Self pay/uninsured**	2/31 (6.5%)	NS	0/12 (0.0%)	NS	16/427 (3.7%)
- **Undisclosed**	2/31 (6.5%)	NS	1/12 (8.3%)	NS	4/427 (0.9%)
Ethnicity					
- **Caucasian**	17/31 (54.8%)	NS	8/12 (66.7%)	NS	289/427 (67.8%)
- **African-American**	13/31 (42.0%)	NS	4/12 (33.3%)	NS	104/427 (24.3%)
- **Mixed**	1/31 (3.2%)	NS	0/12 (0.0%)	NS	25/427 (5.8%)
- **Asian**	0/31 (0.0%)	NS	0/12 (0.0%)	NS	8/427 (1.9%)
- **Undisclosed**	0/31 (0.0%)	NS	0/12 (0.0%)	NS	1/427 (0.2%)
Evidence of one prenatal care visit	31/31 (100.0%)	NS	12/12 (100.0%)	NS	419/427 (98.1%)

[1] Some women delivered multiple infants/placentae in which one placenta was found to be infected, while the other was not (n = 3); or placentae were found to contain both ureaplasmas and other microorganisms (n = 4). As a consequence, data from n = 7 women were excluded from this analysis

[2] NS – Not statistically significant

[3] Gravida - total number of clinical pregnancies

[4] Parity - the total number of viable offspring resulting from all clinical pregnancies

Data analysed by ANOVA tests to identify differences among each group/cohort above.

Adverse pregnancy outcomes

There were no differences in the incidence of adverse pregnancy outcomes between women infected with *Ureaplasma* spp. within the chorioamnion, those infected with other microorganisms within the chorioamnion and those women in whom no infection was identified (Table 4.9).

Table 4.9. Pregnancy outcomes for women (n = 470) with chorioamnion infection caused by *Ureaplasma* spp. or other microorganisms, compared to those histories of women who were not exposed to chorioamnion infection

	***Ureaplasma* spp. detected (n = 31[1])**	**Significance**	**Other microorganisms detected (n = 12[1])**	**Significance**	**No microorganisms detected (n = 427[1])**
At least one sign/symptom of infection[2]	3/31 (9.7%)	NS[3]	1/12 (8.3%)	NS	29/427 (6.8%)
Previous history of chorioamnionitis documented	3/31 (9.7%)	NS	1/12 (8.3%)	NS	15/427 (3.51%)
Hypertension	2/31 (6.4%)	NS	3/12 (25.0%)	NS	114/427 (26.7%)
Pregnancy induced hypertension	3/31 (9.7%)	NS	3/12 (25.0%)	NS	115/427 (26.7%)
Cervical incompetence	21/31 (67.7%)	NS	6/12 (50.0%)	NS	230/427 (53.9%)
Antibiotics administered during labour[4]	16/31 (51.6%)	NS	8/12 (66.7%)	NS	238/427 (55.7%)
Preterm premature rupture of membranes (pPROM)	12/31 (38.7%)	NS	5/12 (41.7%)	NS	158/427 (37.0%)
Mode of delivery					
- **Vaginal**	27/31 (87.1%)	NS	11/12 (91.7%)	NS	290/427 (67.9%)
- **Cesarean**	4/31 (12.9%)	NS	1/12 (8.3%)	NS	135/427 (31.6%)
- **Undisclosed**	0/31 (0.0%)	NS	0/12 (0.0%)	NS	2/427 (0.5%)

[1] Some women delivered multiple infants/placentae in which one placenta was found to be infected, while the other was not (n = 3); or placentae were found to contain both ureaplasmas and other microorganisms (n = 4). As a consequence, data from n = 7 women were excluded from this analysis

[2] Signs and symptoms of infection included: maternal temperature > 38 °C, uterine or abdominal tenderness, foul-smelling vaginal discharge, maternal tachycardia (> 120 bpm) or fetal tachycardia (> 160 bpm)

[3] NS – not statistically significant

[4] While the antibiotic dose and type were not recorded, antibiotics were administered to women more than 3 hours prior to delivery.

Data analysed by ANOVA tests to identify differences among each group/cohort above.

Adverse neonatal outcomes

The outcomes of neonates born after pregnancies were affected by chorioamnionitis due to *Ureaplasma* spp. and other microorganisms were also compared to neonates delivered from pregnancies in which no chorioamnion infection was identified.

The most significant finding of this current study was that chorioamnion infection with *Ureaplasma* spp., but not caused by other microorganisms, was associated with the development of histological chorioamnionitis ($p < 0.001$). While there was no difference in the maternal or fetal stages of inflammation between any of these cohorts, pregnancies exposed to *Ureaplasma* spp. had a higher incidence of chorioamnionitis (68.4%) overall. Interestingly, the presence of ureaplasmas within the chorioamnion was not always associated with inflammation and 33.3% of placentae demonstrated no histological chorioamnionitis. Of the *Ureaplasma* spp.-infected placentae, 34.2% showed evidence of mild chorioamnionitis (maternal grade 1) and 31.6% of placentae demonstrated severe histological chorioamnionitis (maternal grade 2 or 3) (Figure 4.4).

Chorioamnion infection with microorganisms other than *Ureaplasma* spp. was associated with the development of RDS ($p = 0.016$). While there was a higher incidence of sequelae in neonates exposed to *Ureaplasma* spp. chorioamnion infection and also to other organisms, there was no difference in the mean length of stay in the neonatal ward for these babies, when compared to the stay for babies who were not exposed to chorioamnion infection.

Table 4.10. Adverse outcomes for neonates (n= 527) delivered after pregnancies affected by chorioamnionitis caused by *Ureaplasma* spp. and other microorganisms, compared to outcomes for neonates who were not exposed to infection during pregnancy

	***Ureaplasma* spp. detected (n = 38[1])**	**Significance**	**Other microorganisms detected (n = 15[1])**	**Significance**	**No microorganisms detected (n = 478[1])**
Gestational age at delivery (mean, range)	35.4 ± 0.3 (32 - 40)	NS [2]	35.9 ± 0.7 (33 - 41)	NS	25.6 ± 0.1 (32 - 41)
Apgar score [3]– 1 minute (mean, range)	7.9 ± 0.3 (1 - 9)	NS	7.2 ± 0.6 (1 - 9)	NS	7.8 ± 0.1 (1 - 10)
Apgar score [3] – 5 minutes (mean, range)	8.8 ± 0.1 (7 - 9)	NS	8.5 ± 0.3 (5 - 9)	NS	8.8 ± 0.1 (2 - 10)
Birth weight (mean, range)	2489.6 ± 88.53 (1380 - 3873)	NS	2901.3 ± 180.3 (1865 - 3925)	NS	2700.6 ± 51.4 (1060 - 4530)
Placental weight (mean, range)	445.2 ± 20.5 (260 - 711)	NS	432.4 ± 30.2 (199 - 617)	NS	428.6 ± 5.8 (132 - 1099)
Chorioamnionitis [4] in current pregnancy	26/38 (68.4%)	< 0.001	4/15 (26.7%)	NS	90/478 (18.8%)
- **Maternal stage inflammation**	1.6 ± 0.1 (1 - 3)	NS	1.5 ± 0.1 (1 - 2)	NS	1.2 ± 0.1 (1 - 3)
- **Fetal stage inflammation**	2.4 ± 0.1 (1 - 3)	NS	3[5]	NS	2.2 ± 0.1 (1 - 3)
Male : Female	15 : 23	NS	7 : 8	NS	252 : 225[6]
Continuous positive airway pressure (CPAP)	8/38 (21.0%)	NS	4/15 (26.7%)	NS	56/478 (11.7%)
Features of RDS[7] < 24 hours after birth	9/38 (23.7%)	NS	4/15 (26.7%)	NS	93/478 (19.4%)
Required oxygen support for > 6 hrs	7/38 (18.4%)	NS	4/165 (26.7%)	NS	43/478 (9.0%)
Diagnosed RDS[7]	6/38 (15.8%)	NS	3/15 (20.0%)	0.016	43/478 (9.0%)
Length of Stay	6.5 ± 1.2 (1 - 37)	NS	7.9 ± 2.2 (1 - 24)	NS	6.1 0.3 (1 - 43)

[1] Some placentae (n = 4) were found to have polymicrobial infections and therefore could not be grouped into a single cohort in the above table. These placentae were excluded from this analysis

[2] NS – not statistically significant

[3] Apgar - a measure of neonatal health upon delivery and measures of appearance, pulse, reflex, activity and respiration.

[4] Chorioamnionitis was determined by US pathologists according to (Redline *et al.* 2003)

[5] only one placenta in this group was graded, therefore there was no SEM or range

[6] The sex of one infant was not disclosed

[7] RDS – respiratory distress syndrome

Data analysed by ANOVA tests to identify differences among each group/cohort above.

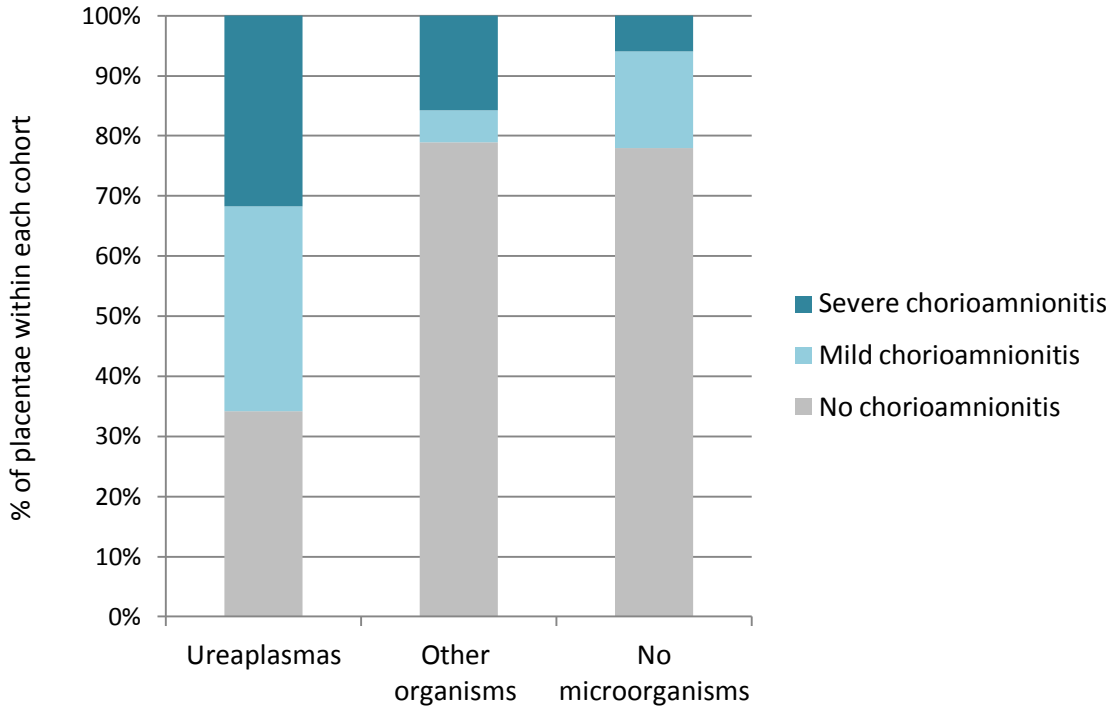


Figure 4.4. Severity of histological chorioamnionitis in pregnancies affected by chorioamnion infection with *Ureaplasma* spp. (n = 38), other microorganisms (n = 15) and those pregnancies in which no chorioamnion infection was identified (n = 478). Some placentae (n = 4) were not included in this analysis, due to conflicting microbiology results. Chorioamnionitis was determined by US pathologists (blinded to outcome) and graded according to Redline's criteria (2003)

Discussion

UGT infections in pregnant women are a leading aetiology of PTB, with up to 40% of all PTBs associated with infection (DiGiulio 2012). While there is a strong body of evidence which identifies UGT infections in association with very early PTBs (< 32 weeks), currently no studies have reported the prevalence of UGT infection later in gestation, in pregnancies delivered LPT (32 – 36 weeks) and at term. In this study, for the first time, we have investigated the prevalence of chorioamnion infection in women who delivered LPT or at term and identified that chorioamnion infection was present in 10.6% of all pregnancies studied. This supports the proposal by Goldenberg *et al.* (2011) that 10 – 15% of deliveries >32 weeks are associated with infection. This study confirmed that there were no major differences in the prevalence of adverse outcomes or signs/symptoms of infection in women with chorioamnionitis caused by *Ureaplasma* spp. or other microorganisms and women with no evidence of infection (Table 4.9). This highlights why it is so difficult to identify and treat women with asymptomatic chorioamnionitis during pregnancy.

In this current study we have also identified that a history of chorioamnionitis in previous pregnancies ($p = 0.025$), correlated with histological chorioamnionitis in the current pregnancy ($p < 0.001$; Table 4.6) and that neonates exposed to UGT infection during pregnancy required oxygen or positive pressure support more frequently after delivery ($p = 0.009$; Table 4.7). The results of this study are similar to the findings of other researchers who focused on very PTBs and demonstrated that UGT infection was associated with decreased maternal age (Hillier *et al.* 1988), the development of histological chorioamnionitis (Hillier *et al.* 1988; Aaltonen *et al.* 2007; Hecht *et al.* 2008; Kasper *et al.* 2010) and adverse respiratory outcomes for neonates (Abele-Horn *et al.* 1997b; Cultrera *et al.* 2006; Gwee *et al.* 2013).

Significantly, this current study demonstrated that chorioamnion infection was associated with a history of chorioamnionitis in prior pregnancies, but was not a cause of LPT birth. Previous studies have also demonstrated that the most significant indicator of adverse outcomes in a current pregnancy is a past history of adverse pregnancy outcomes (Goldenberg *et al.* 2008b). Similarly, the most important predictor of LGT *Streptococcus agalactiae* (Group B Streptococcus or GBS) carriage during pregnancy is a prior history of harbouring this microorganism in the LGT during prior pregnancies (Di Renzo *et al.* 2014) and the most important indicator of delivering preterm in a current pregnancy is a prior PTB (Bloom *et al.* 2001). To the best of our knowledge, this is the first study to demonstrate an association between chorioamnion infection in a current pregnancy and a past history of chorioamnionitis. Previous studies have identified microorganisms within the

endometrium of reproductive-age women (Cassell *et al.* 1993b) and based on these findings, we propose that the female UGT (of both pregnant and non-pregnant women) may be colonised with microorganisms for prolonged periods. This may also explain the significant finding we reported for the first time, that women experience recurrent infections and adverse pregnancy outcomes, including histological chorioamnionitis, over multiple pregnancies.

Of the microorganisms isolated, the human *Ureaplasma* spp. were the most prevalent microorganisms identified within the chorioamnion, accounting for more than 70% of all clinical isolates within this study. The other microorganisms isolated within this study included *S. agalactiae* (GBS)*, Bacteroides* spp., *Bifidobacterium* spp., *G. vaginalis*, *E. coli, Propionibacterium* spp. and uncultured bacteria. Previously, studies have reported the presence of contaminating microorganisms in studies of placentae after very early PTBs, either from environmental sources or as a consequence of vaginal delivery (Leitich *et al.* 2003) and so it is first necessary to determine if the microorganisms isolated within our study are consistent with infection/colonisation of the chorioamnion. For this study, we defined 'infection' as the invasion of microorganisms accompanied by a reaction of the host (e.g. inflammation) (Schultz *et al.* 2003; Edwards and Harding 2004) and while many of the microorganisms isolated within our study are often found as normal flora of the female LGT (e.g. *Ureaplasma* spp., *S. agalactiae, Bacteroides* spp., *Bifidobacterium* spp., *G. vaginalis* and *Propionibacterium* spp.) (Anderson *et al.* 2013), ascending invasive infections are the most accepted route of intraamniotic infections (Kim *et al.* 2009). Therefore, the presence of these microorganisms within the chorioamnion is not unexpected and we further demonstrated that the presence of these microorganisms was associated with chorioamnionitis, strongly suggesting that these microorganisms found within chorioamnion tissue are consistent with infection and not contamination. Additionally, many of the microorganisms identified within our study have been implicated as a cause of intraamniotic infection in previous studies of very PTBs and have also been associated with bacterial vaginosis and a two-fold increase in the likelihood of delivering preterm (Nejad and Shafaie 2008). While 70% of the women in this study delivered vaginally, we identified no *Lactobacillus* spp. within any chorioamnion tissue specimens. *Lactobacillus* spp. have been isolated in more than 90% of vaginal specimens and the presence of this microorganism within placental tissue is consistent with contamination and not infection. Taken together, these results suggest that the aseptic sampling techniques used within our study prevented the contamination of chorioamnion tissues and that the microorganisms detected within our study are aetiological agents of chorioamnion infection and inflammation.

Within this current study, we also sought to determine the microorganisms most commonly associated with adverse pregnancy and neonatal outcomes. Women infected with *Ureaplasma* spp. were significantly younger (24.1 ± 07 yrs) than those women infected with other microorganisms (26.3 ± 1.3 yrs) or who were uninfected (27.8 ± 0.3 yrs; p = 0.002).

For neonates, the diagnosis of respiratory distress syndrome was correlated with chorioamnion infection caused by other microorganisms (p = 0.016) but not with *Ureaplasma* spp. RDS is an acute respiratory condition associated with poor neonatal lung compliance following birth and is commonly reported for neonates delivered in the LPT period (Ramachandrappa and Jain 2009). Studies have demonstrated poor respiratory outcomes for neonates exposed to infections at < 32 weeks of gestation (Benstein *et al.* 2003; Viscardi *et al.* 2006; Payne *et al.* 2010; Collins *et al.* 2013; Eun *et al.* 2013) and it is interesting that in this current study there was no association between *Ureaplasma* spp., the most common isolate in our study, and RDS. However, this is not surprising as previous studies in an ovine model have demonstrated that chronic fetal exposure to *Ureaplasma* spp. *in utero* resulted in improved neonatal lung gas volumes and increased production of surfactant, indicating that lung maturation was occurring (Moss *et al.* 2005; Kramer *et al.* 2009; Knox *et al.* 2010; Robinson *et al.* 2013). There are currently no studies which demonstrate similar respiratory outcomes in human neonates, and so further studies which test nasopharyngeal aspirates to determine if ureaplasmas are present at this site may determine if *Ureaplasma* spp. affect the LPT neonatal lung.

The most significant finding of this current study was that chorioamnion infection with *Ureaplasma* spp., but not other microorganisms, was associated with the development of histological chorioamnionitis (p < 0.001). While we saw no significant association between the incidence of histological chorioamnionitis and LPT birth or the presence of other microorganisms; we did see a significant association between the presence of *Ureaplasma* spp. within the chorioamnion and chorioamnionitis (67.5%; p < 0.001). Previous studies have reported an association between infection with ureaplasmas and the development of histological chorioamnionitis (Table 4.11). Originally, Shurin *et al.* (1975) demonstrated that vaginal colonisation with *Ureaplasma* spp. was significantly associated with the development of histological chorioamnionitis during pregnancy, and these researchers suggested that it may be possible for these microorganisms to access the UGT at the time of pregnancy. Kundsin *et al.* (1984) identified that 53% of chorioamnion specimens contained *Ureaplasma* spp., and of these, 60% had evidence of histological chorioamnionitis.

Table 4.11. Previous of studies which demonstrate an association between *Ureaplasma* spp. and histological chorioamnionitis. While the prevalence of *Ureaplasma* spp. varies within these studies, the development of histological chorioamnionitis was consistently between 40 – 100% of all pregnancies, regardless of the gestational age at delivery

Author	Gestational age	Specimen	Number	*Ureaplasma* spp. positive[1]	*Ureaplasma* spp. with chorioamnionitis[1]	*Ureaplasma* spp. without chorioamnionitis[1]
Shurin *et al.* (1975)	all births	Vaginal cultures	244	54 (22.1%)	32 (59.2%)	22 (40.7%)
Yoon *et al.* (2003)	≤ 35 weeks	Amniotic fluid	257	23 (9.0%)	15/20 (75%)	5/20 (25%)
Park *et al.* (2013)	< 34 weeks	Amniotic fluid	213	35 (16.4%)	7/16 (43.7%)	-
Berger *et al.* (2009)	< 28 weeks	Amniotic fluid or placental tissue	435	32 (7.3%)	11/25 (44.0%)	3/46 (6.5%)
Goldenberg *et al.* (2008)	23 – 32 weeks	Cord blood	351	43 (12.2%)	33 (76.9%)	-
Viscardi *et al.* (2008)	< 33 weeks	Cord blood	313	46 (14.7%)	30 (65.0%)	-
Kundsin *et al.* (1984)	< 37 weeks	Placental tissue	156	53 (34.0%)	32 (60.4%)	21 (39.6%)
Hillier *et al.* (1988)	< 37 weeks	Placental tissue	94	32 (34.0%)	19/29 (65.5%)	10/65 (15.4%)
Van Marter *et al.* (2002)	< 36 weeks	Placental tissue	206	58 (28.1%)	51 (87.9%)	7 (12.1%)
Miralles *et al.* (2005)	< 33 weeks	Placental tissue	14	6 (42.8%)	6 (100%)	-
Egawa *et al.* (2007)	< 32 weeks	Placental tissue	83	4 (4.8%)	4 (100%)	-
Hecht *et al.* (2008)	< 28 weeks	Placental tissue	1292	-	7 (70%)	3 (30%)
Olomu *et al.* (2009)	< 28 weeks	Placental tissue	866	52 (6%)	33 (65%)	-
Namba *et al.* (2010)	≤ 32 weeks	Placental tissue	151	63 (42%)	52 (83.0%)	11 (17.0%)
Sweeney *et al.* (current study)	> 32 weeks	Chorioamnion tissue	535	42 (7.8%)	28/42 (66.6%)	14/42 (33.3%)

[1] Information not available for all placentae within study. Available data has been shown from each study

Since these first reports, other studies have consistently demonstrated that the presence of *Ureaplasma* spp. in amniotic fluid (Yoon *et al.* 2003a; Berger *et al.* 2009; Park *et al.* 2013) and chorioamnion tissues (Kundsin *et al.* 1984; Hillier *et al.* 1988; Miralles *et al.* 2005; Egawa *et al.* 2007; Hecht *et al.* 2008; Berger *et al.* 2009; Olomu *et al.* 2009; Namba *et al.* 2010) correlated with histological chorioamnionitis. The prevalence of histological chorioamnionitis within *Ureaplasma* spp. positive placentae varied from 43.7% to 100% (Table 4.11). Similarly, several studies have also found that *Ureaplasma* spp. isolated from cord blood was associated with histological chorioamnionitis (Goldenberg *et al.* 2008a; Viscardi *et al.* 2008) and the presence of *Ureaplasma* spp. within cord blood may indicate a systemic spread of these organisms into the umbilical cord blood supply (Table 4.11). The major limitation of these studies was that they focused on the very early PTBs (< 32 weeks), when the majority of infections are often polymicrobial (Hillier *et al.* 1988; Jalava *et al.* 1996; Hecht *et al.* 2008; Onderdonk *et al.* 2008; Marconi *et al.* 2011). As a result, researchers have not been able to confidently claim that these microorganisms are true aetiological agents of histological chorioamnionitis. However, in our large study of LPT and term placentae we have demonstrated for the first time that infection with *Ureaplasma* spp. is independently associated with the development of histological chorioamnionitis, regardless of gestational age (LPT or term).

While the presence of *Ureaplasma* spp. in this study was associated with the development of histological chorioamnionitis, we also assessed if the numbers of ureaplasmas present had an effect on the development of histological chorioamnionitis. Interestingly, there was no significant difference in the mean CFU of ureaplasmas present within placentae for pregnancies with or without histological chorioamnionitis. Within this study, we were only able to sample small sections of the placenta (not the entire placenta itself), which may be why we do not see a clear relationship between the numbers of microorganisms present and the severity of inflammation, these findings may also suggest that there are other factors which influence the development of histological chorioamnionitis. These findings are again in contrast to other studies, which have demonstrated a correlation between the mean CFU of *Ureaplasma* spp. and the severity of histological chorioamnionitis (Jacobsson *et al.* 2009; Kasper *et al.* 2010; Kacerovsky *et al.* 2011). Further studies that investigate the presence and concentration of innate and adaptive immune factors are required to determine if there are other host and/or microbe factors which may influence the development of chorioamnionitis.

UGT infection was demonstrated in ~11% of pregnancies within our current study. However, the question remains as to the other likely causes or aetiologies of birth in the LPT period. Some known precursors and risk factors (aside from infection) include: spontaneous preterm labour or pPROM, cervical incompetence, preeclampsia/eclampsia, placenta previa/accrete, multiple gestation pregnancies, intrauterine growth restriction, prior Cesarean section delivery, chorioamnionitis, fetal distress and/or adverse maternal outcomes requiring medically indicated preterm deliveries (Goldenberg *et al.* 2008b; Holland *et al.* 2009). Within our study population, many of these factors (which may predispose to PTB) were present. Of the women in our study, only 3.4% had evidence of a prior preterm delivery, while 32.9% experienced pPROM and 26.7% underwent Cesarean section delivery. Only 2.0% of these Cesarean section deliveries were medically indicated; while for the remainder it was not always clear if these deliveries were elective or medically indicated. Cervical incompetence was prevalent within our study population and was present in 48.2% of women. Preeclampsia and haemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome were also present in 16.1% and 1.1% of our study participants. Other important factors, such as fetal distress, intrauterine growth restriction, placental abruption and non-reassuring fetal signs were present in this study; however their incidence was quite low (3.2%, 4.1%, 2.2% and 1.1%, respectively). By contrast, 22.6% of women in our study had evidence of histological chorioamnionitis. Multiple gestation pregnancies were also quite common in this study, with 54 sets of twins and two sets of triplets all delivered in the LPT period. Given the presence of these factors, it is probable that a combination of these various precursors/risk-factors contribute to delivery in the LPT period, and as such, the aetiology of LPT birth is likely to be multifactorial. Similarly, Holland *et al.* (2009) investigated those LPT births which were deemed to be unavoidable and those which were elective and identified that >80% of all LPT births were unavoidable, due to outcomes similar to those mentioned above (preeclampsia, HELLP syndrome, placental abruption, intrauterine growth restriction etc.).

To the best of our knowledge, this is the first study to report the prevalence of chorioamnion infection in the LPT period and ~11% of LPT and term pregnancies were affected by infection. Greater than 70% of all PTBs worldwide occur in the LPT period (Laws *et al.* 2010; March of Dimes 2012); therefore, the number of infants potentially exposed to chorioamnion infection in this period is far greater (1.3 million per year) than the number of infants delivered at less than 32 weeks of gestation (702 000; See table 4.12). These findings highlight that irrespective of gestation, infection during pregnancy is an important finding. UGT infection at any stage of pregnancy can have severe

adverse outcomes for the mother and for the child and these findings should be a major consideration for obstetricians.

Table 4.12. Estimated number of pregnancies affected by intraamniotic infection in both early and late preterm gestation

	Early PTB (< 31 weeks)	Late PTB (32 – 36 weeks)
Prevalence in the preterm population[1]	15.6%	84.4%
Total number of pregnancies[2]	2.3 million	12.7 million
Rate of UGT infection[3]	Approx. 30%	10.4%
Number of births potentially affected by infection[4]	702 000	1.3 million

[1] Statistics from Li *et al* 2011

[2] Number of births estimated by multiplying the total number infants delivered preterm (15 million annually (Bick 2012) by the total prevalence (percentage) of each population.

[3] Early PTB rate of intraamniotic infection as quoted in (March of Dimes 2012), while LPT birth rate has been identified within the present study.

[4] Calculated values based on proportion of intraamniotic infection within each populations.

While this study has greatly extended our understanding of chorioamnion infection in the LPT and term period, there are some limitations within this study which must be addressed. More than 50% of women in this study were treated with intrapartum antibiotics and the dosage and identity of these antibiotics were not recorded within the database. However, we utilised both culture and molecular detection and identification techniques and we isolated both cultivable and non-cultivable bacteria. The use of antibiotics for prolonged period (> 3 hrs) during labour may have decreased the overall numbers of microorganisms isolated from these placentae and therefore the true rate of chorioamnion infection in the LPT period may in fact be higher than is reported within this study. While we were able to isolate/detect microorganisms, including uncultured bacterium, from these placentae, further investigation using more sensitive techniques, such as deep sequencing, may be useful in determining the true prevalence of infection in the LPT and term period, in order to fully characterise infection throughout gestation.

The major strength of this study is that chorioamnion tissue was the specimen of choice. The use of this tissue, coupled with the proven aseptic collection techniques meant that we were able to isolate microorganisms from the chorioamnion and that the presence of these microorganisms was directly correlated with chorioamnionitis at the same anatomical site. Additionally, the low prevalence of

polymicrobial infections within this data set meant that we were able to clearly demonstrate differences in the sequelae incurred by women and infants infected with *Ureaplasma* spp. or other microorganisms, which has not always been possible in previous studies focused on PTBs, which occur at less than 32 weeks of gestation. Finally, histological chorioamnionitis was graded by United States pathologists based on Redline's criteria (Redline *et al.* 2003), which is a well-accepted and highly published method for grading chorioamnionitis. The presence of chorioamnionitis was determined prior to any microbiological testing, thereby reducing any chance of influencing the data set. This study also confirmed that LPT infants experience more adverse sequelae than their term-born counterparts, including respiratory distress syndrome, CPAP ventilation or respiratory support in the form of required oxygen support for > 6 hours in the first 24 hours of life. These findings correlate with other studies that are now prevalent within the literature (Adams-Chapman 2006; Darnall *et al.* 2006; Engle and Kominiarek 2008; Colin *et al.* 2010; Hibbard *et al.* 2010; Berard *et al.* 2012; Boyle and Boyle 2013).

Conclusion

The presence of chorioamnion infection in LPT and term placentae is an important finding and should be a major consideration for obstetricians. This current study demonstrated that chorioamnion infection directly correlates with inflammation of the chorioamnion (chorioamnionitis) and adverse pregnancy and neonatal outcomes, including neonatal respiratory sequelae. We also determined that the presence of current chorioamnion infection correlated with a history of chorioamnionitis in prior pregnancies, confirming that this is a risk factor and suggests that these organisms may colonise/infect the endometrium for chronic periods.

This is also the first study to determine that *Ureaplasma* spp. were independently associated with the development of histological chorioamnionitis. Interestingly, while the prevalence of histological chorioamnionitis was higher in *Ureaplasma* spp.-affected pregnancies, there was no correlation between the number of ureaplasma CFU and the severity of inflammation, suggesting that other host and/or microbe interactions may influence the development of histological chorioamnionitis. Further studies which focus specifically on the host/microbe interactions and the development of histological chorioamnionitis are required to fully elucidate the pathogenesis of *Ureaplasma* spp. infections during pregnancy.

Chapter Five:

Characterisation of *Ureaplasma* spp. from late preterm and term placentae

Introduction

Ureaplasma spp. are prevalent colonisers of the lower genital tract (LGT) and are found in up to 80% of females and 50% of males (Cassell *et al.* 1993b; Volgmann *et al.* 2005). These organisms are considered to be of low virulence; however they are capable of causing serious infections. During pregnancy, *Ureaplasma* spp. can ascend and invade the upper genital tract (UGT) and are associated with spontaneous abortion and miscarriage (Robertson *et al.* 1986; Joste *et al.* 1994), preterm birth (PTB) (Hillier *et al.* 1988; Gerber *et al.* 2003; Goldenberg *et al.* 2008a) and preterm premature rupture of membranes (pPROM) (Kacerovsky *et al.* 2011). Previous studies have also identified *Ureaplasma* spp. as the most prevalent microorganisms isolated from placentae with histological chorioamnionitis (Hillier *et al.* 1988; Eschenbach 1993; Aaltonen *et al.* 2007; Berger *et al.* 2009; Kasper *et al.* 2010; Namba *et al.* 2010; Czikk *et al.* 2011); in Chapter Four we have demonstrated for the first time that *Ureaplasma* spp. are independently associated with histological chorioamnionitis, regardless of gestational age (late preterm or at term). These microorganisms are also capable of causing severe neonatal outcomes, including sepsis (Waites *et al.* 1993), meningitis (Gwee *et al.* 2013), bronchopulmonary dysplasia (BPD) (Viscardi *et al.* 2002; Kasper *et al.* 2010; Sung *et al.* 2010) and an increased risk of developing of cerebral palsy (Berger *et al.* 2009).

While up to 42% of women may have *in utero* infection with *Ureaplasma* spp. during pregnancy (Miralles *et al.* 2005), the presence of these microorganisms is not always associated with the development of histological chorioamnionitis or adverse pregnancy outcomes. In particular, a study by Gerber *et al.* (2003) identified that of the women with intraamniotic infections caused by *Ureaplasma* spp. (n = 29), only 21% developed pPROM, 24% experienced PTB and more than 70% went on to deliver at term with no apparent adverse pregnancy outcomes. It is currently unclear why only some women experience adverse pregnancy outcomes, but it has been suggested that virulent species or serovars of *Ureaplasma* spp. may be associated with adverse pregnancy outcomes. The human *Ureaplasma* spp. are categorised into two distinct species and 14 serovars: *U. parvum* is comprised of serovars 1, 3, 6 and 14; while *U. urealyticum* is comprised of serovars 2, 4, 5 and 7 - 13 (Robertson *et al.* 2002) and studies have identified some ureaplasma serovars as being associated with adverse pregnancy or neonatal outcomes. *U. urealyticum* serovar 4 has been associated with recurrent miscarriages (Naessens *et al.* 1988), while *U. urealyticum* serovar 9 was found to be the most prevalent ureaplasma serovar isolated from tracheal and gastric aspirates of infants (Eun *et al.* 2013). By contrast, Sung *et al.* (2011) detected *U. parvum* serovars 3 and 6 as the most prevalent serovars in respiratory secretions of infants, and their presence was associated with the development of BPD. Furthermore, *U. parvum* serovars 3 and 6 have been identified as the most

common ureaplasma isolates from the LGT of asymptomatic women (Knox *et al.* 1997; Knox *et al.* 2003); while *U. parvum* serovar 6 was found to be associated with adverse pregnancy outcomes (Knox *et al.* 1998). Interestingly, *U. parvum* serovars 3 and 6 were found to be the most prevalent ureaplasma serovars within semen samples from infertile men in an Australian population and *U. parvum* serovar 6 was found to be the most adherent to sperm and was not always removed by assisted reproductive technology washing procedures (Knox *et al.* 2003). In an ovine model of intraamniotic infection with *U. parvum* serovars 3 or 6, low passage clinical isolates obtained from these infertile men were intraamniotically injected into pregnant ewes and induced chorioamnionitis and colonised the fetal lung tissue (Moss *et al.* 2005; Moss *et al.* 2008; Collins *et al.* 2010; Knox *et al.* 2010; Dando *et al.* 2012; Collins *et al.* 2013; Robinson *et al.* 2013). In these studies, the presence of *Ureaplasma* spp. was not always associated with histological chorioamnionitis and this is similar to what has been reported in human pregnancies affected by ureaplasmas. These results demonstrate that different ureaplasma serovars may be more prevalent in geographically distinct study populations. Different methods were also used to detect, speciate and serotype these clinical isolates. Because of the variability in results of these studies, the question remains as to whether virulence/pathogenicity is species- or serovar-specific; or if it is the lack of consistent speciating/serotyping methods that has resulted in the variation reported by these studies. By designing a simple and effective diagnostic assay, which effectively speciates and serotypes clinical isolates, it may be possible to determine if there are 'virulent' species or serovars of *Ureaplasma* spp., or it may be that there are other virulence determinants that play an important role in the development of adverse pregnancy and neonatal outcomes.

Culture for *Ureaplasma* spp. remains the gold-standard method for detection of these microorganisms within clinical specimens (DiGiulio 2012). However, this is often time-consuming and difficult to perform in clinical and diagnostic laboratories, as these fastidious organisms do not grow on standard bacteriological media. Instead specialised transport and culture media is required and these are not often available commercially and therefore must be produced 'in house'. To improve the detection of *Ureaplasma* spp., conventional and real-time PCR assays have been developed (Knox and Timms 1998; Yi *et al.* 2005; Xiao *et al.* 2010); however, not all of these assays are designed for high-throughput laboratories, they require highly stringent parameters and they are time-consuming. The *Ureaplasma* spp. are not routinely screened for during pregnancy or in placentae after delivery, despite the strong association between *Ureaplasma* spp. and adverse pregnancy outcomes (Australian Government Department of Health and Ageing 2012). By designing and

optimising a detection tool which speciates and serotypes the human *Ureaplasma* spp., it may be possible to identify 'virulent' ureaplasma species and/or serovars.

Others have suggested that 'virulence' may not be limited to particular *Ureaplasma* spp., but that the maternal immune responses may also be crucial to the development of adverse sequelae. Dando *et al.* (2012) demonstrated that chronic intraamniotic *Ureaplasma* spp. infections in an ovine model resulted in the production of anti-ureaplasma IgG antibodies within the maternal serum in some ewes, but not in others. The presence of serum IgG antibodies was correlated with an increased expression of interleukin (IL)-1β, IL-6 and IL-8, but decreased expression of TNF-α and IL-10 within chorioamnion tissue. Similarly, in humans it has been demonstrated that different immune responses were detected, depending on the ethnicity of women (Peltier *et al.* 2012). Chorioamnion tissue derived from Caucasian (n = 6) or African-American (n = 5) women were exposed to *Ureaplasma* spp. Exposure of chorioamnion tissue *in vitro* to *U. parvum* resulted in elevated levels of TNF-α in the Caucasian-derived tissues; while in African-American-derived tissues, *U. parvum* was associated with low levels of IL-10. Additionally, *U. urealyticum* infection induced elevated levels of IL-10 and IL-1β in Caucasian tissues, but induced no immune response in Africa-American chorioamnion tissue (Peltier *et al.* 2012). These findings suggest that differences in the maternal immune response may influence the development and severity of disease and warrant further investigation.

It has also been proposed that women may experience different pregnancy outcomes, depending on antigenic variation of the pathogen. The multiple banded antigen (MBA) is the major antigen recognised by the host immune system in response to ureaplasma infection and is composed of two major domains: an upstream conserved region that is similar in all *Ureaplasma* spp. serovars, and a downstream region that is composed of tandem repeating units which contain both cross-reactive and serovar-specific epitopes (Zheng *et al.* 1995; Zheng *et al.* 1996). Importantly, it is this surface-exposed region that has been shown to vary in size *in vivo* (Zheng *et al.* 1994), and numerous studies by our group have demonstrated *mba*/MBA size variation within a well-established ovine model of ureaplasma intraamniotic infection (Knox *et al.* 2010; Dando *et al.* 2012; Robinson *et al.* 2013). In particular, a study by Knox *et al.* (2010) reported variation in the severity of inflammation of sheep tissues, including the chorioamnion. After pregnant ewes were intraamniotically injected with the same dose of *Ureaplasma* spp., this study showed that after delivery some sheep had no evidence of histological chorioamnionitis, whilst others had moderate or severe inflammation. Interestingly, it was identified that the number of *mba*/MBA size variants expressed by these ureaplasmas was

inversely correlated with the severity of inflammation and histological chorioamnionitis. Based on these findings, it was hypothesised that MBA size variation may be a mechanism by which *Ureaplasma* spp. modulate the host immune response or evade host immune detection/eradication (Knox *et al.* 2010). Importantly, it is yet to be determined if ureaplasmas isolated from human pregnancies are able to vary their surface-exposed MBA and if this has any effect on pregnancy outcomes.

For this current study, we hypothesised that the host may not always recognise *Ureaplasma* spp., due to variation of the surface-exposed MBA. We further hypothesised that MBA variation may be associated with differences in the host immune response. The aims of this study were to (i) develop and optimise a real-time PCR and high resolution melt (HRM) assay to speciate and serotype *U. parvum* clinical isolates and to identify any 'virulent' *U. parvum* serovars; (ii) to assess the presence of MBA/*mba* variation occurring in clinical isolates; and (iii) to characterise the cytokines, chemokines and growth factors within cord blood in response to chorioamnion infection with *Ureaplasma* spp. and other microorganisms. The presence of *Ureaplasma* species and serovars, the degree of MBA/*mba* variation and the levels of cytokines, chemokines and growth factors were correlated with the development of adverse pregnancy and neonatal outcomes.

Materials and Methods:

All methods pertaining to this chapter are summarised in Chapter Three.

Briefly, *Ureaplasma* spp. clinical isolates that were isolated from late preterm placentae (Chapter Four) were stored (see Chapter Three, section 3.4) for use in these experiments. American type culture collection (ATCC) strains of *U. parvum*, *U. urealyticum* and other microorganisms (detailed in Chapter Three, section 3.5) were also used in this chapter.

For real-time PCR and HRM assays, *Ureaplasma* spp. clinical isolates, ATCC strain ureaplasmas and other bacterial isolates were cultured (Chapter Three, sections 3.5 and 3.6.1) and the DNA extracted (Chapter Three, section 3.8). DNA template from each clinical isolate, ATCC strain ureaplasmas and other microorganisms were tested using designed PCR primers (Chapter Three, sections 3.15 and 3.16) targeting the *mba* gene and were validated for their specificity and serotyping abilities (Chapter Three, sections 3.17 and 3.18). Once the ability of the real-time PCR assays to speciate and serotype *U. parvum* serovars 1, 3, 6 and 14 was confirmed, ureaplasma clinical isolates were then serotyped using these designed assays (Chapter Three, section 3.19). Serotyping results were then compared to the results of *mba* gene sequencing (as per Chapter Three, sections 3.9.2, 3.10, 3.11 and 3.12).

The presence or absence of *mba*/MBA size variation of these *Ureaplasma* spp. clinical isolates was also assessed. The proteins of these clinical isolates were extracted (Chapter Three, section 3.6.1) and MBA protein size variation was assessed by western blot (Chapter Three, section 3.13). *mba* gene size variation was also assessed following DNA extraction (Chapter Three, section 3.8.1) and PCR assays which targeted the downstream repetitive region of the *mba* gene (Chapter Three, section 3.14).

Statistical analysis

Data are presented as the mean value, plus the standard error of the mean (SEM). *Ureaplasma* spp. spp. and serovars were correlated with pregnancy outcome data (maternal demographic data, maternal pregnancy outcomes and neonatal outcomes) to determine if some *Ureaplasma* spp. and serovars were more 'virulent' than others. Additionally, the presence/absence of *mba/*MBA size variation was correlated with pregnancy outcome data (as above) and the concentrations of cord

blood cytokines, chemokines and growth factors. Data was analysed using analysis of variance (ANOVA) tests. Statistical significance was accepted as $p < 0.05$.

Results

PCR Primers

Four PCR primer pairs (see Table 5.1) were designed to specifically amplify *U. parvum* at different regions of the *mba* gene (see Supplementary Figure 8.1). Each of the forward (F) primers was optimised with each reverse (R) primer, to produce a total of 16 PCR assays. Six of these primer pairs (see Table 5.2) amplified *U. parvum* serovars without cross-reactivity to the *U. urealyticum* serovars (2, 4, 5, 7 - 13), or to any other microorganisms tested. The primer pairs which demonstrated cross-reactivity were excluded from further analysis and six successful primer pairs were further investigated by real-time PCR.

Table 5.1. Novel PCR primers targeting areas of the multiple banded antigen (*mba*) gene, designed for use in real-time PCR and high resolution melt assays

Primer name	Primer Sequence	Tm	% AT content
UpuF	5' CTAATAATGTTATTGATAATGCAG 3'	55 °C	75%
UpmbaR	5' GTTTTCAATTTCGTAAACTGC 3'	55 °C	67%
UpuF2	5' TTATAATAAAAAATATCTAATAATG 3'	56 °C	92%
UpmbaR2	5' CCAGCTCCAACTAAGGTAAC 3'	56 °C	50%
UpuF3	5' TTATATAATTAAAAGTGCAAGTGC 3'	57 °C	75%
UpmbaR3	5' TTGTTCATTAGGTTTTGGTTCACGA 3'	57 °C	64%
UpuF4	5' GTGCTAAATAAAAAGTATTTGC 3'	57 °C	73%
UpmbaR4	5' CCTGAAGTCTTGATTAATCCAC 3'	57 °C	59%

U. parvum real-time PCR assay optimisation

Using real-time PCR, each of the six PCR assays (UpuF/UpmbaR, UpuF2/UpmbaR, UpuF2/UpmbaR4, UpuF3/UpmbaR4, UpuF4/UpmbaR and UpuF4/UpmbaR4) again demonstrated amplification of only *U. parvum* serovars 1, 3, 6 and 14 and these assays produced a single fluorescent 'peak', consistent with the production of a single PCR amplicon.

The high resolution melt (HRM) curve data generated for each of the six primer pairs were scrutinised and only a single PCR primer pair was able to differentiate the ATCC *U. parvum* serovars 1, 3, 6 and 14 according to the differentiation criteria defined in the methods chapter (see Chapter Three, Section 3.14). The PCR assay utilising UpuF2 and UpmbaR primers demonstrated specificity and no cross-reactivity to any other microorganisms tested (including *U. urealyticum*; Figure 5.1A).

This assay produced a single amplicon as evidenced by the standard melting graph (Figure 5.1B) and differentiated *U. parvum* serovars 1, 3, 6 and 14 by HRM (Figure 5.1C and 5.1D). This real-time PCR and HRM assay was selected for further analysis of *U. parvum* clinical isolates.

Table 5.2. Comparison of real-time PCR assays for the amplification and differentiation of *U. parvum* serovars. Of the 16 assays optimised, only six were able to successfully amplify *U. parvum* serovars and one assay was also able to differentiate *U. parvum* serovars 1, 3, 6 and 14 by high resolution melt.

Primer Pairs	Amplicon Size	Specificity [1]	Cross-reactivity [2]	Serotyping ability [3]
UpuF / UpmbaR	429 bp	Yes	No	No
UpuF / UpmbaR2	314 bp	No	Yes - Kp	-
UpuF / UpmbaR3	594 bp	No	Yes - Uu	-
UpuF / UpmbaR4	631 bp	No	Yes - Kp	-
UpuF2 / UpmbaR	445 bp	Yes	No	Yes
UpuF2 / UpmbaR2	330 bp	No	Yes - Uu	-
UpuF2 / UpmbaR3	610 bp	No	Yes - Uu	-
UpuF2 / UpmbaR4	647 bp	Yes	No	No
UpuF3 / UpmbaR	373 bp	No	Yes - Uu	-
UpuF3 / UpmbaR2	258 bp	No	Yes - Uu	-
UpuF3 / UpmbaR3	538 bp	No	Yes - Sa, Kp	-
UpuF3 / UpmbaR4	575 bp	Yes	No	No
UpuF4 / UpmbaR	353 bp	Yes	No	No
UpuF4 / UpmbaR2	238 bp	No	Yes - Uu	-
UpuF4 / UpmbaR3	518 bp	No	Yes - Uu	-
UpuF4 / UpmbaR4	555 bp	Yes	No	No

[1] Specificity – amplifies *U. parvum* serovars only.

[2] Cross-reactivity – other species that were amplified when the PCR assay was not specific to *U. parvum*. Uu – *U. urealyticum*, Kp – *Klebsiella pneumoniae*, Sa – *Streptococcus agalactiae*.

[3] Serotyping ability – the ability of each primer pair to amplify only *U. parvum* serovars 1, 3 6 and 14; and to differentiate each of the four serovars by HRM analysis (see Chapter three, section 3.18 for serotyping guidelines).

Efficacy of *U. parvum* real-time PCR and HRM assay for serotyping of clinical isolates

Clinical isolates of *U. parvum* (n = 55) were tested using the real-time PCR and HRM assay (with primers UpuF2 and UpmbaR), and of these isolates, 31 (56.4%) were amplified using the optimised assay. Ureaplasmas that had been cultured *in vitro* were detected and serotyped using this assay; however, many of the clinical specimens, which were not cultured (original clinical specimen) contained low DNA concentrations and were not always detected or typed (HRM plots were affected) using this assay.

Each cultured clinical isolate produced a single fluorescent 'peak' by standard melt, consistent with the production of a single amplicon. These fluorescent 'peaks' often matched, or were very similar to *U. parvum* serovars 1, 3, 6 and 14 ATCC positive controls and the amplicons produced by these clinical isolates were of the correct size, when compared to positive control *U. parvum* serovars (ATCC serovars 1, 3, 6 and 14).

The standard melt analysis and HRM profiles produced by each clinical isolate demonstrated similarities to the HRM profiles produced by ATCC *U. parvum* serovars 1, 3, 6 and 14 (Fig 5.2A). The clinical isolates were serotyped and assigned to a serovar based on the results of HRM (Figure 5.2B) and difference plots (Figure 5.2C)

For example, clinical specimens 122 and 429 produced HRM profiles that corresponded to *U. parvum* serovar 6 and these clinical isolates were identified as *U. parvum* serovar 6. Similarly, clinical isolate 498B produced a HRM profile that was highly similar to *U. parvum* serovar 1 (Figure 5.2C). The serovar identity of 498B was confirmed using the software to be *U. parvum* serovar 1. When *U. parvum* serovar 1 ATCC strain was designated as the baseline genotype, the HRM curve of clinical isolate 498B was plotted within 5 fluorescent units (the cut-off, indicated by the red dotted line) of serovar 1 baseline controls and this confirmed that this clinical isolate was *U. parvum* serovar 1.

Of the 31 clinical isolates which were successfully amplified, 20/31 (64.5%) were serotyped successfully using the designed real-time PCR and HRM assay (Supplementary Table 8.2). Unfortunately, in some instances clinical isolate HRM curves did not correlate with any of the *U. parvum* positive controls or were outside of the ± 5 fluorescent unit cut-off for successful serotyping and so these clinical isolates were unable to be successfully serotyped. It was hypothesised that the erroneous results seen may be due to differences (additional SNPs) in the genetic sequences amplified and so we selected a small group of clinical isolates for sequencing to confirm this theory. However, PCR amplicons generated from the real-time PCR and HRM were not found to contain any SNPs within their sequenced amplicons (data not shown).

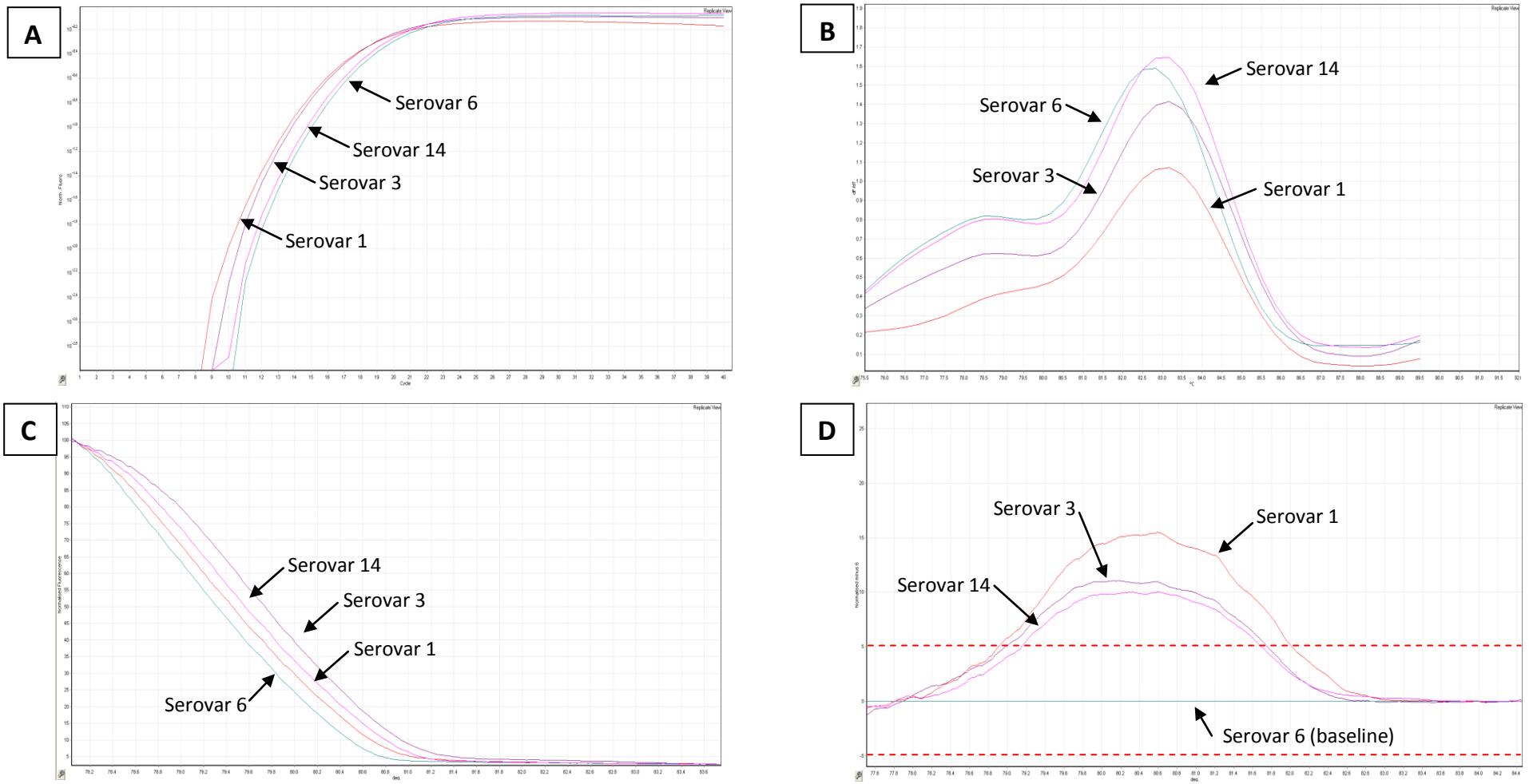


Figure 5.1. Real-time PCR and HRM assays performed on ATCC strains of *U. parvum* serovars 1, 3, 6 and 14. Real-time PCR amplification (panel A) produced strong amplification of *U. parvum* serovars only, while standard melt analysis (panel B) demonstrated a single fluorescent 'peak', consistent with amplification of the gene target. Normalised melt curves (panel C) demonstrated differences in the melt profiles of *U. parvum* serovars 1, 3, 6 and 14, which were further differentiated by difference plots (panel D)

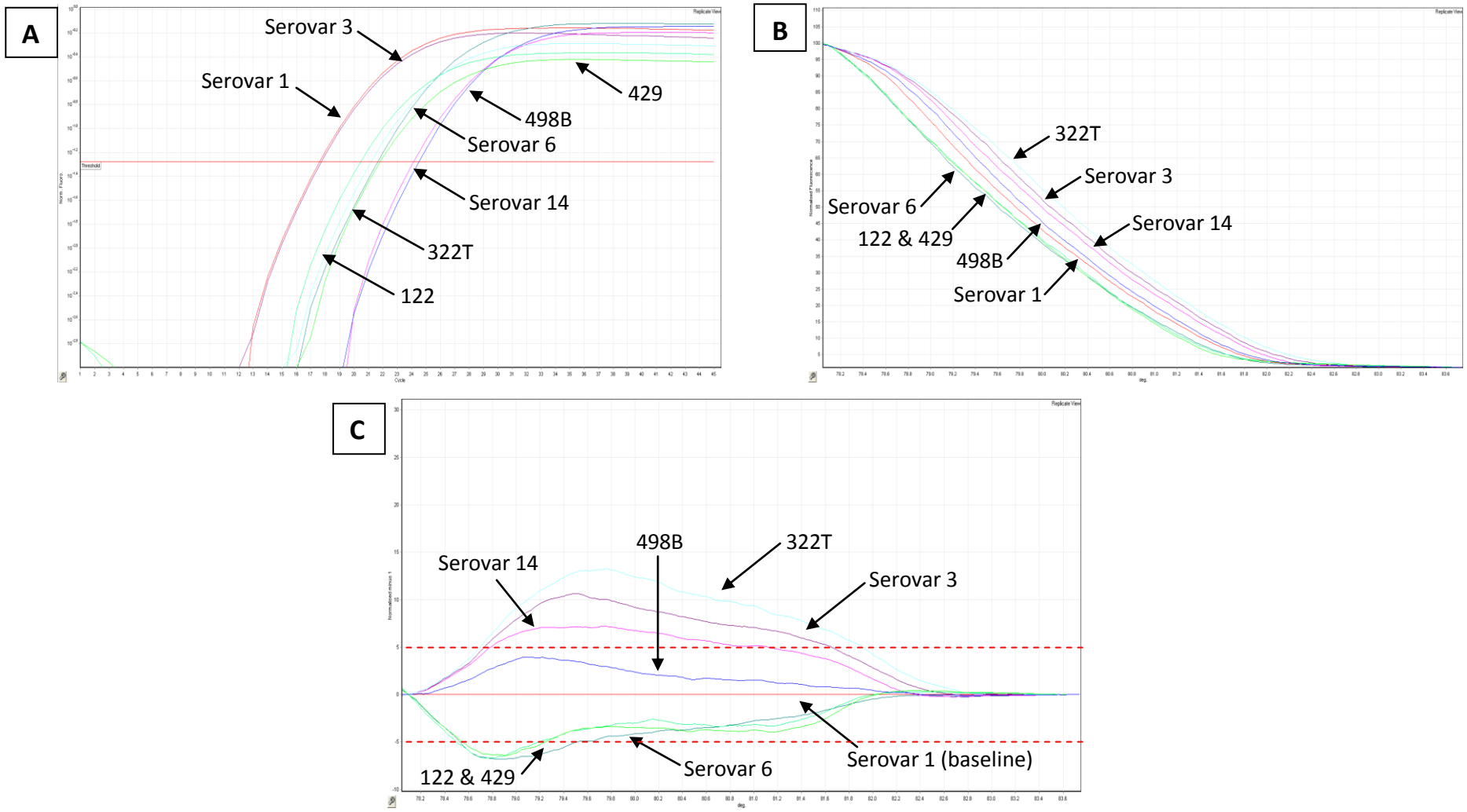


Figure 5.2. Real-time PCR and HRM assay performed on *U. parvum* ATCC strains and clinical isolates. PCR amplification (panel A) demonstrated strong amplification of *U. parvum* clinical isolates. Normalised melt curves (panel B) demonstrated similarities to *U. parvum* clinical isolates which were able to successfully serotype 64.5% of clinical isolates tested when difference plots (panel C) were analysed. Representative data is shown

Ureaplasma species associated with adverse pregnancy and neonatal outcomes

The pregnancy and neonatal outcomes for participants whose placentae were infected with *U. parvum* and *U. urealyticum* were compared to determine if the different ureaplasma species were associated with adverse outcomes.

Women from whom *U. urealyticum* (n = 6) was isolated in the chorioamnion had a lower maternal age (22.8 ± 2.0 yrs) than those women in whom *U. parvum* (n = 36; 24.7 ± 0.8 yrs, p = 0.046; Table 5.3) was identified. Almost all women (> 97%) with ureaplasma infection attended at least one prenatal care visit during their pregnancy (Table 5.3).

Women who were infected with *U. parvum* or *U. urealyticum* within the chorioamnion had no differences in pregnancy outcomes (Table 5.4); however, women infected with *U. urealyticum* reported a higher prevalence of histological chorioamnionitis in previous pregnancies (33.3%), compared to women who were infected with *U. parvum* (5.6%, p = 0.031; Table 5.3). Those women who were infected with *U. urealyticum* also had a higher incidence of pPROM (66.7%), when compared to those infected with *U. parvum* during their current pregnancies (30.6%, p = 0.06; Table 5.4).

For those babies born to women infected with *Ureaplasma* spp. during pregnancy, there were no differences in the neonatal outcomes for infants infected with *U. parvum,* compared to those infected with *U. urealyticum* (Chapter Nine, Suppmentary Table 9.3).

Table 5.3. Maternal demographic data for women whose chorioamnion was infected with *U. parvum* and *U. urealyticum* during pregnancy.

	***U. parvum* (n = 36)**	***U. urealyticum* (n = 6)**	**Significance**
Maternal Age	24.7 ± 0.8 (17 – 32)	22.8 ± 2.0 (19 – 32)	0.046
Gravida[1]	1.9 ± 0.2 (1 – 5)	3.2 ± 0.3 (2 – 4)	NS[2]
Parity[3]	1.7 ± 0.2 (1 – 4)	2.3 ± 0.4 (1 – 4)	NS
Marital Status			
- **Married**	12/36 (33.3%)	1/6 (16.7%)	NS
- **Single**	24/36 (66.7%)	5/6 (83.3%)	NS
Medical Insurance			
- **Private**	14/36 (38.9%)	1/6 (16.7%)	NS
- **Medicaid**	16/36 (44.4%)	5/6 (83.3%)	NS
- **Self pay/uninsured**	4/36 (11.1%)	0/36 (0.0%)	NS
- **Unknown**	2/36 (5.6%)	0/36 (0.0%)	NS
Ethnicity			
- **Caucasian**	22/36 (61.1%)	1/6 (16.7%)	NS
- **African-American**	12/36 (33.3%)	5/6 (83.3%)	NS
- **Asian**	0/36 (0.0%)	0/6 (0.0%)	NS
- **More than one race**	2/36 (5.6%)	0/6 (0.0%)	NS
Evidence of prenatal care	35/36 (97.2%)	6/6 (100.0%)	NS

[1] Gravida – total number of clinical pregnancies

[2] NS - Not statistically significant

[3] Parity – total number of viable offspring resulting from pregnancies

Data was analysed by ANOVA

Table 5.4. Maternal pregnancy outcomes for women whose chorioamnion was infected with *U. parvum* and *U. urealyticum* during pregnancy

	***U. parvum* (n = 36)**	***U. urealyticum* (n = 6)**	**Significance**
Signs/symptoms of infection[1]	4/36 (11.1%)	1/6 (16.7%)	NS[2]
Chorioamnionitis documented previously	2/36 (5.6%)	2/6 (33.3%)	0.031
Chorioamnionitis in current pregnancy[3]	22/36 (61.1%)	5/6 (83.3%)	NS
- **Maternal Stage**	1.5 ± 0.1 (1 – 3)	1.6 ± 0.2 (1 – 2)	NS
- **Fetal Stage**	2.0 ± 0.2 (1 – 3)	3.0 ± 0.0[4]	NS
Antibiotics administered < 3hrs prior to delivery	15/36 (41.7%)	4/6 (66.7%)	NS
PTL/cervical incompetence	16/36 (44.4%)	4/6 (66.7%)	NS
preterm premature rupture of membranes (pPROM)	11/36 (30.6%)	4/6 (66.7%)	NS

[1] Signs or symptoms of infection included: maternal temperature > 38 °C, uterine or abdominal tenderness, foul-smelling vaginal discharge, maternal tachycardia (> 120 bpm) or fetal tachycardia (> 160 bpm)

[2] NS - Not statistically significant

[3] Chorioamnionitis was determined by US pathologists and graded for the severity of inflammation according to (Redline *et al.* 2003)

[4] No range or standard error of the mean was available for this data point, only one infant had evidence of amnion involvement

Data was analysed by ANOVA

U. parvum serovars associated with adverse pregnancy and neonatal outcomes

We also compared the pregnancy and neonatal outcomes for women infected with the different *U. parvum* serovars (1, 3, 6; n = 31) (Table 5.5).

Women who were infected within the chorioamnion with *U. parvum* serovar 3 were younger (21.4 ± 0.9 yrs) than women in whom *U. parvum* serovars 1 and 6 were identified (26.4 ± 1.1 yrs and 26.0 ± 1.2 yrs, respectively; p = 0.024. Table 5.5). Women who were infected with *U. parvum* serovar 1 were more likely to be married (p = 0.006), while women infected with *U. parvum* serovars 3 and 6 were more frequently unmarried (p = 0.008).There were no other differences in the demographic data between these three groups of women, nor were there any differences in the incidence of adverse pregnancy or neonatal outcomes (Chapter Nine, Supplementary Tables 8.4 and 8.5).

Table 5.5. A comparison of the demographic data of women infected with *U. parvum* serovars 1, 3 and 6 (n = 31[1]).

	Serovar 1 (n = 10)	Serovar 3 (n = 9)	Serovar 6 (n = 12)	Significance
Maternal Age	26.4 ± 1.1 (20 – 32)	21.4 ± 0.9 (17 – 26)	26.0 ± 1.4 (19 – 32)	0.024
Gravida[2]	2.1 ± 0.2 (1 – 4)	1.7 ± 0.4 (1 – 4)	1.9 ± 0.4 (1 – 5)	NS[3]
Parity[4]	2 ± 0.2 (1 – 4)	1.4 ± 0.2 (1 – 2)	1.8 ± 0.3 (1 – 4)	NS
Marital Status				
- **Married**	8/10 (80.0%)	3/9 (33.3%)	1/12 (8.3%)	0.006
- **Single**	2/10 (20.0%)	6/9 (66.7%)	11/12 (91.7%)	0.008
Medical insurance				
- **Private**	7/10 (70.0%)	2/9 (22.2%)	5/12 (41.7%)	NS
- **Medicaid**	2/10 (10.0%)	2/9 (22.2%)	7/12 (58.3%)	NS
- **Self pay/uninsured**	1/10 (10.0%)	3/9 (33.3%)	0/12 (0.0%)	NS
- **Unknown**	0/10 (0.0%)	0/10 (0.0%)	0/12 (0.0%)	NS
Ethnicity				
- **Caucasian**	9/10 (90.0%)	7/9 (77.8%)	6/12 (50.0%)	NS
- **African-American**	1/10 (10.0%)	2/9 (22.2%)	5/12 (41.7%)	NS
- **Asian**	0/10 (0.0%)	0/9 (0.0%)	0/12 (0.0%)	NS
- **More than one race**	0/10 (0.0%)	0/9 (0.0%)	1/12 (8.3%)	NS
Evidence of prenatal care	10/10 (100.0%)	9/9 (100.0%)	11/12 (91.7%)	NS

[1] Five of the *U. parvum* clinical isolates were not serotyped by the real-time PCR and HRM assay

[2] Gravida - total number of clinical pregnancies

[3] NS – not statistically significant

[4] Parity – total number of viable offspring resulting from pregnancies.

Data was analysed by ANOVA

Characterisation of the *Ureaplasma* spp. multiple banded antigen (*mba*/MBA) by PCR and western blot

Western blot of the MBA

Western blot analyses of the *U. parvum* serovars 1, 3 and 6 ATCC strains demonstrated MBA protein bands of approximately 80 kDa; 90 kDa and 70 kDa, respectively (Figure 5.3). *U. urealyticum* serovars 2, 4, 5 7 – 13 produced MBA protein bands of 70 kDa; 80 kDa; 70 kDa; 80 kDa; 100 kDa; 90 kDa; 75 kDa; 80 kDa; 75 kDa and 70 kDa, respectively (serovars 8 and 10 shown in Figure 5.3).

The MBA proteins expressed by clinical isolates were compared to the ATCC strains and variation in the size of MBA proteins ranged from 55 kDa – 100 kDa. The MBA proteins expressed by some clinical isolates demonstrated no variation, i.e. these proteins were the same size as the antigens expressed by ATCC strain serovars (Serovar 1 isolates: 1A, 1B, 262T, 507; Serovar 3 isolates: 33A, 33B, 322T, 325; and Serovar 6 isolates: 334A, 334B, 364A; Figure 5.3). For other clinical isolates, variation in the size of their MBA proteins was demonstrated. These clinical isolates demonstrated either a 'single MBA variant', which was considered to be an individual protein band that differed in size when compared to the corresponding ATCC strain controls (Serovar 1 isolates: 43, 301, 483T, 498A, 498B; Serovar 3 isolates: 44A, 44B, 314T, 365, 435; Serovar 6 isolates: 27, 50, 55B, 122, 182, 310T and 429; Serovar 8 isolate: 8; and Serovar 10 isolate 300); or in some cases, 'multiple MBA variant' bands were seen, where more than one MBA band was identified by western blot (Serovar 1 isolates: 290T, 473T; Serovar 6 isolates: 182, 429; Figure 5.3).

For some clinical ureaplasma isolates (258, 297, 351, 438, 480T and 510T), we were unable to demonstrate MBA protein expression with any of the 14 serovar-specific antisera tested, and therefore we were unable to determine if MBA size variation occurred.

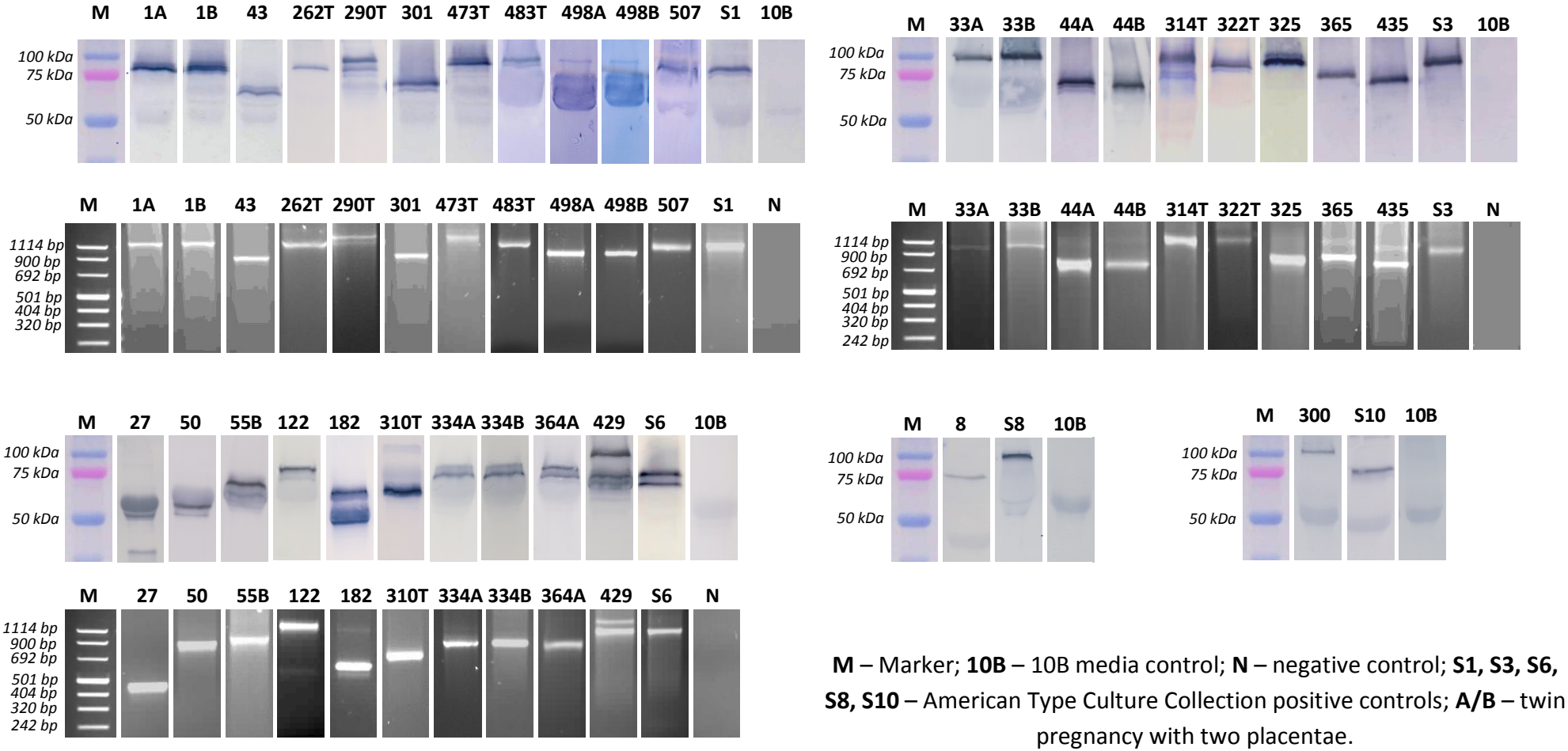
PCR of the *mba*

To assess if MBA size variation (as demonstrated in western blots) was also occurring within the gene (*mba*) encoding the MBA protein, we performed PCR assays which amplified the downstream repetitive region of the *mba* for clinical isolates of serovar 1, serovar 3 and serovar 6. Whilst western blots were performed on serovar 8 and serovar 10 clinical isolates, it was not possible to amplify the downstream region of the *mba* gene in *U. urealyticum* isolates due to differences in the gene regions targeted by available PCR primers (Figure 5.3).

The ATCC strain *U. parvum* serovars 1, 3 and 6 produced PCR amplicons of approximately 900 bp, 950 bp and 800 bp, respectively (Figure 5.3). Similar to the results seen in western blots, we identified variation in the size of amplicons produced by *U. parvum* clinical isolates, and these amplicons ranged in size from 350 bp to 1200 bp. The variation in size of the PCR amplicons produced by *U. parvum* ATCC strains and the clinical isolates directly correlated with the variation in size seen in MBA proteins in western blots. Clinical isolates which demonstrated 'no *mba* variation' had amplicons which were the same size as the ATCC strain controls. Furthermore, those clinical isolates which demonstrated a 'single *mba* variant' or 'multiple *mba* variants' produced *mba* amplicons, which directly correlated with the size and number of MBA proteins expressed by each clinical isolate (Figure 5.3).

U. parvum serovar 6 clinical isolate 480T did not produce an *mba* amplicon when tested using primers that amplified either *U. parvum* serovars 1 and 6; or *U. parvum* serovars 3 and 14. Due to the absence of a downstream *mba* amplicon and the absence of an MBA protein by western blot, a PCR assay targeting the upstream region of the *mba* was performed. This assay confirmed the presence of the upstream conserved portion of the *mba* gene.

Figure 5.3. Variation of the *mba* gene and MBA protein was detected by conventional PCR and western blot. Variation was identified by running each protein and PCR amplicon sample with both an ATCC strain positive control and a molecular weight marker. Images were then cropped and displayed at their correct molecular weight and sorted according to their serovar groupings.



M – Marker; **10B** – 10B media control; **N** – negative control; **S1, S3, S6, S8, S10** – American Type Culture Collection positive controls; **A/B** – twin pregnancy with two placentae.

mba/MBA variation and its association with adverse pregnancy outcomes

The pregnancy and neonatal outcomes of women from whom *Ureaplasma* spp. with no *mba*/MBA variation was isolated were compared to the outcomes for pregnancies in which a single *mba*/MBA size variant was isolated, or those in which multiple *mba*/MBA size variants were isolated.

There were no differences in the maternal demographic data associated with women in which *mba*/MBA variation was identified, when compared to those women in whom no *mba*/MBA variation was identified (Chapter Nine, Supplementary Table 9.6)

However, a major finding of this study was that *mba*/MBA variation as demonstrated by western blot and PCR was associated with the prevalence of histological chorioamnionitis. Placentae from which no *mba*/MBA variation was seen demonstrated a higher incidence of histological chorioamnionitis (81.8%), compared to placentae from which ureaplasmas that expressed only a single *mba*/MBA variant were isolated (43.7%, $p = 0.023$; Table 5.6).

There were no differences in the pregnancy or neonatal characteristics that were associated with *mba*/MBA variation.

Table 5.6. Comparison of pregnancy outcomes in pregnancies with *Ureaplasma* spp. infection and *mba*/MBA variation

	No *mba*/MBA variants[1] (n = 11)	Single *mba*/MBA variant (n = 16)	Multiple *mba*/MBA variants (n =4)	Significance
Signs/symptoms of infection[2]	2/11 (18.2%)	0/16 (0.0%)	1/4 (25.0%)	NS[3]
Chorioamnionitis documented previously	0/11 (0.0%)	1/16 (6.2%)	1/4 (25.0%)	NS
Chorioamnionitis in current pregnancy[4]	9/11 (81.8%)	7/16 (43.7%)	3/4 (75.0%)	0.023
- **Maternal Stage**	1.5 ± 0.2 (1 - 3)	1.1 ± 0.1 (1 - 2)	2 ± 0.5 (1 - 3)	NS
- **Fetal Stage**	2.5 ± 0.3 (1- 3)	2 ± 0.3 (1- 3)	1.5 ± 0.3 (1 - 2)	NS
Antibiotics administered < 3 hours prior to delivery	5/11 (45.4%)	8/16 (50.0%)	1/4 (25.0%)	NS
Cervical incompetence	3/11 (27.3%)	8/16 (50.0%)	2/4 (50.0%)	NS
preterm premature rupture of membranes (pPROM)	3/11 (27.3%)	7/16 (43.7%)	0/4 (0.0%)	NS

[1] *mba/*MBA size variation was determined by conventional PCR and western blot targeting the *mba* gene and its expressed protein (MBA). Data was analysed using ANOVA tests.

[2] Signs and symptoms of infection included: maternal temperature > 38 °C, uterine or abdominal tenderness, foul-smelling vaginal discharge, maternal tachycardia (> 120 bpm) or fetal tachycardia (> 160 bpm)

[3] NS – Not statistically significant

[4] Chorioamnionitis was determined by US pathologists and the severity of inflammation was graded according to (Redline *et al.* 2003).

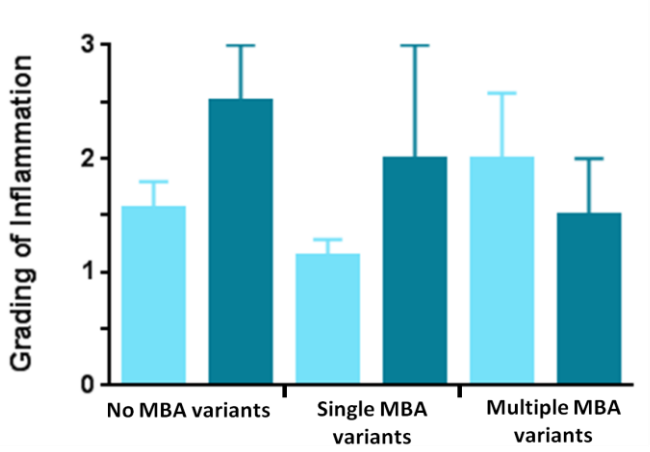


Figure 5.4. There was a trend between the presence of *Ureaplasma* spp. MBA variation (both single and multiple MBA variants) and a reduction in inflammation within the fetal (amnion; shown in dark green) portion of the chorioamnion membranes. Data are presented as mean, plus or minus the standard error of the mean (SEM).

Cytokines, chemokines and growth factors

The presence of cytokines, chemokines and growth factors within cord blood was also tested for any correlation with the development of adverse pregnancy and neonatal outcomes. BioPlex assays detecting IL-6, IL-8, monocyte chemoattractant protein (MCP)-1 and granulocyte colony-stimulating factor (G-CSF) were performed by our collaborators and provided for analysis within this project.

Cord blood sampled from pregnancies with chorioamnion infection, regardless of the aetiology of the infection, demonstrated significantly higher concentrations of IL-8 (536.9 pg/mL) and G-CSF (403.0 pg/mL), compared to concentrations in pregnancies in which no chorioamnion infection was detected (56.6 pg/mL and 231.7 pg/mL, p = 0.03 and p= 0.04, respectively; Figure 5.5).

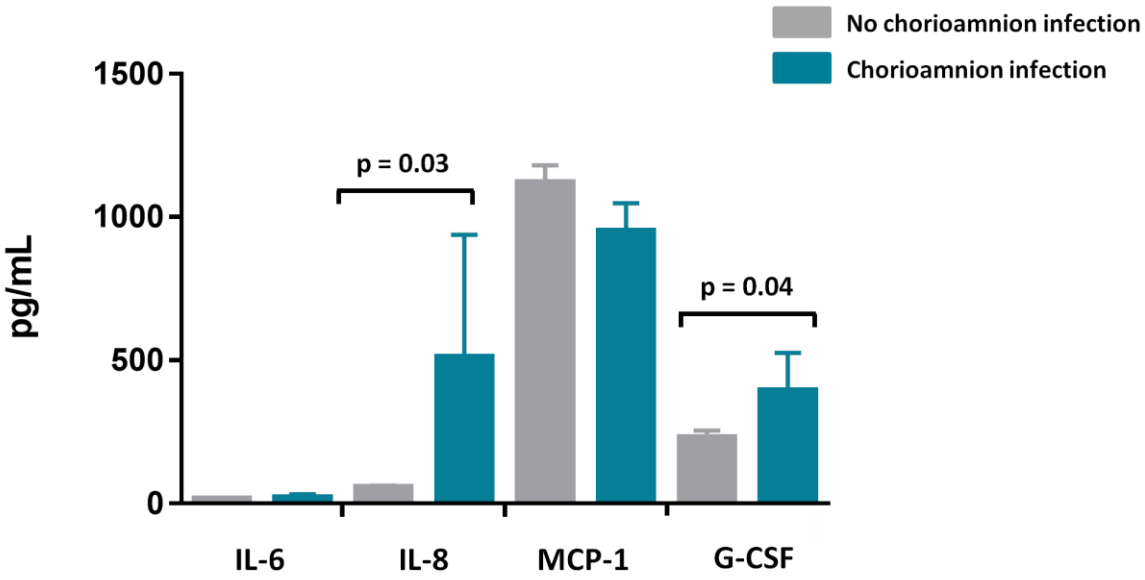


Figure 5.5. A comparison of the concentrations of cytokines, chemokines and growth factors within cord blood obtained from women with and without chorioamnion infection. Data was analysed by ANOVA and are presented as the mean, plus or minus the standard error of the mean (SEM).

The detection of *Ureaplasma* spp. within the chorioamnion was associated with higher concentrations of G-CSF (p = 0.02) within cord blood, but not with the factors IL-6, IL-8 and MCP-1. By contrast, cord blood collected from pregnancies in which the chorioamnion was infected by microorganisms (other than *Ureaplasma* spp.) demonstrated higher levels of IL-8 (p = 0.01, Figure 5.6).

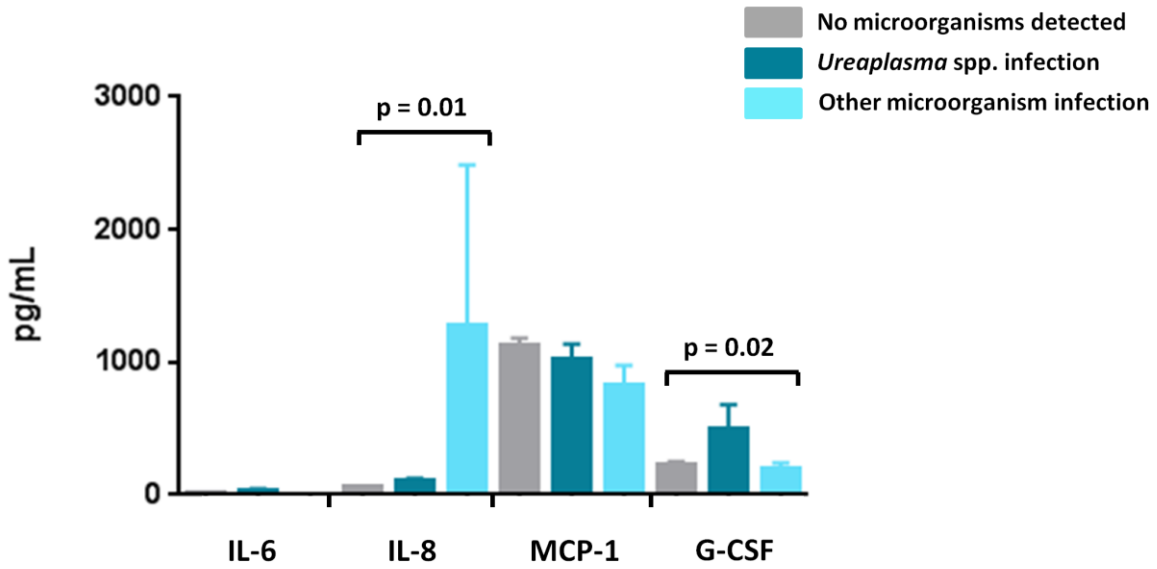


Figure 5.6. Comparison of the concentrations of cytokines, chemokines and growth factors within cord blood of pregnancies with chorioamnion infections caused by *Ureaplasma* spp. or other microorganisms; compared to pregnancies in which no infection was detected. Data was analysed by ANOVA and are presented as the mean, plus or minus the standard error of the mean (SEM)

We also assessed if cord blood cytokines, chemokines and growth factor concentrations correlated with the presence or absence of histological chorioamnionitis. In Chapter Four, we demonstrated that infection with *Ureaplasma* spp. (but not other microorganisms) was associated with the development of histological chorioamnionitis. Pregnancies which were affected by histological chorioamnionitis demonstrated elevated levels of MCP-1 (p = 0.05) and G-CSF (p = 0.008; Figure 5.7).

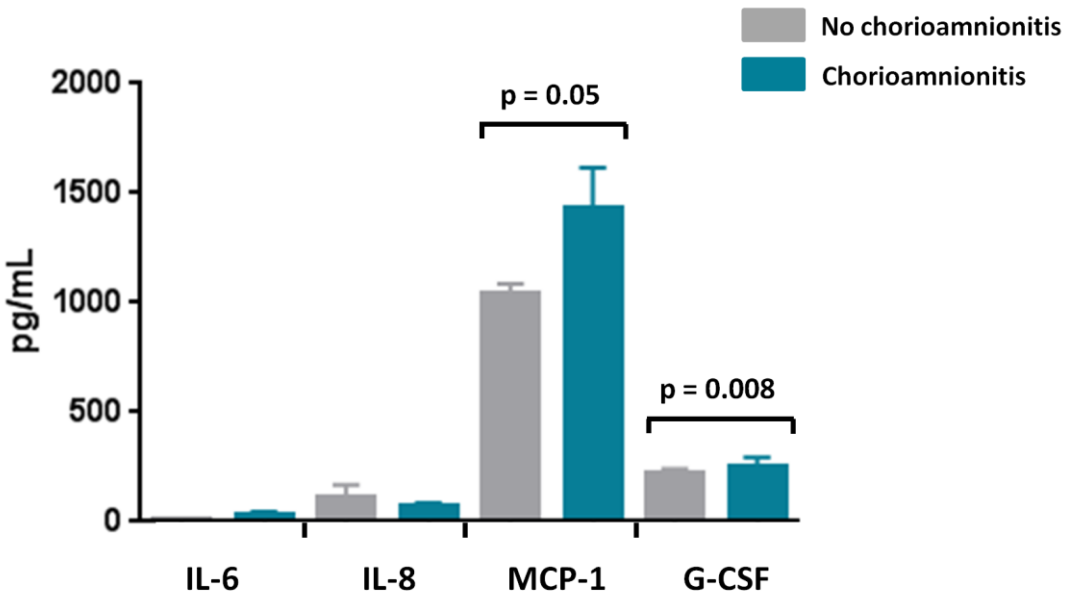


Figure 5.7. Cytokines, chemokines and growth factor concentrations within cord blood from pregnancies exposed to histological chorioamnionitis and those which had no evidence of chorioamnionitis. Data was analysed by ANOVA and are presented as the mean, plus or minus the standard error of the mean (SEM)

Due to similarities in cord blood G-CSF levels in *Ureaplasma* spp.-infected placentae (Figure 5.6) and placentae with histological chorioamnionitis (Figure 5.7), we also assessed if *mba*/MBA variation was associated with variation in cord blood cytokine concentrations.

Concentrations of IL-8 and G-CSF were elevated in placentae, which were infected with ureaplasmas that demonstrated no *mba*/MBA variation, compared to placentae which were ureaplasma-infected but demonstrated a single or multiple *mba*/MBA variants (p = 0.044, p = 0.008, respectively; Figure 5.8). By contrast, levels of MCP-1 were elevated in placentae which were infected with *Ureaplasma* spp. which demonstrated *mba*/MBA variation, when compared to those pregnancies which were infected with ureaplasmas but demonstrated no *mba*/MBA variation.

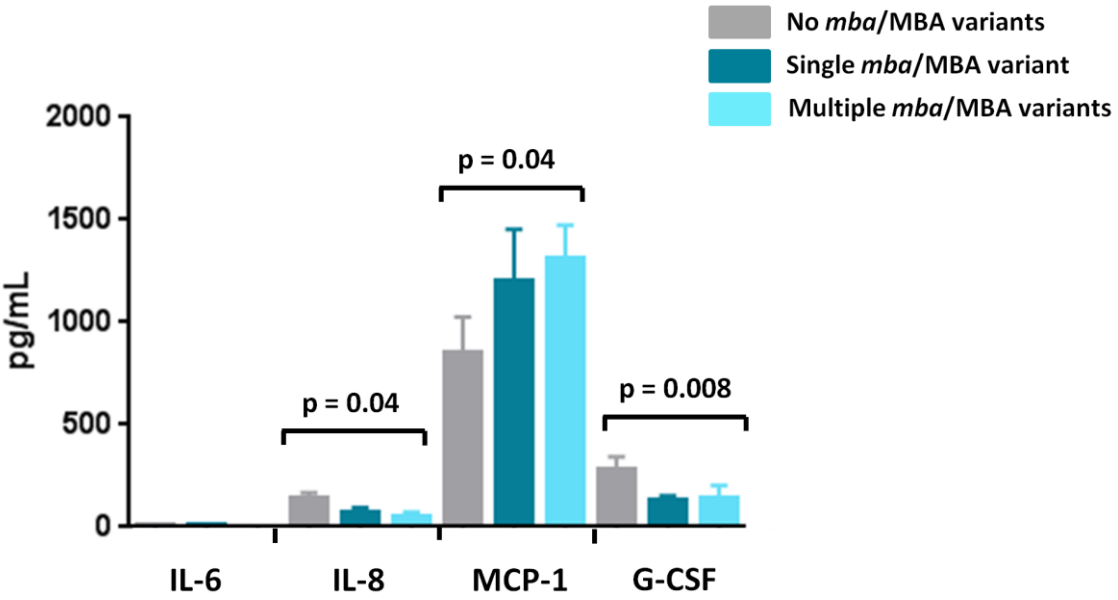


Figure 5.8. Correlations of cytokines, chemokines and growth factors present in cord blood from *Ureaplasma* spp.-infected pregnancies. Concentrations of immune factors differed in pregnancies which demonstrated no *mba*/MBA variation (n = 11) and those which expressed a single (n = 16) or multiple (n = 4) *mba*/MBA variants. Data was analysed by ANOVA and are presented as the mean, plus or minus the standard error of the mean (SEM)

Discussion

The pathogenic role of *Ureaplasma* spp. as an aetiological agent of adverse pregnancy outcomes is controversial. These microorganisms have been isolated from the amniotic fluid and placentae of women experiencing PTB and the development of histological chorioamnionitis (Hillier *et al.* 1988; Aaltonen *et al.* 2007; Berger *et al.* 2009; Namba *et al.* 2010); however, *Ureaplasma* spp. have also been isolated from women who delivered at term with no apparent adverse outcomes (Gray *et al.* 1992; Gerber *et al.* 2003). To investigate the pathogenesis of *Ureaplasma* spp. within human pregnancies, we conducted a prospective study to investigate the prevalence of *Ureaplasma* spp. and serovars in late preterm and term pregnancies and also to characterise the variation of the MBA surface-exposed protein. We correlated the presence of ureaplasmas with adverse pregnancy and neonatal outcomes, including the development of histological chorioamnionitis. Furthermore, the presence of cytokines, chemokines and growth factors within cord blood was also correlated with the presence of ureaplasmas.

In this current program of research (Chapter Four), we demonstrated that *Ureaplasma* spp. were the most prevalent bacteria detected within the chorioamnion of women who delivered late preterm (LPT) or at term, and were identified in 42/535 (7.8%) of placentae. *U. parvum* was the most prevalent ureaplasma species present, accounting for 85.7% of all ureaplasma isolates. Furthermore, we have now identified in this current chapter that *U. parvum* serovars 1, 3 and 6 were the most prevalent ureaplasma serovars within placentae obtained from women who delivered LPT or at term. The women who were infected/colonised with *U. urealyticum* had a higher prevalence of prior chorioamnionitis (Table 5.4); but we have demonstrated that regardless of the *Ureaplasma* spp. or serovar isolated within chorioamnion tissue, the presence of ureaplasmas was associated with histological chorioamnionitis. Chorioamnionitis was documented in 61.1% of *U. parvum*-infected placentae and 83.3% of *U. urealyticum*-infected placentae. Similarly, we demonstrated no difference in the prevalence of chorioamnionitis in placentae infected with *U. parvum* serovars 1, 3 or 6 (70.0%, 55.5% and 58.3%, respectively) and no major differences in adverse pregnancy or neonatal outcomes were seen for women infected with these different serovars. These findings are consistent with previous studies that have shown *Ureaplasma* spp. to be associated with chorioamnionitis (Hillier *et al.* 1988; Aaltonen *et al.* 2007; Egawa *et al.* 2007; Hecht *et al.* 2008), but these ureaplasmas may also be isolated from placentae with no evidence of chorioamnionitis and the pregnancy may continue until term delivery (Gray *et al.* 1992; Gerber *et al.* 2003; Perni *et al.* 2004).

One of the most significant findings of this current study was that *Ureaplasma* spp. isolated from placentae of human pregnancies demonstrated differences in their MBA proteins. Of the isolates obtained from chorioamnion tissue, some expressed no MBA variants (and their proteins were the same size as ATCC strain serovars), while other clinical isolates expressed a single or multiple MBA size variants (as evidenced by single or multiple MBA bands which differed in size, when compared to ATCC strain serovar controls). This study also demonstrated that regardless of the *Ureaplasma* species or serovar that were present, the lack of *mba*/MBA variation was associated with a higher incidence of histological chorioamnionitis and a more severe inflammatory response within the fetal (amnion) membranes (grade 2.5; Table 5.6, Figure 5.4). By contrast, when *mba*/MBA variation occurred (either a single or multiple MBA variants), this was associated with a lower incidence of histological chorioamnionitis and a decreased inflammatory response within the fetal (amnion) membrane (grade 2.0 and 1.5, respectively; Table 5.6. Figure 5.4). Previous studies have demonstrated that the severity of histological chorioamnionitis varied depending on the numbers of *Ureaplasma* spp. present within the amniotic fluid or chorioamnion (Jacobsson *et al.* 2009; Kasper *et al.* 2010; Kacerovsky *et al.* 2011). However, in Chapter Four, we did not find an association between histological chorioamnionitis and the numbers of ureaplasmas present within the chorioamnion (Chapter Four, Table 4.4) but in this current study, we demonstrated that *mba*/MBA variation was associated with the severity of inflammation and histological chorioamnionitis, particularly within the fetal membranes.

Antigenic variation is an important mechanism used by pathogenic microorganisms to mediate the interactions between bacteria and their environment. Variation of antigens/proteins are thought to be an essential strategy for successful invasion, infection and to assist the microorganisms to survive in the presence of a host immune response *in vivo* (Darmon and Leach 2014). Antigenic variation can occur either through size variation (as demonstrated in our current study) or by selective "switching on/off" of a particular protein (often referred to as phase variation). Antigen variation is not a unique trait of *Ureaplasma* spp. and many different microorganisms possess the ability to vary their surface-exposed antigens. It has been shown that other *Mycoplasma* spp. are able to vary the size of their surface-exposed proteins, such as the MgpB and MgpC adhesins of *M. genitalium*, the Vpma protein of *M. agalactiae* and the Vaa putative adhesin protein of *M. hominis* (Glew *et al.* 2000; Glew *et al.* 2002; Chopra-Dewasthaly *et al.* 2008; Citti *et al.* 2010). Antigen variation in *Ureaplasma* spp. and other *Mycoplasma* spp. are regulated by two major mechanisms. The first mechanism involves DNA slipped strand mispairing and/or nucleotide insertion/deletion in tandem repeating units (Citti *et al.* 2010). The second mechanism involves phase variation or the selective “switching on/off” and

this involves DNA rearrangements mediated by site-specific recombinases (Citti *et al.* 2010). Importantly, both size and phase variation of the *Ureaplasma* spp. MBA have been demonstrated previously (Zheng *et al.* 1992; Zheng *et al.* 1994; Zheng *et al.* 1995; Monecke *et al.* 2003; Zimmerman *et al.* 2009; Knox *et al.* 2010; Zimmerman *et al.* 2011; Dando *et al.* 2012; Robinson *et al.* 2013; Zimmerman *et al.* 2013).

Ureaplasma *mba*/MBA variation has been reported in a sheep model of intraamniotic *Ureaplasma* spp. infection (Knox *et al.* 2010; Dando *et al.* 2012; Robinson *et al.* 2013). Knox *et al.* (2010) demonstrated the first evidence of MBA variation within an ovine sheep model. In this study, the same number of *Ureaplasma* spp. were injected into the amniotic fluid of pregnant ewes, but differences in the severity of histological chorioamnionitis was observed. While some ewes developed mild/moderate or severe histological chorioamnionitis, in other sheep there was no evidence of histological chorioamnionitis, despite the presence of high numbers of *Ureaplasma* spp. within the amniotic fluid. This study also identified an inverse correlation between the number of MBA size variants and the severity of histological chorioamnionitis: in sheep amniotic fluid which contained five or fewer MBA size variants, severe histological chorioamnionitis was seen in placentae; however, sheep amniotic fluid which contained nine or more MBA size variants had mild or no evidence of chorioamnionitis. It has also been demonstrated that the degree of MBA size variation was reliant on the duration of infection, with no variation seen after 3 days of infection, some variation seen after 7 days of infection (Robinson *et al.* 2013) and numerous size variations observed following chronic (69 day) infections of the amniotic fluid (Knox *et al.* 2010; Dando *et al.* 2012; Robinson *et al.* 2013). Interestingly, Dando *et al.* (2012) demonstrated that MBA variation was not limited to 'virulent' or 'avirulent' strains of *Ureaplasma* spp. Strains that were associated with severe chorioamnionitis (a 'virulent' strain) or the absence of chorioamnionitis (an 'avirulent' strain) were injected into the amniotic cavity of pregnant ewes. Whilst the size of the MBA varied throughout gestation, even within the same animal, this study showed no difference in the propensity for the 'virulent' and 'avirulent' strains of *Ureaplasma* spp. to vary their surface-exposed MBA. In this current PhD study, we have demonstrated the presence of MBA size variants within the chorioamnion of women who delivered LPT or at term. This study is also the first to demonstrate that there is a relationship between the variation of the *Ureaplasma* spp. MBA and the severity of inflammation within the UGT in human pregnancies. Some ureaplasmas demonstrated multiple MBA size variants, which suggests *in vivo* colonisation/infection of the UGT for 'sufficient time' to generate these MBA size variants or the presence of a host selective pressure on the ureaplasmas present.

Phase variation of the *Ureaplasma* spp. MBA has also been demonstrated in several studies. "Switching on/off" of the MBA has been demonstrated following serial *in vitro* passaging of ureaplasmas with high concentrations of MBA-specific antibodies (Monecke *et al.* 2003; Dando *et al.* 2012). This "switching off" of the MBA was shown to be reversible, as ureaplasmas were again able to express the MBA lipoprotein following the removal of these antibodies. Zimmerman *et al.* (2009) further investigated the role of MBA phase variation and identified that a DNA inversion event between the MBA and an intergenic region downstream of a hypothetical gene (UU376) may serve as the mechanism by which MBA expression could be "switched off". These researchers also identified another gene (UU172), which may also serve as an additional DNA inversion site for "switching on/off" of the MBA (Zimmerman *et al.* 2011). This same group also further elucidated a putative tyrosine recombinase (XerC), which they proposed as the mediator of "on/off switching" of the MBA (Zimmerman *et al.* 2013) and similar tyrosine recombinases have been shown to mediate phase variation in other *Mycoplasma* spp. (Chopra-Dewasthaly *et al.* 2008). In our current study, a number of clinical isolates did not produce an MBA band by western blot when tested with the 14 different anti-*U. parvum* or *U. urealyticum* serovar-specific antisera. *U. urealyticum* clinical isolates 258, 297, 351 and 438 (n = 4) did not demonstrate the presence of an MBA in individual western blots probed with each of the ten *U. urealyticum* antisera. Similarly, a single *U. parvum* clinical isolate 480T did not produce an MBA band by western blot in response the four different *U. parvum* antisera, nor was an *mba* amplicon produced by PCR assays targeting the downstream repetitive region of the *mba* gene. These results are consistent with phase variation and the "switching off" of the MBA antigen and the *mba* gene. In 4/5 (80.0%) of placentae from which these ureaplasmas were isolated, there was evidence of histological chorioamnionitis; however, the maternal grading of inflammation was mild (mean 1.2) and only one placentae demonstrated inflammation of the fetal (amnion) portion of the chorioamnion membrane. Another possible explanation is that these isolates may be significantly different to the known ATCC strains of *Ureaplasma* spp. and therefore cannot be detected by the methods used within this study. Recent molecular subtyping of *Ureaplasma* spp. clinical isolates has shown extensive horizontal gene transfer (Xiao *et al.* 2011b; Paralanov *et al.* 2012) and this may account for the inability to type these strains (and could be a contributing factor as to why we were unable to fully characterise these isolates). Non-typeable clinical isolates of *Ureaplasma* spp. have been reported previously (Knox *et al.* 1998; Echahidi *et al.* 2002; Knox *et al.* 2003) and so it is not surprising that we were unable to fully characterise all of the clinical isolates within our study. Regardless, this is an interesting finding and is potentially the first report of phase variation and "switching off" of the MBA *in vivo*.

Significantly higher concentrations of IL-8 and G-CSF were detected within cord blood in association with chorioamnion infection, regardless of the aetiology (ureaplasmas or other microorganisms), when compared to pregnancies with no infection (Figure 5.5). The presence of other microorganisms (not including *Ureaplasma* spp.) was associated with a higher concentration of IL-8 within cord blood (1269.4 pg/mL vs. 60.6 pg/mL for those who were uninfected; p = 0.01; Figure 5.6); while the presence of *Ureaplasma* spp. was associated with a higher concentration of G-CSF (489.3 pg/mL vs. 230.2 pg/mL than for uninfected placentae; p = 0.023; Figure 5.6). Higher concentrations of G-CSF were also present in cord blood when histological chorioamnionitis was present within placentae (Figure 5.7). G-CSF is a cytokine that affects the proliferation and differentiation of neutrophil progenitors and previous studies have identified elevated levels of G-CSF associated with histological chorioamnionitis (Boggess *et al.* 1997; Hoskins *et al.* 1997). In Chapter Four, we demonstrated that the presence of *Ureaplasma* spp. was associated with histological chorioamnionitis ($p < 0.01$). In this current chapter, we further demonstrated that the presence of *Ureaplasma* spp. or the presence of histological chorioamnionitis is significantly associated with elevated levels of the cytokine G-CSF. This strengthens the finding of an association between ureaplasmas and the development of histological chorioamnionitis *in utero* and indicates that this cytokine may be a biomarker of asymptomatic chorioamnionitis during pregnancy.

The concentrations of cytokines, chemokines and growth factors in cord blood were also correlated with ureaplasma MBA variation detected within chorioamnion clinical isolates. When no ureaplasma *mba*/MBA variation was detected in clinical isolates (identified within chorioamnion tissue), higher concentrations of IL-8 were detected within the cord blood (137.7 pg/mL, p = 0.04), when compared to those placentae which were infected with ureaplasmas that demonstrated *mba/*MBA size variation. By contrast, clinical ureaplasma isolates that demonstrated a single or multiple MBA variants were associated with lower levels of IL-8 (73.7 pg/mL and 46.8 pg/mL, respectively; p = 0.044) within cord bloods obtained from ureaplasma-infected placentae. Other studies have also shown elevated levels of IL-8 in amniotic fluid of women with chronic histological chorioamnionitis (Ogge *et al.* 2011), in the umbilical cord blood of pregnancies complicated by preterm premature rupture of membranes and histological chorioamnionitis (Andrys *et al.* 2010) and the increased transcription of IL-8 within placental tissues in association with prostaglandin synthesis and preterm labour (Phillips *et al.* 2014). IL-8 is an important cytokine involved in the migration of neutrophils and granulocytes towards the site of infection (Bickel 1993). Neutrophil infiltration of *Ureaplasma* spp.-infected placentae has been demonstrated previously and is associated with the development of

inflammation and the development of histological chorioamnionitis in a sheep model of intraamniotic ureaplasma infection (Dando *et al.* 2012). Within cord blood, the reduction of this inflammatory mediator would result in a decreased likelihood of the host mounting an immune response against these intraamniotic pathogens. The novel finding that G-CSF is elevated in cord blood when the placentae are infected with ureaplasmas that do not demonstrate MBA variation may be a biomarker of more severe chorioamnionitis and certainly requires further investigation.

An interesting trend in the levels of G-CSF in association with MBA size variation was also observed. Despite the low numbers in our study groups, we were able to identify that low levels of G-CSF were present in ureaplasma-infected placentae, which also demonstrated size variation of the MBA. These placentae demonstrated approximately 40% lower concentrations of G-CSF, when compared to placentae which were infected with ureaplasmas that did not demonstrate any MBA size variation. It is important to note that while this difference was not statistically significant, it may be due to the small cohort sizes available. Again, further studies are required to confirm these findings.

Studies in a rodent model of *Ureaplasma* spp. urogenital infection have also shown an association between adverse sequelae and host immune responses. Reyes *et al.* (2009) infected Fisher F344 rats with *U. parvum* and sampled bladder tissues at two weeks post-infection. Two major sequelae were seen in *Ureaplasma*-infected rats: moderate sequelae in the form of urinary tract infection (UTI) and inflammation and the more severe sequelae of UTI with struvite crystals. The cytokine profiles within the bladder tissues obtained from these animals were significantly different. There was no significant differences in the microbial load (number of CFU) of *Ureaplasma* spp. in bladder tissues; however, levels of IL-18, MCP-1 and interferon (IFN)-γ were elevated in those animals with moderate sequelae (UTI and inflammation). By contrast, animals with severe sequelae (UTI and struvite crystals) demonstrated significant decreases in IL-18, MCP-1, IFN-γ, while IL-1α, IL-1β and growth related oncogene/keratinocyte chemoattractant (GRO/KC - the rat analogue of human IL-8) were significantly elevated. This is similar to the findings of our current study, which demonstrated high levels of IL-8 within cord blood when no MBA variation was present; but when these organisms varied their surface-exposed MBA, the levels of this cytokine were significantly reduced. The results of this study further confirm that the numbers of ureaplasmas within tissue are not a critical factor in the development of pathology, however, the host inflammatory response appears to be a key determinant of the severity of inflammatory response and the development of adverse sequelae (Reyes *et al.* 2009).

A real-time PCR and HRM assay to speciate and serotype *U. parvum* clinical isolates was also designed and optimised. This assay successfully speciated and serotyped 64.5% of *U. parvum* clinical isolates but a further 11 isolates could not be subtyped. A number of conventional and real-time PCR assays that detect and subtype *U. parvum* and *U. urealyticum* have been published, however, many of these assays are time-consuming and laborious (Cao *et al.* 2007a; Cao *et al.* 2007b) to perform, multiple reactions are required to type the 14 known serovars and these are not all based on the same gene. These assays are not easily transferred into clinical and diagnostic laboratories and many laboratories still diagnose human infections using culture and PCR designed to detect rather than serotype.

Knox *et al.* (1998) designed conventional PCR assays that serotyped the *U. parvum* serovars based on polymorphisms within a fragment of the *mba* gene. Conventional PCR assays and sequencing of the *mba* in this study confirmed that the upstream conserved portion of the *mba* demonstrates serovar-specific differences in each of the four ATCC *U. parvum* serovars, however, additional SNPs/polymorphisms were also found in some clinical isolates. Kong *et al.* (2000) designed conventional PCR assays that were able to differentiate groups of *U. parvum* and *U. urealyticum* serovars based on 16S rRNA gene, the 23S rRNA intergenic spacer region, urease gene subunits and the *mba* gene. These assays detected and identified three groups within the *U. parvum* species: serovar 1, serovars 3/14 and serovar 6. The 10 *U. urealyticum* serovars were also detected and differentiated into three groups: (i) serovars 2, 5, 8 and 9; (ii) serovars 4, 10, 12 and 13; and (iii) serovars 7 and 11. These reactions were also laborious to complete and required post-PCR analyses such as agarose gel electrophoresis, which increased the risk of post-PCR contamination. Real-time PCR assays that detect, speciate and subtype the ureaplasmas simultaneously would be the most advantageous for use within a clinical laboratory setting.

The major advantage of real-time PCR compared to conventional PCR assays are accessibility, sensitivity and cost-effectiveness. The real-time and rapid nature of real-time PCR is also a major benefit in clinical and diagnostic laboratories, when time-to-diagnosis is of great importance. Real-time PCR assays have been developed to speciate and/or serotype the human *Ureaplasma* spp. (Mallard *et al.* 2005; Yi *et al.* 2005; Cao *et al.* 2007a; Cao *et al.* 2007b). Xiao *et al.* (2010) created real-time PCR assays to differentiate *U. parvum* and *U. urealyticum* serovars. An initial real-time PCR assay differentiated *U. parvum* and *U. urealyticum* species, followed by 14 monoplex real-time PCR assays that serotyped all 14 serovars belonging to *Ureaplasma* spp. Each assay was specifically optimised for $MgCl_2$ concentration, primer concentration and annealing temperature. Some assays

also utilised touch-down protocols in order to gain specificity and used numerous gene targets, some of which are hypothetical or have unknown functions and are yet to be fully characterised (Glass *et al.* 2000; Xiao *et al.* 2010). While these assays were able to serotype numerous clinical isolates, the combination of specific assay cycling parameters, coupled with the additional 'speciating' PCR, followed by the 14 monoplex serotyping real-time PCR assays means that these assays are not suited to a high-throughput clinical and diagnostic laboratory. This large number of real-time PCR reactions (most of which would be done in duplicate or triplicate) significantly increase the cost and time associated with testing and serotyping ureaplasma clinical isolates and is predominantly a research tool.

Our research group previously developed a real-time PCR and high resolution melt (HRM) assay that was able to amplify and differentiate *U. parvum* and *U. urealyticum* species; and that was also able to serotype the four *U. parvum* serovars. HRM is a post-PCR analysis in which the temperature of each tube is increased in precise increments to 'melt' the amplicon. By amplifying the upstream conserved region of the *mba* gene, this assay speciated and serotyped 20/31 (64.5%) of clinical isolates; however, some clinical isolates had additional SNPs and these organisms were unable to be typed successfully (Sweeney *et al.* (2010) unpublished findings). More recently, Payne *et al.* (2014) designed a real-time PCR and HRM assay very similar to the unpublished work within our research group. The assay previously optimised in our group and by Payne *et al.* (2014) amplified the upstream conserved region of the *mba* gene. However, the major shortcomings of both of these assays is that amplification produced two distinct fluorescent 'peaks', consistent with the amplification of more than one amplicon. Therefore, these assays required further optimisation.

The real-time PCR and HRM assay designed within the current study is superior to previous real-time PCR and HRM assays and this assay specifically amplifies and speciates the four *U. parvum* serovars (Serovar 1, 3, 6 and 14; Figure 5.1). Only a single specific amplicon is produced and we have demonstrated the utility of real-time PCR and HRM for the speciation and differentiation of ureaplasma clinical isolates. While some isolates were unable to be successfully typed, this is consistent with other studies and further supports the proposal that there may be additional ureaplasma serovars, or that horizontal gene transfers affects our ability to fully characterise *Ureaplasma* serovars.

The major advantage of this assay is that it targets a ureaplasma-specific gene and polymorphisms within this gene are able to distinguish each of the *U. parvum* serovars, which account for > 90% of

all clinical isolates (Knox *et al.* 1997; Knox *et al.* 2003; Sung *et al.* 2010). By selecting a well-characterised gene, we have been able to create an easy and rapid method for the speciation and serotyping of *U. parvum* clinical isolates. The use of the *mba* gene is also more likely to reflect the antigenic differences in clinical isolates, as it is based on the MBA protein, the antigen which represents the basis for the current serotyping scheme (Robertson *et al.* 2002). Whilst this assay has been found to be a fast and easy way to serotype *U. parvum* clinical isolates, there are some limitations of the assay. Firstly, clinical specimens may contain a mixture of serovars and these may not be distinguished by this assay and additional assays which target specific serovars (such as those developed by Knox *et al*. 1997) would be required to identify mixtures. Furthermore, it is important to have similar initial concentrations of DNA amplicons for the successful serotyping of *U. parvum* clinical isolates. A recent study by Ng *et al.* (2014) demonstrated that the melting temperatures and curves seen by HRM are dependent on DNA starting concentration and that variation in this starting concentration of DNA may result in the inability to serotype some isolates. In an attempt to increase the sensitivity of this assay, some researchers have added $MgCl_2$ following PCR cycling and prior to HRM analysis; this has been shown to reduce the variability of fluorescence and increase the ability to successfully 'type' clinical isolates (Ng *et al.* 2014). Within our study, we standardised the amount of DNA template added to each real-time PCR and HRM assay and this is a major strength of our study. However, uncultured clinical isolates could not always be serotyped using this real-time PCR and HRM assay due to low concentrations of template within the specimen, which affected HRM curves. The addition of $MgCl_2$ in different concentrations following PCR cycling did not improve the ability of the assay to serotype the clinical isolates. Additionally, an important consideration in the use of speciating and serotyping assays for *Ureaplasma* spp. is that as yet there appears to be no intrinsically ‘virulent’ or ‘avirulent’ *Ureaplasma* species or serovars and we demonstrated no major differences in adverse pregnancy or neonatal outcomes amongst the different species (*U. parvum* or *U. urealyticum*) and prevalent serovars (1, 3 and 6).

Conclusions:

This is the first prospective study to demonstrate MBA variants within the chorioamnion of women who delivered LPT or at term. Regardless of the *Ureaplasma* species or serovar present, when MBA variation occurred there was a decreased incidence of histological chorioamnionitis, particularly in the amnion (fetal) membranes. This is also the first study to demonstrate differences in cord blood cytokines, chemokines and growth factors in association with MBA variation of ureaplasmas. The cytokine IL-8 and growth factor G-CSF were significantly higher in *Ureaplasma* spp.-infected

placentae which demonstrated no MBA variation. By contrast, placentae which were infected with ureaplasmas which varied their surface-exposed MBA were associated with significantly lower levels of IL-8 and G-CSF in the corresponding cord blood.

The ability of *Ureaplasma* spp. to vary their surface-exposed MBA is consistent with the evasion of host immune detection and eradication. *Ureaplasma* spp. have been isolated from within the amniotic sac as early as 15 weeks of gestation (Cassell *et al.* 1993b; Gerber *et al.* 2003) and animal models have confirmed that ureaplasmas may survive and replicate in the amniotic cavity, even in the presence of an immune response, establishing chronic infections *in vivo*. It is likely that prolonged intraamniotic infections with *Ureaplasma* spp. will result in adverse neonatal outcomes, due to the prolonged exposure of the fetus to ureaplasmas *in utero*. Infection with *Ureaplasma* spp. has been associated with bronchopulmonary dysplasia (BPD), sepsis, meningitis and pneumonia. In severe cases, ureaplasmas have also been associated with the development of adverse CNS outcomes and cerebral palsy at 1 year of age (Berger *et al.* 2009).

The findings of this study further suggest that it is unlikely that there are 'virulent' *Ureaplasma* species or serovars; however, the host-microbe interactions (as evidenced by MBA size variation) and the host immune response (as evidenced by cord blood cytokines) may determine the severity of inflammation and pathology.

Chapter Six:

Ex vivo model of *U. parvum* chorioamnion tissue infection

Introduction

Preterm birth (PTB); delivery < 37 weeks of gestation, accounts for 10% of all births worldwide (Bick 2012; March of Dimes 2012). Despite an increased awareness and strategies for managing women at risk of delivering preterm, the rates of PTB continue to increase in almost every country. With 70% of neonatal morbidity and up to 50% of neonatal and infant mortality associated with PTB, there is a need to understand how these births occur and, in the long-term, to prevent these births from occurring (Goldenberg *et al.* 2008b). Infection of the female upper genital tract (UGT) is a major finding in women, and 40% of women who deliver preterm have evidence of UGT infections (Goldenberg *et al.* 2002; DiGiulio 2012). Of the pathogens associated with PTB, the human *Ureaplasma* species (*U. parvum* and *U. urealyticum*) are the bacteria most frequently isolated from the UGT of women experiencing PTB, histological chorioamnionitis, funisitis and preterm premature rupture of membranes (pPROM) (Hillier *et al.* 1988; Cassell *et al.* 1993b; Knox *et al.* 1997; Novy *et al.* 2009; DiGiulio 2012).

It is commonly believed that microorganisms within the female lower genital tract (LGT) may ascend and cause UGT infections (Goldenberg *et al.* 2000b). These microorganisms, often normal flora of the LGT, ascend through the cervix to infect/colonise the choriodecidual space and the chorioamnion. Within the chorioamnion, microorganisms (including *Ureaplasma* spp.) are able to elicit strong immune responses, including increased expression or production of inflammatory mediators such as cytokines, chemokines, growth factors, matrix metalloproteinases and prostaglandins (Goldenberg *et al.* 2002; Goldenberg *et al.* 2005). The induction of pro-inflammatory mediators activate and recruit granulocytes, including neutrophils and monocytes and macrophages to the site of infection, amplifying the inflammatory response and it is this inflammation that has been attributed to adverse pregnancy outcomes, such as histological chorioamnionitis, funisitis and pPROM. The general pathways that may lead to PTB or adverse pregnancy outcomes have been described previously (Goldenberg *et al.* 2002; Behrman 2007; Challis *et al.* 2009); however, we do not yet fully understand the host immune responses that are specifically triggered during infection with *Ureaplasma* spp.

Previous studies have demonstrated that the *Ureaplasma* spp. multiple banded antigen (MBA) is the major pathogen-associated molecular pattern (PAMP) of ureaplasmas and this surface-exposed lipoprotein binds/interacts with Toll-like receptors (TLRs) 1, 2 and 6, activating nuclear factor (NF)-kB and the production of the proinflammatory cytokines (Shimizu *et al.* 2008). Immune responses to

Ureaplasma spp. UGT infections have been investigated previously using animal models, including mice (von Chamier *et al.* 2012; Allam *et al.* 2014), sheep (Moss *et al.* 2005; Knox *et al.* 2010; Dando *et al.* 2012; Robinson *et al.* 2013) and non-human primates (Yoder *et al.* 2003; Viscardi *et al.* 2006; Novy *et al.* 2009); however, the results of these studies may not always align with human host immune responses, as the signalling pathways which lead to PTB in animals are often different to those in humans (Mitchell and Taggart 2009). This limits the usefulness of these animal models in investigating the role of the host immune response during *Ureaplasma* spp. infections.

More recently, host immune responses to infection have been studied using human gestational tissues. The placenta is a critical research specimen that offers researchers a unique opportunity to study the immune responses produced by both the maternal and fetal tissues upon exposure to stimuli. Previous studies have utilised chorioamnion tissue explants to study the host immune responses to ureaplasma infections. Abrahams *et al.* (2013) exposed chorioamnion explants to heat-killed *U. parvum* and *U. urealyticum* and demonstrated significant increases in mRNA expression of TLR 8 when stimulated with *U. parvum* but not with *U. urealyticum*. A study by Aaltonen *et al.* (2007), similarly stimulated human chorioamnion explants with heat-killed *Ureaplasma* spp. and demonstrated significant increases in the production of TNF-α, IL-10 and prostaglandin (PG)-E_2. Similarly, Menon *et al.* (2009) measured the cytokine and prostaglandin responses after primary chorioamnion tissues were exposed to ureaplasmas inactivated by heat and demonstrated that stimulation with ureaplasmas resulted in significant increases in TNF-α and IL-10. Other studies have also shown key cytokines to be increased following stimulation with heat-inactivated *Ureaplasma* spp., with levels of IL-1β, TNF-α, IL-8, IL-10 and PGE_2 being significantly increased upon exposure to these pathogenic microorganisms (Estrada-Gutierrez *et al.* 2010; Peltier *et al.* 2012). However, each of these studies utilised ureaplasmas that had been heat-killed; which destroyed their viability and surface-exposed lipoproteins of these microorganisms. These changes were likely to affect how these microorganisms interacted with chorioamnion tissue cells and may also have affected TLR signalling; therefore, the immune responses seen within these studies may not truly reflect what occurs *in vivo*. In addition, each of these studies utilised primary chorioamnion explants incubated in well-plates, and so the origin of these immune responses could not be determined (i.e. they were unable to determine if these cytokines were produced by the maternal or fetal interfaces) and so further studies are required to fully elucidate human host immune responses to *Ureaplasma* spp.

Infection of the chorioamnion may be modelled more accurately using an *ex vivo* Ussing chamber system. Keelan *et al.* (2009) adapted this Ussing chamber model, which had traditionally been used

to study the permeability and secretory functions of the gut epithelia (Heyman *et al.* 1988; Foitzik *et al.* 1997; Hotz *et al.* 1998), to study host immune responses with chorioamnion tissues. Intact chorioamnion tissue was suspended within an Ussing chamber, creating a barrier and the maternal (chorion) and fetal (amnion) surfaces of this tissue were separately perfused with cell culture media, to model the *in vivo* physical and immunological barrier properties of this tissue. The 'maternal' membrane was exposed to stimuli (*Escherichia coli* lipopolysaccharide [LPS]) over a 20 hour period and aliquots of the perfused cell culture media were collected over time from both compartments. Perfused media were subsequently tested for the presence of immune modulators by enzyme-linked immunosorbent assays (ELISA). Despite only the maternal membranes being exposed to LPS, increased concentrations of macrophage-derived chemokine (MDC), transforming growth factor (TGF)-β, TNF-α and PGE_2 were detected in both the 'maternal' and 'fetal' perfusates, which confirms that immune responses were elicited separately in both the chorion and amnion cells. This model was validated as an accurate model with which to study host immune responses to infections (Keelan *et al.* 2009); however, as yet no studies have utilised live microorganisms using this model.

Using this *ex vivo* model of chorioamnion infection, we sought to investigate the host-microbe interactions upon exposure to a *U. parvum* serovar 6 clinical isolate. We hypothesised that the ureaplasmas would infiltrate the chorioamnion and activate specific immune responses within the maternal and fetal membranes. By investigating the immune responses to *Ureaplasma* spp., this study may elucidate how ureaplasmas activate the host immune system and may identify key immune modulators that are present during ureaplasmas infections. Furthermore, the results of this work may provide additional evidence of biomarkers that could be used in future to identify or predict UGT infections with ureaplasmas.

Materials and Methods

All methods pertaining to this chapter are documented in Chapter Three. Briefly, four placentae were obtained from women delivering at term and who had no complications. The placentae were processed within one hour of delivery (Chapter Three, section 3.22). In each of the four experiments, chorioamnion tissue was excised from the placenta and washed with sterile phosphate buffered saline (PBS). Six membrane discs were cut, and these were each sandwiched between semi-rigid mesh and suspended within six Ussing chambers. The 'maternal' and 'fetal' compartments of each Ussing chamber were separately perfused with phenol red-containing ('maternal' compartment) or phenol red-free ('fetal' compartment) M199 media. Media within the maternal compartment was also supplemented with FITC-dextran, as an indicator of membrane permeability or integrity throughout each Ussing chamber experiment. After the Ussing chambers had equilibrated, the maternal membrane was stimulated with (a) 2 x 10^7 CFU live *U. parvum* serovar 6 clinical isolate 429 inocula (Chapter Three, section 3.20; n = 2 chambers), (b) 2 x 10^7 CFU UV-inactivated (non-replicative, but with all surface-antigens intact) *U. parvum* serovar 6 clinical isolate 429 (n = 2 chambers); or (c) vehicle control (n = 2 chambers). At 0, 2, 4, 8, 20 and 30 hours, perfused media was sampled from each maternal and fetal compartment and snap frozen. At the conclusion of each placenta experiment, the remaining media was drained and snap frozen and chorioamnion tissue was divided and fixed for histology or snap frozen. A small amount of chorioamnion tissue was also homogenised and cultured within 10B broth, in order to detect and characterise live ureaplasmas.

Stored maternal and fetal perfusates were analysed at the conclusion of all experiments for: (i) the presence and number of viable ureaplasmas within the maternal and fetal compartments over time; (ii) FITC-dextran fluorescent intensity within both maternal and fetal perfusates, as a measure of membrane integrity or permeability; (iii) the cytokine, chemokine and growth factor responses within maternal and fetal compartments over time; (iv) chorioamnion tissue pathology at the conclusion of each 30 hour experiment; and (v) the production of matrix metalloproteinases (MMPs) at the conclusion of each tissue experiment.

Statistical analyses

Data are presented as the mean value, plus the standard error of the mean (SEM). Cytokine responses in maternal and fetal perfusates and post-infection pathology scores were analysed using analysis of variance (ANOVA) and data analysed/plotted using GraphPad Prism.

Results

Optimisation of *U. parvum* growth in M199 cell culture media

The growth of *U. parvum* serovar 6 clinical isolate 429 in M199 media, supplemented with animal serum and urea at concentrations reported for term human amniotic fluid, was assessed. This growth was compared to the growth of ureaplasmas in 10B broth, the ideal *in vitro* growth medium for these bacteria (Shepard and Lunceford 1976).

No ureaplasma growth was detected by culture in serum deficient M199 media; however, there were differences in the ureaplasma growth rate and the concentration of ureaplasmas after the ureaplasmas were incubated in 10B broth and in M199 media supplemented with either fetal calf serum or horse serum. Higher concentrations of ureaplasmas were achieved in M199 media containing fetal calf serum, when compared to the growth in M199 media supplemented with horse serum (Figure 6.1). The highest concentration of ureaplasmas was achieved after growth in 10B broth at 20 hours. However, as expected, the concentration of ureaplasmas cultured in 10B broth decreased between 20 and 30 hours due to the accumulation of a metabolic by-product (ammonia) produced by ureaplasmas, resulting in an alkaline pH shift and the death of the ureaplasmas.

Based on these results, we determined that M199 media supplemented with 10% fetal calf serum was the optimal growth media for use in *ex vivo* experiments, as this tissue culture medium would support the growth of ureaplasmas and would also maintain the viability of chorioamnion tissue during these experiments.

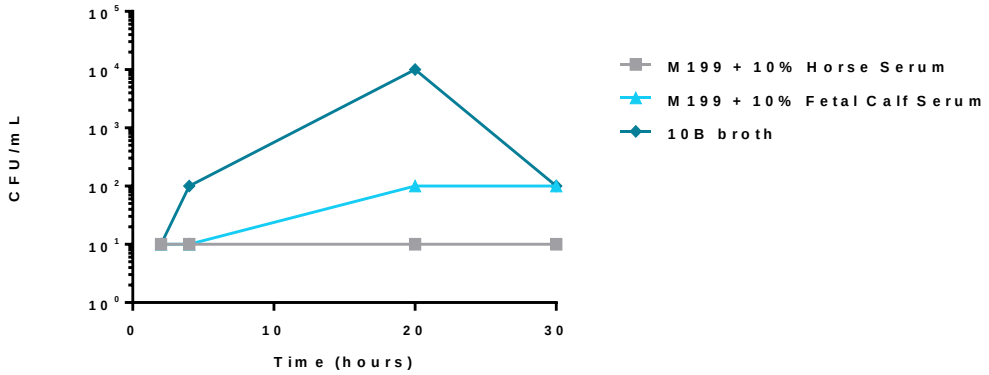


Figure 6.1 *U. parvum* growth in M199 cell culture media supplemented with fetal calf serum or horse serum. Optimal growth of ureaplasmas was achieved in M199 media supplemented with 10% fetal calf serum. Experiments were performed in duplicate and are plotted and analysed as the mean ± standard error of the mean (SEM). Replicate data from each experiment were remarkably similar and error bars were too small to be plotted on log-scale graphs (Raw data shown in Chapter Nine, page 224)

Optimisation of HEC-1A cytokine production after exposure to *U. parvum*

The HEC-1A endometrial cell line was maintained in media supplemented with 10% fetal calf serum at 20% O_2 and 5% CO_2 and exposed to 2 x 10^4 CFU or 2 x 10^7 CFU of *U. parvum* serovar 6 clinical isolate 429. The cell culture supernatant was sampled at 0, 8, 20 and 30 hours post-infection to assess cytokine production in response to these two different infectious doses of *U. parvum*.

The pooled supernatants from duplicate experiments were tested by BioPlex assays for IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ and TNF-α. The HEC-1A cells exposed to high (2 x 10^7 CFU) and low (2 x 10^4 CFU) doses of *U. parvum* serovar 6 clinical isolate 429 demonstrated differences in the production of the cytokines IL-8, GM-CSF and IL-10 (Figure 6.2) over time. The highest levels of these cytokines were elaborated by HEC-1A cells exposed to high dose *U. parvum* serovar 6 at 20 and 30 hours post-infection. Concentrations of IL-8, IL-10 and GM-CSF were significantly upregulated in HEC-1A cells exposed to high dose *U. parvum* ($p = 0.01$, $p = 0.006$ and $p = 0.025$, respectively); while elevated levels of IL-8 were also seen in HEC-1A cells exposed to low dose *U. parvum* clinical isolates ($p = 0.02$; Figure 6.2). Based on these preliminary findings, a high dose inoculum (2 x 10^7 CFU) of our *U. parvum* clinical isolate was used subsequently for the placental *ex vivo* experiments.

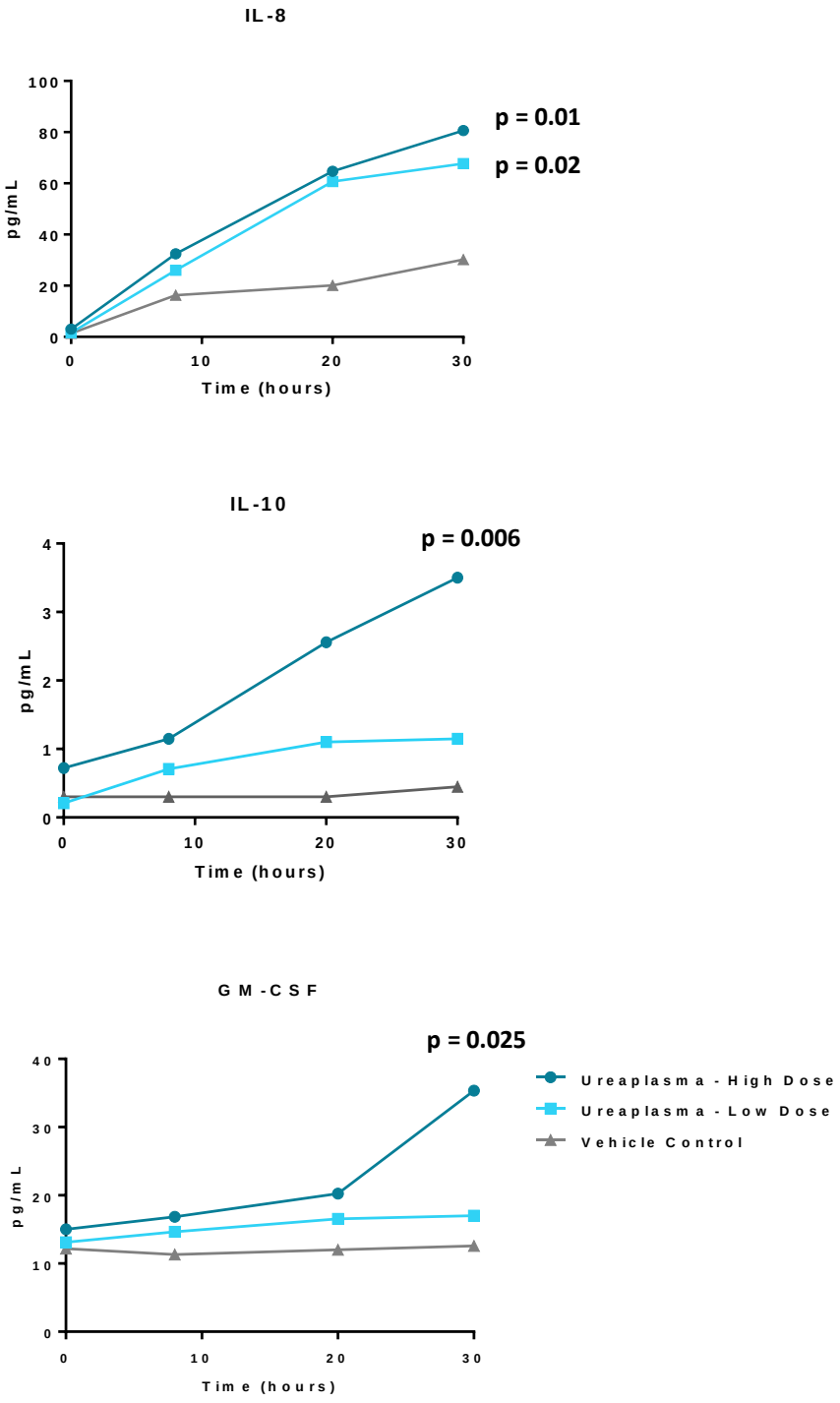


Figure 6.2 High dose (2 x 10^7 CFU) of *U. parvum* serovar 6 clinical isolate elicited a strong immune response in the HEC-1A endometrial cell line, with elevated levels of IL-8, IL-10 and GM-CSF. Pooled culture supernatants from duplicate experiments were analysed by BioPlex assays for cytokines IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ and TNF-α. Data are presented as the mean value of replicate (n = 2) samples that were pooled (to n = 1) for analysis.

Optimisation of *U. parvum* growth under different oxygen tensions

Experiments to determine the optimal oxygen tension to best replicate the *in vivo* environment within the placenta and also to support the growth of *U. parvum* serovar 6 were then performed. No *U. parvum* growth was detected when incubated under 3% oxygen. However, similar growth patterns were observed when ureaplasmas were grown under 5% and 20% oxygen. The highest concentrations of ureaplasmas were achieved when ureaplasmas were grown under 8% oxygen (Figure 6.3).

Based on these findings, it was determined that 8% oxygen would be the ideal oxygen tension for the growth of ureaplasmas in the *ex vivo* Ussing chamber model and this oxygen tension was also similar to the oxygen tensions (range = 5 - 8%) achieved within placentae *in vivo* (Kay 2011).

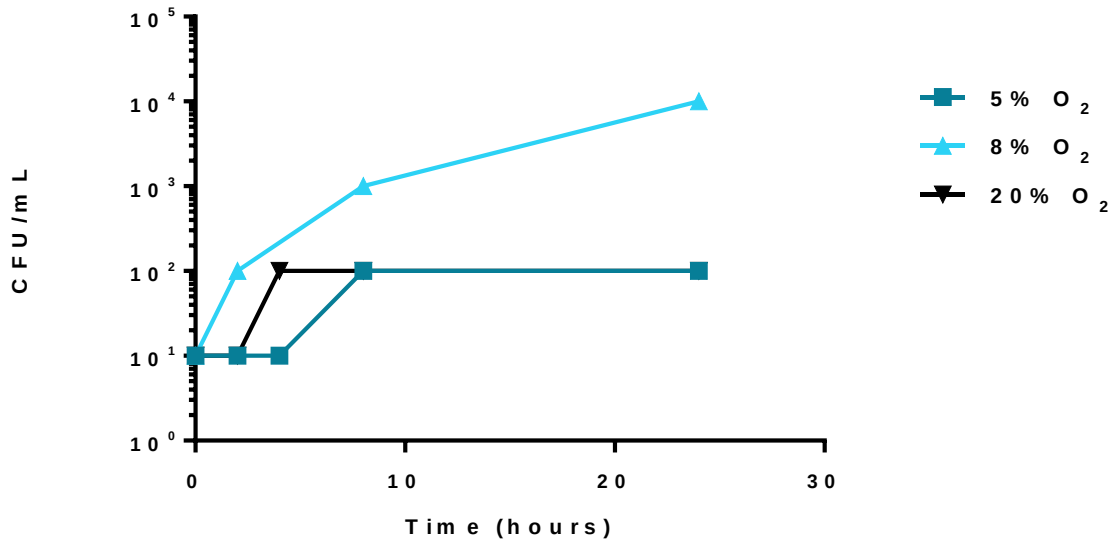


Figure 6.3. Growth of *U. parvum* under different oxygen tensions. The highest concentration of growth was achieved when ureaplasmas were cultured in supplemented M199 media under 8% oxygen. Experiments were performed in duplicate and data are presented as the mean, plus or minus the standard error of the mean. Replicate data from each experiment was remarkably similar, and therefore error bars were too small to be plotted graphs (Raw data shown in Chapter Nine, page 243).

Ex vivo Ussing chamber experimental model results

Culture of *U. parvum* from chorioamnion tissues and perfusates

Chorioamnion

When setting up the Ussing chamber experiments, a portion of chorioamnion tissue from each of the four placentae were snap frozen for subsequent culture and 16S rRNA PCR. No *Ureaplasma* spp. or other microorganisms were detected within these chorioamnion tissues prior to commencing Ussing chamber experiments.

Maternal and fetal perfusates

After the completion of each placental experiment, the perfusates were also tested by culture. No viable ureaplasmas were detected within any of the maternal or fetal perfusates from Ussing chambers in which chorioamnion membranes were exposed to vehicle controls or to the UV-inactivated *U. parvum.*

At the completion of each experiment (t = 30 hours), *U. parvum* was cultured from each of the maternal perfusates (n = 2 for each of the 4 placentae tested) that were initially inoculated with live high-dose *U. parvum*. The numbers of *U. parvum* (CFU/mL) increased exponentially between 0 – 4 hours; but the concentration of ureaplasmas did not differ significantly for the remainder of each 30 hour experiment (Figure 6.4). In these same Ussing chambers, no ureaplasma growth was detected in the fetal perfused media that was collected at 0, 2, 4, 8 and 20 hours post-inoculation; however, at the final 30 hour time point ureaplasmas were detected within each of the fetal perfusates (n = 2 for each of the 4 placentae tested (average: 2.53×10^4 CFU).

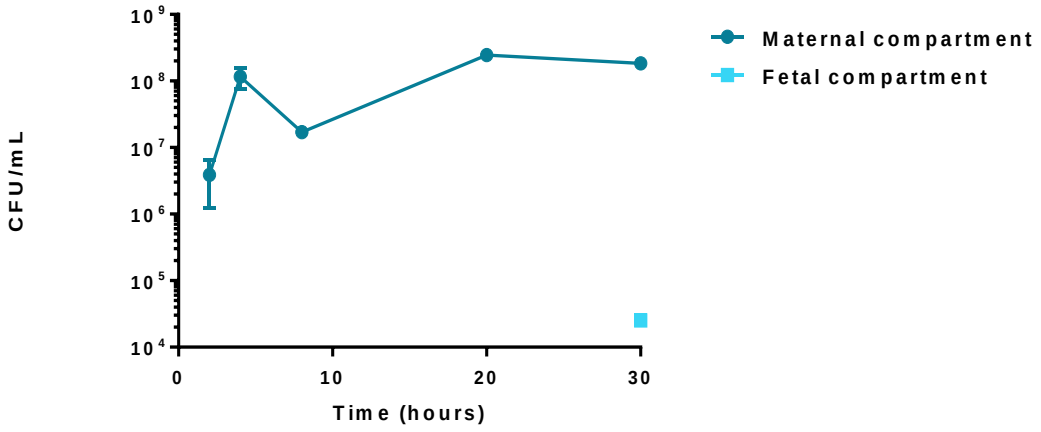


Figure 6.4. The number of *U. parvum* CFU did not differ significantly between replicate chambers or in each of the 4 placental experiments. Live ureaplasmas were subsequently cultured from each of the maternal perfusates (collected at 2, 4, 8, 20 and 30 hours). *U. parvum* was only cultured from the fetal perfusates that were collected at 30 hours post-infection (ureaplasmas were inoculated into the maternal compartment). Data are presented as the mean, plus or minus the standard error of the mean (SEM)

Western blot of *U. parvum* MBA protein at 30 hours post-infection

Uncultured perfusates collected from both the maternal and fetal compartments were centrifuged and tested by western blot. The initial inoculum (*U. parvum* serovar 6 isolate 429) was also tested by western blot and demonstrated two distinct MBA protein bands. Western blot analysis of uncultured maternal and fetal perfusates demonstrated the same MBA protein bands as the initial inoculum, with two clear MBA bands present in each compartment (Figure 6.5).

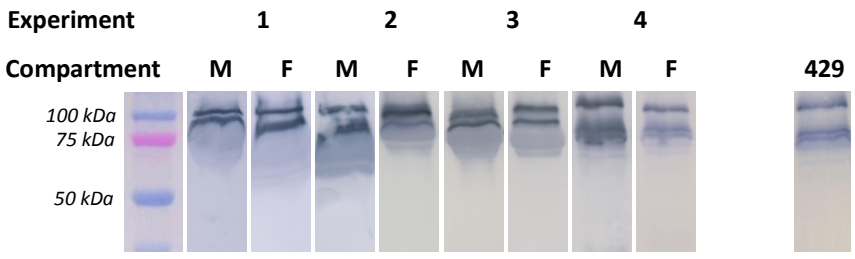


Figure 6.5. Western blot demonstrating the MBA proteins detected in the maternal and fetal (M – maternal, F – fetal) compartments at 30 hours post-infection. No MBA antigen variation was demonstrated, when compared to the initial inoculum of *U. parvum* serovar 6 clinical isolate 429. Each perfusate was tested (n = 2 for each of the 4 placentae). Representative results are shown for each compartment of one Ussing chamber per experiment.

Response of chorioamnion tissue upon stimulation with *U. parvum*

Integrity of chorioamnion tissue within the Ussing chambers

The integrity of chorioamnion tissue within each of the six Ussing chambers was evaluated for the duration of each 30 hour placental experiment (n = 4). FITC-dextran was added to each maternal compartment at the commencement of the experiments and the accumulation of FITC-dextran within the fetal perfusates was assayed as an indicator of membrane integrity (accumulation of FITC-dextran within the fetal compartment is an indicator of membrane permeability or leakage).

The fetal perfusates collected from Ussing chambers in which the maternal membrane was exposed to UV-inactivated *U. parvum* or vehicle control demonstrated no significant chorioamnion leakage or rupture over the course of the 30 hour *ex vivo* experiments, averaging approximately 1 – 1.5% per hour (Figure 6.6). By contrast, the concentration (% of total transfer) of FITC-dextran within fetal perfusates increased significantly at 30 hours after the maternal membrane was exposed to live ureaplasmas (p = 0.006; Figure 6.6).

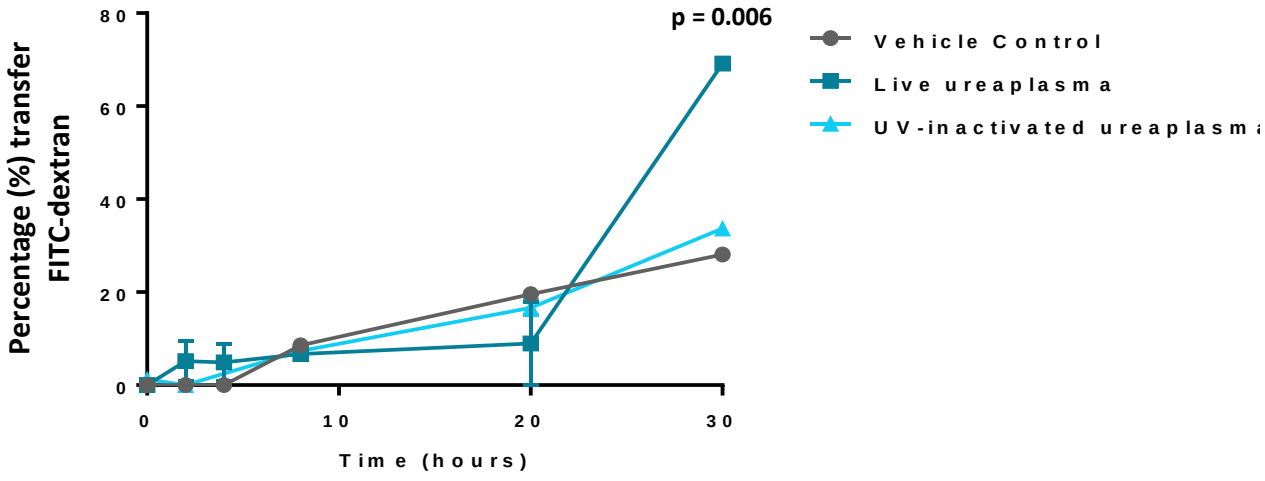


Figure 6.6. Chorioamnion membrane permeability as measured using FITC-labelled dextran. Transfer of FITC-dextran from the maternal compartment to the fetal compartment (presented as percentage transfer) during placental experiments (duplicate chambers for n = 4 experiments). Significant increases in FITC-dextran transfer were demonstrated at 30 hours post-infection in the Ussing chambers in which the maternal side of chorioamnion membranes were exposed to live *U. parvum*. Data are presented as the mean, plus or minus the standard error of the mean (SEM)

Histological analysis of chorioamnion tissues

Chorioamnion tissue from each placentae (n = 4) was fixed prior to the start of each placental experiment and these tissues were embedded, sectioned and stained with haematoxylin and eosin. These chorioamnion tissues demonstrated no significant pathology prior to the commencement of each Ussing chamber *ex vivo* experiment.

Upon the completion of *ex vivo* experiments, chorioamnion tissues were again fixed, embedded and sectioned before staining with haematoxylin and eosin. Chorioamnion membranes that were exposed to live *U. parvum* demonstrated major differences in the overall tissue structure, when compared to tissues that were exposed to UV-inactivated *U. parvum* or vehicle controls. Very few studies histologically grade/score chorioamnion tissues for evidence of membrane rupture and so a pathology scoring system was developed for this study. Researchers blinded to outcome were given randomised images of chorioamnion tissue and the images were scored according to criteria described in Chapter Three, section 3.20.

Chorioamnion tissues exposed to vehicle controls or to UV-inactivated *U. parvum* demonstrated no significant differences in the grading of the tissue pathology after each experiment (mean score 1.5 and 1.75, respectively; Figure 6.7A and C). However, the chorioamnion tissues exposed to live *U. parvum* (duplicate tissues for n = 4 experiments) showed significant detachment/separation of the chorion and amnion membranes (mean score: 3; Figure 6.7B) and this was consistent with deterioration of the chorioamnion membranes as measured by the transfer of FITC-dextran at 30 hours post-infection (detailed above).

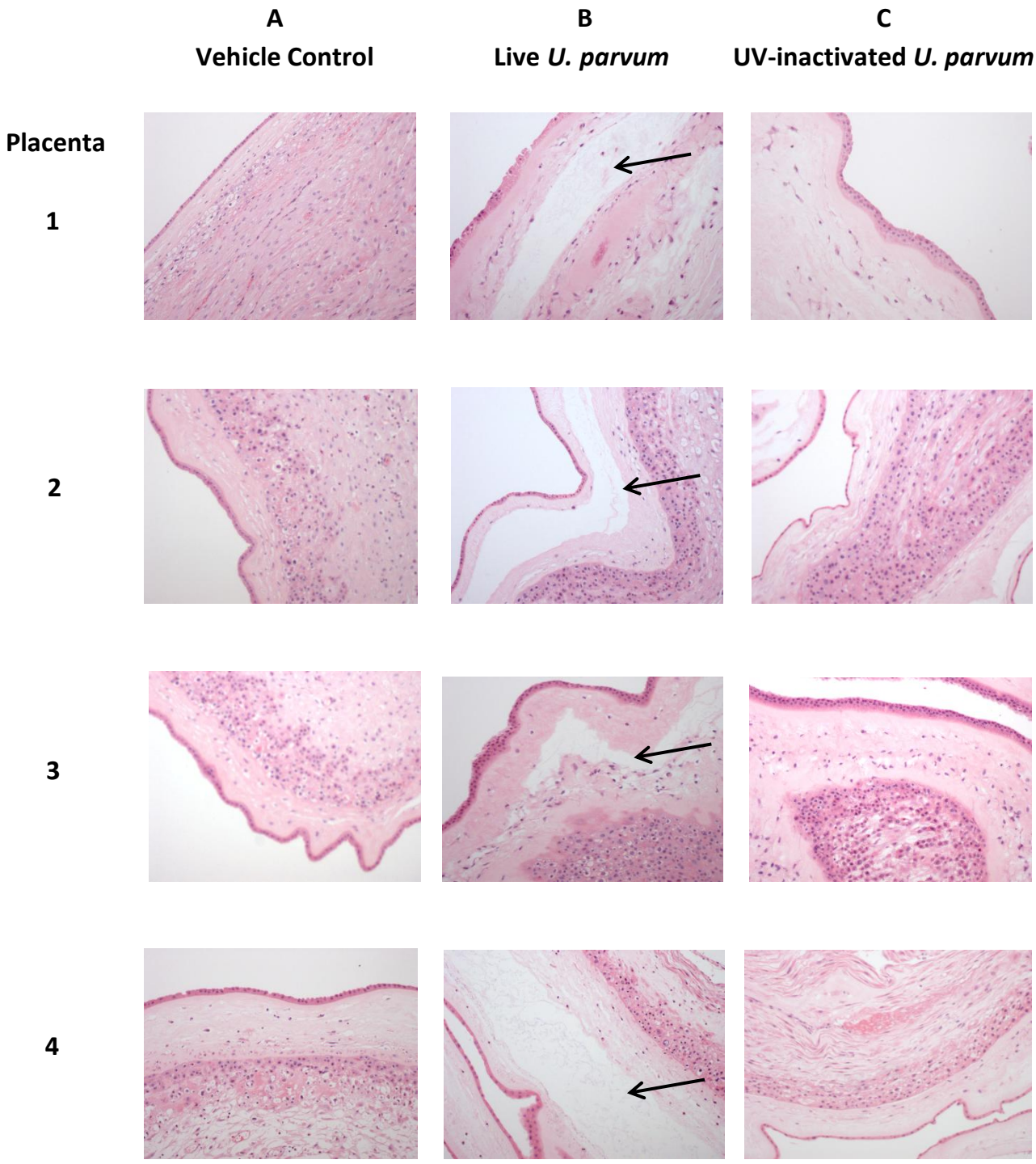


Figure 6.7. Haematoxylin and eosin stained tissue sections exposed to (A) vehicle control, (B) live *U. parvum* and (C) UV-inactivated *U. parvum*. Following exposure to live ureaplasma (30 hours), chorioamnion tissues demonstrated detachment/separation of the chorion and amnion (black arrow). All images shown at x 100 total magnification

Cytokine responses of human chorioamnion tissue exposed to live and UV-inactivated ureaplasmas

Duplicate perfusates collected from maternal and fetal compartments of Ussing chamber experiments stimulated with live or UV-inactivated *U. parvum* and vehicle controls were tested for the presence of IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF IFN-γ and TNF-α using BioPlex assays. This assay was chosen based on results of previous human *in vitro* and *in vivo* studies and included cytokines, chemokines and growth factors which have been reported to be upregulated in response to *Ureaplasma* spp. infections (Aaltonen *et al.* 2007; Jacobsson *et al.* 2009; Kacerovsky *et al.* 2011; Kacerovsky *et al.* 2013).

No significant differences were demonstrated in the concentrations of IL-2, IL-4, IL-6, IL-8, GM-CSF, IFN-γ and TNF-α in either the maternal or fetal perfusates that were exposed to live *U. parvum,* UV-inactivated *U. parvum* or vehicle control in supplemented M199 media. However, the concentrations of IL-10 were significantly higher within the maternal perfusates after exposure to live *U. parvum* for 20 and 30 hours (20.4 ± 2.8 pg/mL, p = 0.046; Figure 6.8) than the levels achieved when membranes were exposed to the vehicle control (7.2 ± 0.2 pg/mL) or UV-inactivated *U. parvum* (4.94 ± 0.9 pg/mL). No significant differences in the concentrations of IL-10 were seen in fetal perfusates.

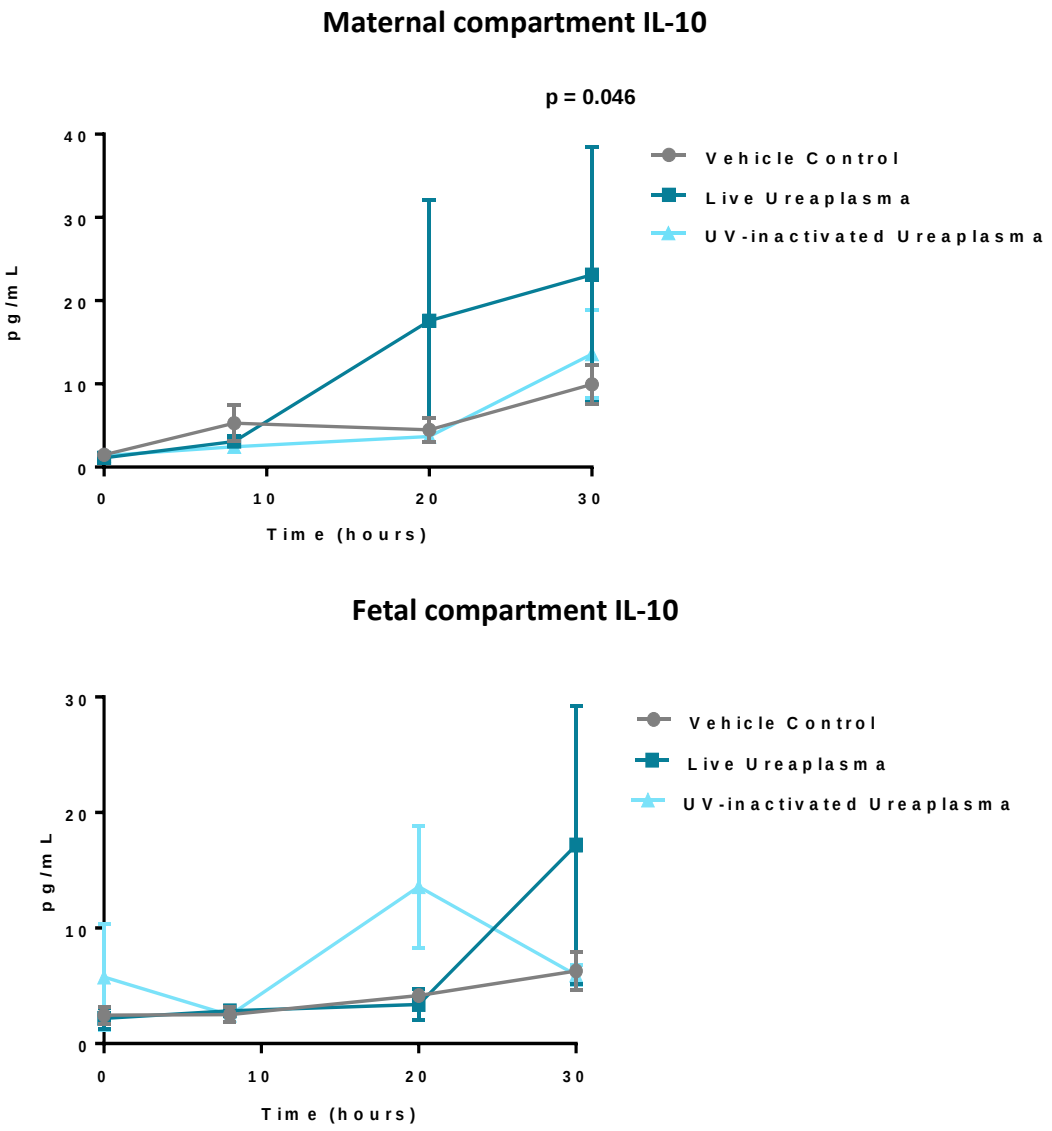


Figure 6.8. Cytokine responses of duplicate maternal and fetal perfusates from n = 4 placental experiments. Concentrations of IL-10 were significantly increased at 20 and 30 hours post-infection within the maternal compartment of Ussing chambers when the maternal side of chorioamnion membranes were stimulated with live *U. parvum*. Data are presented as the mean, plus or minus the standard error of the mean (SEM)

Gelatin zymography for matrix metalloproteinase (MMP)-9 activity

For each placental experiment, maternal and fetal perfusates (x 2) from each Ussing chamber were pooled per condition (vehicle control, live *U. parvum* or UV-inactivated *U. parvum*). One sample in total per Ussing chamber was assessed by gelatin zymography for MMP-9 activity.

No MMP-9 activity was detected in pooled maternal and fetal perfusates at 0 hours, regardless of treatment (Figure 6.9); however, at 30 hours post-infection Ussing chamber perfusates demonstrated differences in MMP-9 activity. MMP-9 activity was detected in the perfusates of only one Ussing chamber stimulated with vehicle controls at 30 hours post-infection. Ussing chambers exposed to live or UV-inactivated *U. parvum* demonstrated upregulation of MMP-9 activity (Table 6.1). MMP-9 activity in pooled perfusates from n = 4 placental experiments showed prominent gelatinase activity, the presence of clear bands and MMP-9 activity was detected in 6/8 (75.0%) perfusates. Perfusates from UV-inactivated *U. parvum* Ussing chambers showed lesser gelatinase activity, less prominent bands and 4/8 (50.0%) zymographs (Figure 6.9).

Table 6.1. MMP-9 activity at 30 hours post-exposure in each Ussing chamber condition for n = 4 placental experiments. No MMP-9 activity was detected in pooled maternal and fetal perfusates at 0 hours. MMP-9 activity in pooled maternal and fetal perfusates at 30 hours is expressed as the total number of chambers positive for MMP-9 per placental experiment (n = 4).

Placental experiment	Vehicle control	Live *Ureaplasma* spp.	UV-inactivated *Ureaplasma* spp.
1	0/2	1/2	1/2
2	0/2	1/2	2/2
3	1/2	2/2	0/2
4	0/2	2/2	1/2

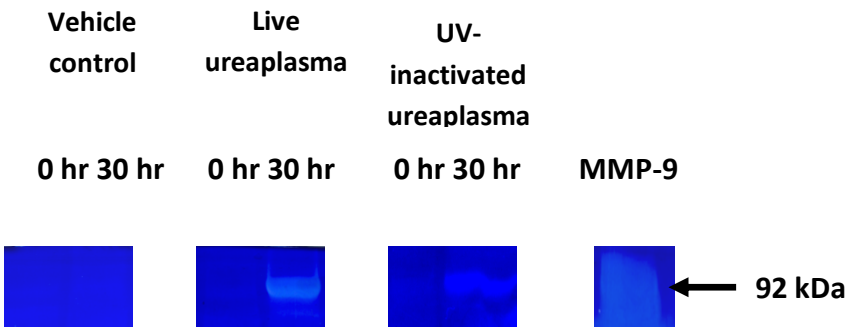


Figure 6.9. Gelatinase activity of matrix metalloproteinase (MMP)-9. While variable responses in MMP-9 activity was seen, overall there was a trend to upregulation of MMP-9 activity in perfusates exposed to live *U. parvum,* those exposed to UV-inactivated *U. parvum* infected chambers at the end of each 30 hour experiment. Representative results are shown from n = 4 experiments, alongside a 92 kDa MMP-9 positive control (kindly provided by Dr Eliza Whiteside).

Discussion

The human host immune response is integral for the success of pregnancy and is involved in all aspects of gestation, from preparation of the endometrium for implantation to immunotolerance of the fetus and initiation of normal term labour (Racicot *et al.* 2014). Any disruptions to these tightly regulated processes may contribute to adverse pregnancy outcomes, such as PTB and pPROM (Challis *et al.* 2009). Currently, the immune mechanisms which lead to PTB and adverse pregnancy outcomes are not well understood and this is particularly true for infection with *Ureaplasma* spp. To better understand the immune responses within host tissues during ureaplasma infections, we conducted a pilot study using an *ex vivo* Ussing chamber model to investigate the specific immune responses of the chorioamnion membranes (the chorion and the amnion) following stimulation with live or UV-inactivated (non-replicative) *U. parvum*.

The most significant finding of this current study was that concentrations of IL-10 were only elevated at 20 and 30 hours post-infection within the maternal compartment after the maternal interface of the chorioamnion was stimulated with live *U. parvum* (Figure 6.8). While we also saw an increase in the production of IL-10 within the fetal compartment at 30 hours post-infection, this was not significant; suggesting that the responses seen are specific to the tissues that were initially exposed to the pathogen. While the production of IL-10 has been identified previously; within fibroblasts, resident macrophages and amnion epithelial cells of human chorioamnion tissues (Huleihel *et al.* 2003), within cell culture supernatants collected from chorioamnion tissue explants stimulated with ureaplasmas (Aaltonen *et al.* 2007; Menon *et al.* 2009; Peltier *et al.* 2012) and also within amniotic fluid of women experiencing UGT ureaplasma infections (Kacerovsky *et al.* 2013); these increases were often accompanied by simultaneous increases in pro-inflammatory cytokines, including IL-1β, IL-8 and TNF-α. In this pilot study, we tested for the presence of both IL-8 and TNF-α but there were no significant increases in the production of these key pro-inflammatory cytokines. Despite this, our preliminary data suggests that IL-10 may be an important determinant of the host immune response to *U. parvum* infection and is the first cytokine produced by the chorioamnion. IL-10 is an anti-inflammatory cytokine that plays a crucial role in the regulation of inflammatory pathologies and other research has also demonstrated a role for IL-10 in immunomodulation and immunosuppression of inflammatory responses (Moore *et al.* 2001; Saraiva and O'Garra 2010).

In Figure 6.10, the major potential pathways that may be activated by *Ureaplasma* spp. infections of the UGT are summarised. Within the placenta and chorioamnion, pattern recognition receptors

(PRRs) are present on a range of cell types, including the chorioamnion and on the surface of macrophages (Hofbauer cells) (Abrahams 2008). These PRRs are able to actively sense and respond to pathogens by recognising their pathogen-associated molecular pattern (PAMP), such as the MBA lipoprotein of *Ureaplasma* spp. (Shimizu *et al.* 2008; Triantafilou *et al.* 2013), and recognition of the MBA by PRRs (e.g. TLRs) (Abrahams 2008) may stimulate different immune responses. Macrophages within the chorioamnion are capable of polarisation into M1 or M2 phenotypes, which respond to the presence of microorganisms in different ways (Mantovani *et al.* 2005). Macrophage polarisation to an M1 phenotype results in the production of a TH1 immune response, generation of cytokines (e.g. IL-1β, TNF-α and interferon-γ), reactive oxygen species and the capacity to present antigens to antigen presenting cells (APCs); while M2 macrophage polarisation results in a TH2 immune response, the production of (predominantly immunosuppressive) cytokines (e.g. IL-4 and IL-10), generation of nitric oxide, increases in cell apoptosis and antibody-mediated host responses (Brown *et al.* 2014) and both M1 and M2 macrophage phenotypes within the placenta during pregnancy are shown to be associated with adverse pregnancy outcomes. For example, some strains of *Toxoplasma gondii* can actively induce M2 macrophage polarisation and minimal inflammatory responses within murine placentae, despite high numbers of parasites colonising the placenta. By contrast, other *T. gondii* strains injected into mice at the same dose induced M1 macrophage polarisation and high levels of inflammation within the placenta, which led to high intrauterine growth retardation (Liu *et al.* 2013; Kong *et al.* 2015). While pregnancy is a predominantly TH2 condition, which can further be regulated by fluctuation in estrogen levels (Behrman 2007); within our pilot study, we reported higher concentrations of IL-10 in chorioamnion tissues exposed to live *U. parvum* and this was consistent with a potential TH2 immune response.

IL-10 has been shown to interact with NF-kB at the transcriptional level, inhibiting the activation of NF-kB and subsequently halting the production of pro-inflammatory cytokines by macrophages (Janeway 2005). In addition, during each of our four *ex vivo* chorioamnion experiments, we showed no significant increases in the production of the pro-inflammatory cytokines IL-2, IL-4, IL-6, IL-8, IFN-γ or TNF-α in response to stimulation of primary chorioamnion tissues with live *U. parvum*. While within our experiments, we were unable to determine if M2 macrophage polarisation was occurring and if this was the factor which led to high levels of IL-10 within *ex vivo* chorioamnion tissue; this would be the next logical step in extending this work and would provide further novel information in the role of the host immune response to *Ureaplasma* spp.

By contrast, some studies have demonstrated that ureaplasma infections may signal the production of pro-inflammatory immune responses. Studies which utilise heat-killed *Ureaplasma* spp. have demonstrated the production of pro-inflammatory cytokines IL-1B, IL-8 and TNF-a within a range of placental cell types (Aaltonen *et al.* 2007; Menon *et al.* 2009; Peltier *et al.* 2012). Similarly, *in vitro* studies utilising human-derived monocytes obtained from cord blood demonstrated that a secondary infectious challenge with *Ureaplasma* spp. enhanced pro-inflammatory immune responses by blocking the expression of the immunosuppressive cytokine, IL-10 (Manimtim *et al.* 2001). In Chapter Five, we did demonstrated the infiltration of neutrophils (chorioamnionitis) and the production of pro-inflammatory cytokines (IL-8 and G-CSF) within cord blood of placentae infected with *Ureaplasma* spp.; however, the levels of these pro-inflammatory cytokines were correlated with MBA size variation. *In vivo*, when MBA variation occurred, there was a significant reduction in pro-inflammatory cytokines within cord blood; despite the presence of large numbers of ureaplasmas infecting the chorioamnion. Similarly, C57BL/6 mice infected intraamniotically with *U. parvum* demonstrated lower levels of pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α, when compared to BALB/c mice infected with the same strain and dose of *U. parvum* (von Chamier *et al.* 2012). Our findings, along with studies by others suggest that there may be other factors (such as genetic background and antigen variation) which may influence the development of disease.

It may be that induction of pro-inflammatory responses are dependent on the numbers of *Ureaplasma* spp. present within the chorioamnion, and/or the duration of infection. The presence of estrogen may also influence the immune response, and lower levels of estrogen are present earlier in gestation which may account, in part, for why *Ureaplasma* spp. infections in pregnancies which end < 32 weeks have reported predominantly pro-inflammatory immune responses (Li *et al.* 2000; Collins *et al.* 2010; Kasper *et al.* 2010; Kacerovsky *et al.* 2013). Future studies in this area will be important to investigate both pro- and anti-inflammatory immune responses, the presence and expression of PRRs (including TLRs) and additional immune factors, such as apoptosis and macrophage polarisation, which may play a key role in the regulation of host immune responses to UGT *Ureaplasma* spp. infections.

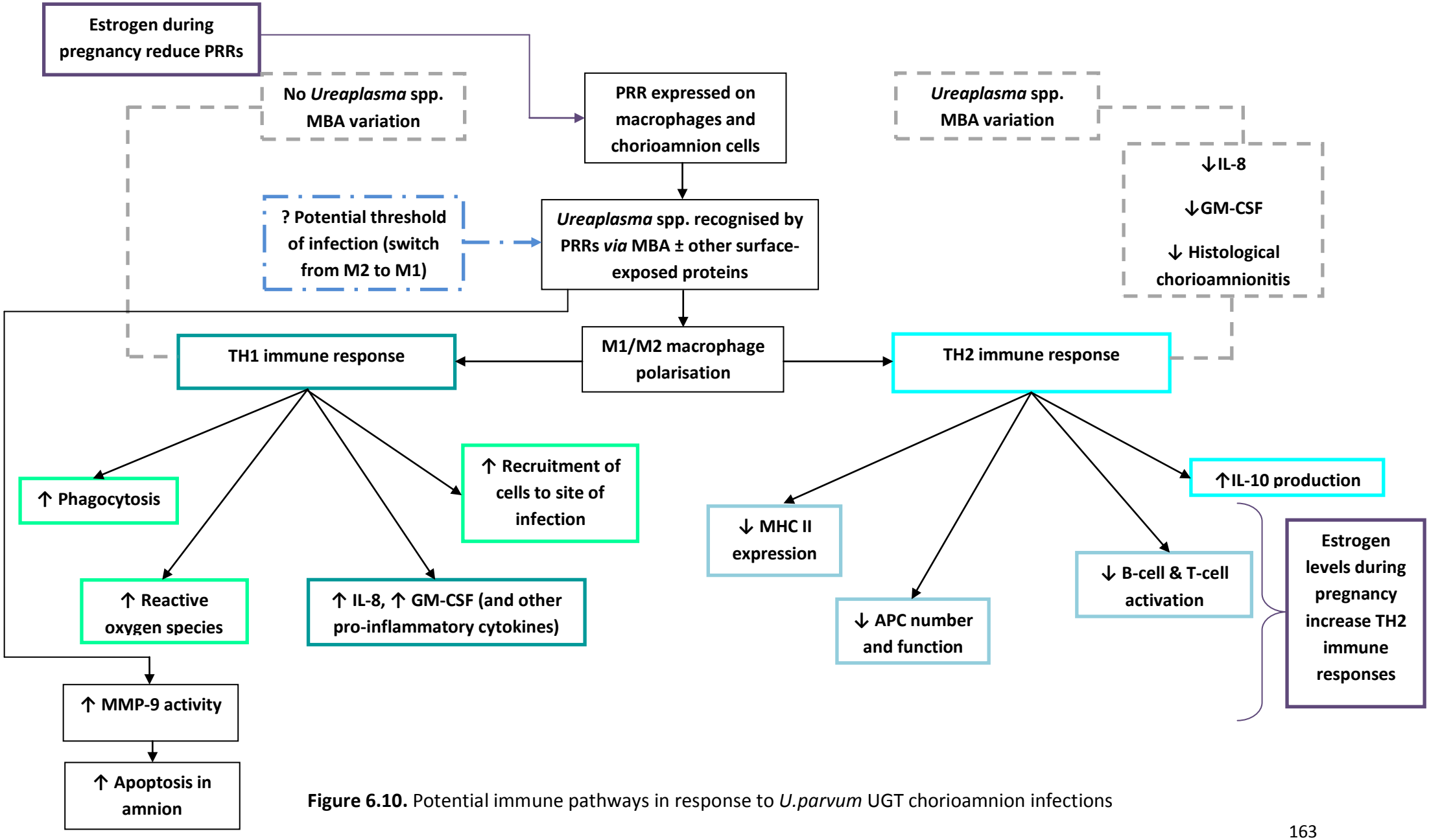


Figure 6.10. Potential immune pathways in response to *U.parvum* UGT chorioamnion infections

Within this pilot study, we also demonstrated that infection with live *U. parvum*, but not UV-inactivated *U. parvum* resulted in chorioamnion tissue damage, including the loss of structure and integrity after only 30 hours of exposure. Histological analysis of chorioamnion tissues infected with live ureaplasmas demonstrated major structural differences, with the amnion being partially detached and a high tissue pathology score (mean: 3). This damage was independently scored by 2 researchers using criteria developed specifically for these experiments. This same tissue pathology was not seen in chorioamnion tissues exposed to UV-inactivated *U. parvum* or those exposed to the vehicle control and this was reflected in lower pathology scores (mean: 1.75 and 1.5, respectively). Previous studies have identified significant pathological changes in UGT and fetal tissues infected with *U. parvum*. Intraamniotic infection with ureaplasmas resulted in increases in macrophages and neutrophils within chorioamnion tissues and structural changes to the neonatal lung within a pregnant sheep model (Moss *et al.* 2008; Collins *et al.* 2010; Knox *et al.* 2010; Dando *et al.* 2012; Robinson *et al.* 2013). Interestingly, within this sheep model, the severity of chorioamnionitis varied between animals, even when the animals received the same ureaplasma inoculum. In this current experiment, after just 30 hours of exposure to ureaplasmas, uniform changes and degradation of the chorioamnion were observed. Within non-human primates, infection with *Ureaplasma* spp. resulted in histological chorioamnionitis, funisitis and altered fetal lung structure (Yoder *et al.* 2003; Viscardi *et al.* 2006; Novy *et al.* 2009). Human pregnancies affected by *Ureaplasma* spp. have also shown changes in pathology, with inflammatory responses within the chorioamnion, cord and altered fetal lung function (Hillier *et al.* 1988; Gerber *et al.* 2003; Pandey *et al.* 2007; Schelonka and Waites 2007; Hecht *et al.* 2008; Viscardi 2010; DiGiulio 2012). Taken together, these studies confirm that *Ureaplasma* spp. are a cause of structural and inflammatory changes within maternal and fetal tissues of the host. In this pilot study, we identified notable pathology and detachment of the amnion membranes, which has not been previously identified within any animal model studies of UGT ureaplasma infections in pregnancy. This finding may be consistent with the initial stages of chorioamnion membrane rupture, often referred to as pPROM when it occurs *in vivo* during gestation. pPROM is a common cause of PTB, affecting up to 30% of all pregnancies which end prematurely (Goldenberg *et al.* 2008b). Studies have also demonstrated a significant correlation between women experiencing pPROM and the presence of UGT infections within these pregnancies (Goldenberg *et al.* 2008b; Kacerovsky *et al.* 2009; Kacerovsky *et al.* 2011); however, no studies have reported on the histological changes to the chorioamnion membranes involved in pPROM. The novel findings generated using this Ussing chamber model suggest that this may be an ideal model with which to further investigate not only the development of pPROM in response to ureaplasmas, but also investigate chorioamnion exposure to other bacteria commonly associated with adverse

pregnancy outcomes. In these current experiments, only the maternal membrane surface was challenged with ureaplasmas and additional experiments in which the fetal membrane surface is exposed to bacteria would further our understanding of these pathological changes during ureaplasma infections.

In this pilot study we assessed the integrity of chorioamnion tissue over time using fluorescently (FITC)-labelled dextran and measuring the transfer from the maternal compartment to the fetal compartment over time. While all Ussing chambers demonstrated similar FITC-dextran transfer at 0, 2, 4, 8 and 20 hours, averaging 1 - 1.5%, there was a significant difference in FITC-dextran transfer at 30 hours but only in the Ussing chambers in which the maternal membranes were stimulated with live *U. parvum* (Figure 6.6). The sharp increase in FITC-dextran transfer seen in these Ussing chambers is consistent with live ureaplasmas negatively affecting the integrity of chorioamnion tissue and this may precede and be associated with membrane rupture (pPROM). This finding that live ureaplasmas can affect the structure and integrity of chorioamnion tissue is novel and also requires further investigation. Other studies have investigated membrane integrity in response to different stimuli but identified no significant alterations in chorioamnion tissue. Assays utilising chorioamnion tissues suspended within a Transwell system demonstrated no alterations in human chorioamnion tissue integrity when stimulated with γ-irradiated *E. coli* over 20 hours; with 99% of fluorescently-labelled beads added to the maternal compartment remaining on this side of the chorioamnion membranes (Stinson *et al.* 2014). The size of these spherobeads is not stated within this study, and so it is unclear if this result is comparable to the results of FITC-dextran within our current study. Keelan *et al.* (2009) stimulated human chorioamnion tissues with *E. coli* LPS when the tissues were suspended within an Ussing chamber system. In this study, FITC-dextran was added to the maternal chamber and transfer of FITC-dextran from the maternal compartment to the fetal compartment occurred at a rate of approximately 1% per hour over the course of each 20 hour experiment. Within our current study, we reported similar FITC-dextran transfer rates for vehicle control and UV-inactivated *U. parvum* Ussing chambers, when compared to the findings of Keelan *et al.* (2009). This further confirms the validity of the results reported within our study. However, the experiments in this current study were conducted for an additional 10 hours. At 30 hours post-infection, just as was observed by Keelan for LPS, there was minimal transfer of FITC-dextran in UV-inactivated *U. parvum* and vehicle control Ussing chambers and no major histological changes were observed within the membrane. This further confirms our finding that only live ureaplasmas negatively affected the structure and integrity of the chorioamnion tissue.

Other studies have identified that infection or exposure to *Ureaplasma* spp. *in utero* can affect the structure and development of the neonatal lung and gut. *Ureaplasma* spp. infections are demonstrated to cause structural changes in fetal lung, including altered lung structure, premature lung maturation, increased neonatal lung pressure-volume curves and fibrosis in fetal sheep or non-human primates (Yoder *et al.* 2003; Viscardi *et al.* 2006; Moss *et al.* 2008; Robinson *et al.* 2013). Using a pregnant ovine model of intraamniotic *Ureaplasma* spp. infection, tissues harvested from ewes intraamniotically injected with ureaplasmas had significant inflammatory responses within the neonatal gut, alterations in the development and structure of the neonatal gut, cellular damage to the villus epithelium and perturbations in the formation of cellular tight junctions within the neonatal gut (Wolfs *et al.* 2013). These findings confirm that *Ureaplasma* spp. can alter the structure and integrity of host tissues. The findings reported within this study that infection with live *Ureaplasma* spp. result in altered chorioamnion membrane structure and integrity may be consistent with the early stages of bacterial invasion of the UGT.

Perhaps the most interesting finding of this study was that *Ureaplasma* spp. can penetrate the chorioamnion membrane, travelling from the maternal compartment to the fetal compartment by the 30 hour time point. While *Ureaplasma* spp. have been isolated from within the amniotic fluid and placentae of women experiencing PTB and pPROM previously (Gerber *et al.* 2003; Jacobsson *et al.* 2009; Kacerovsky *et al.* 2009; DiGiulio *et al.* 2010; DiGiulio 2012), it has not been proven if these microorganisms can traverse the chorioamnion membrane. It has been suggested that these microorganisms may asymptomatically colonise the female UGT prior to pregnancy (Cassell *et al.* 1993b; Cicinelli *et al.* 2012), or that these microorganisms may be present at the time of conception as these microorganisms may adhere to the surface of human sperm and can gain access to the female UGT (Knox *et al.* 2003). However, this current study is the first to demonstrate that *ex vivo* chorioamnion tissue can be penetrated by ureaplasmas and that these microorganisms can pass into the fetal compartment. Winram *et al.* (1998) demonstrated that *Streptococcus agalactiae* (Group B Streptococcus) was able to traverse the chorioamnion membrane in a transwell system; however, in that study the integrity of the membranes were not assessed. The finding that microorganisms may traverse/invade the chorioamnion membrane may have severe implications for the health and wellbeing of the pregnancy and for the neonate. If *Ureaplasma* spp. are able to pass through the chorioamnion membrane and access the UGT during pregnancy, these microorganisms may infect the UGT for chronic periods and currently it is not known what effect chronic *in utero* ureaplasma infections may have on the neonate. Previous evidence suggests that *in utero* infections caused by ureaplasmas are associated with respiratory sequelae and an increased incidence of adverse

neuromotor outcomes and cerebral palsy (Lyon 2000; Kotecha *et al.* 2004; Pandey *et al.* 2007; Berger *et al.* 2009). Combined, these results highlight the need to understand *Ureaplasma* spp. pathogenesis and the effect on pregnancy and fetal outcomes.

The expression of MMP-9 was also upregulated in Ussing chambers in which the maternal side of the membranes were exposed to live *U. parvum*, and in some cases, those exposed to UV-inactivated *U. parvum*. MMP-9 is one of several matrix metalloproteinases whose activation results in the degradation of collagen fibres within tissues, such as the chorioamnion (Strauss 2013). Throughout pregnancy, MMPs are tightly regulated and there is a fine equilibrium of collagen degradation and synthesis to maintain the chorioamnion membrane throughout pregnancy (until labour is triggered) (Goldman *et al.* 2003; Yonemoto *et al.* 2006). However, during UGT infections, the degradative effects of MMPs are increased within the chorioamnion membrane, particularly for MMP-2 and 9 (Strauss 2013). Within this small pilot study, we identified that MMP-9 activity was significantly increased in pooled maternal and fetal perfusates from Ussing chambers stimulated with live *U. parvum*, and some moderate MMP-9 activity was seen in Ussing chambers stimulated with UV-inactivated *Ureaplasma* spp. With only one control chamber displaying evidence of moderate MMP-9 activity after 30 hours, these findings suggest that that ureaplasmas can elicit a response which upregulates the production of degradative MMP-9. Previous studies have provided evidence that MMPs are upregulated during *Ureaplasma* spp. infections. In experimental ureaplasma UGT infections in Sprague Dawley rats, upregulation of MMP-9 was demonstrated (Peltier *et al.* 2007). Human pregnancies complicated by *Ureaplasma* spp. have also shown upregulation of MMP-9 within their amniotic fluid (Kacerovsky *et al.* 2013); however, there are no previous studies that have tested ureaplasma infected and non-infected chorioamnion for the presence and production of MMP-9. Taken together, these preliminary results suggest that MMP-9 was upregulated in chorioamnion tissue exposed to *U. parvum* and this may further facilitate the degradation of the tissues as evidenced by alterations in FITC-dextran membrane integrity assays.

Within this small pilot study, we did not observe any variation in the expression of the *U. parvum* serovar 6 MBA protein over the 30 hour period of each placental experiment. No MBA size variants, in addition to those present within the initial inoculum were observed for the ureaplasmas harvested at the end of each experiment. MBA variation has been reported previously *in vivo* within a pregnant ovine model. MBA size variation was seen after 7 days of infection, but not after 3 days of intraamniotic infection within pregnant sheep (Robinson *et al.* 2013). Within our study, we were only able to expose *Ureaplasma* spp. to chorioamnion tissue for 30 hours and so it was unlikely that

MBA variation would be produced within this short time period. Other experiments have attempted to induce MBA size variation *in vitro* through the use of anti-MBA antibodies. However, in these studies only MBA phase variation (the switching on/off of the MBA lipoprotein) was observed and this required high concentrations of anti-ureaplasma antibodies (Monecke *et al.* 2003; Dando *et al.* 2012). Future experiments which utilise monocytes and macrophages (i.e. those that are activated by exposure to *Ureaplasma* spp.) may be used in an attempt to stimulate/produce MBA size variation to occur.

As with many studies, there are limitations which should also be addressed. Only four placentae were tested in this pilot study, which limits the statistical power of this work. However, in this pilot study we have confirmed the utility of the Ussing chamber for use in *ex vivo* infection studies using live microorganisms. This model allows an additional level of complexity which mimics the role of the chorioamnion *in vivo*. Furthermore, for the first time this Ussing chamber model has been optimised to achieve physiological oxygen tensions and this is a major strength of this study. Previous studies investigating the immune responses to chorioamnion tissue explants have utilised standard cell culture incubators and the oxygen tension in this equipment is unlike the microenvironment within the placenta. It is not clear what effect these differing oxygen tensions have on the immune responses reported within these studies. The novel findings of this pilot study warrant further investigation and also raise further questions. The reason for amnion detachment following stimulation with live *Ureaplasma* spp. is currently unknown and it is not clear if apoptosis may also play a key role in amnion detachment or altered membrane integrity. Additionally, we identified elevated concentrations of MMP-9 and IL-10 in chorioamnion tissues stimulated with live ureaplasmas. By identifying specific mediators worthy of further investigation, it may be possible to identify the signalling pathways which lead to immune responses and pPROM during *Ureaplasma* spp. infections.

Conclusions:

This is the first study to utilise an Ussing chamber model to investigate the host immune responses to live bacteria (*U. parvum*) and we demonstrated that the presence of ureaplasmas was associated with tissue pathology, altered membrane integrity and increases in MMP-9 activity. The preliminary data within this study also demonstrated that the anti-inflammatory cytokine IL-10 was elevated within the maternal compartment in response to live ureaplasma infection, and this TH2-type response may be key to the long-term survival of these pathogens in the female UGT during

pregnancy and the establishment of chronic *in utero* infections. We also demonstrated, for the first time, that live ureaplasmas invade and cross the chorioamnion membrane and although this may be due to the degradative action of these microorganisms on the chorioamnion membrane, this provides a route for exposure of the fetus.

By further investigating the innate immune response of the chorioamnion (including responses after fetal membrane exposure to live ureaplasmas), the expression of TLR receptors, cytokines, chemokines, growth factors and other signalling pathways it may be possible to better understand the host immune response to ureaplasma infections of the female UGT and how this can be controlled for a future better outcome for the fetus.

Chapter Seven:

General Discussion

Discussion

The human *Ureaplasma* spp. are the bacteria most frequently isolated from the female lower genital tract (LGT; 40 - 80%), male LGT (up to 50%) and the female upper genital tract (UGT) (Volgmann *et al.* 2005). These microorganisms are also among the most prevalent organisms isolated from the amniotic fluid (Cassell *et al.* 1983; Gray *et al.* 1992; Jalava *et al.* 1996; Knox *et al.* 1997; Yoon *et al.* 2000; Gerber *et al.* 2003; Yoon *et al.* 2003b; Perni *et al.* 2004; Aaltonen *et al.* 2007; Berger *et al.* 2009; Jacobsson *et al.* 2009; Kasper *et al.* 2010) and placentae (Kundsin *et al.* 1984; Hillier *et al.* 1988; Miralles *et al.* 2005; Egawa *et al.* 2007; Hecht *et al.* 2008; Olomu *et al.* 2009; Namba *et al.* 2010) of women who deliver preterm. However, the pathogenic role of *Ureaplasma* spp. is not always clear as these microorganisms may also be isolated from the amniotic cavity of women who deliver at term with no evidence of adverse pregnancy outcomes (Gray *et al.* 1992; Gerber *et al.* 2003; Perni *et al.* 2004). Because there are different clinical presentations and pregnancy outcomes associated with female genital tract infection with ureaplasmas, it has been proposed that some *Ureaplasma* spp. or serovars may be more 'virulent' than others (Naessens *et al.* 1988; Sung *et al.* 2010; Eun *et al.* 2013) or that some of these microorganisms may possess virulence factors, such as the ability to vary their surface-exposed protein, the multiple banded antigen (MBA), which may modulate host-microbe interactions (Monecke *et al.* 2003; Zimmerman *et al.* 2009; Knox *et al.* 2010; Zimmerman *et al.* 2011; Dando *et al.* 2012; Robinson *et al.* 2013; Zimmerman *et al.* 2013). The host immune response to ureaplasmas during infection may also influence the development of sequelae, such as histological chorioamnionitis or pPROM (Kim *et al.* 2003; Aaltonen *et al.* 2007; Goldenberg *et al.* 2008a; Kasper *et al.* 2010; Oh *et al.* 2010). Given this, research into the pathogenesis of ureaplasma infections and the host-microbe interactions that take place during infection may lead to the identification of biomarkers and ultimately therapeutic strategies aimed at reducing the high rates of preterm birth (PTB) and sequelae in neonates.

Therefore, the overall hypotheses of this PhD program of research were that: (i) *Ureaplasma* spp. can infiltrate the placenta and chorioamnion during pregnancy; and (ii) *Ureaplasma* spp. are an aetiological agent during gestation and may be associated with adverse pregnancy and neonatal outcomes. We further hypothesised that (iii) the host immune system may not always recognise ureaplasmas, as these microorganisms possess the ability to vary the size of their surface-exposed lipoprotein, the MBA. These hypotheses were investigated in human pregnancies and also within an *ex vivo* chorioamnion tissue model of *Ureaplasma* spp. infection. The results of this research project have confirmed that *Ureaplasma* spp. are a major aetiological agent of infection in late preterm (LPT) and term pregnancies; and that the presence of ureaplasmas within the chorioamnion was

independently associated with histological chorioamnionitis. It was also identified that ureaplasma clinical isolates obtained from the chorioamnion of human pregnancies demonstrated differences in the size of their MBA protein and variation of the size of the surface-exposed MBA was associated with a decreased incidence of histological chorioamnionitis. Furthermore, we demonstrated that the presence of *Ureaplasma* spp. infection in human pregnancies was associated with host immune responses and elevated levels of the cord blood cytokine granulocyte colony-stimulating factor (G-CSF); however, when MBA protein variation was present, there was a significant reduction in the levels of G-CSF and interleukin (IL)-8 within cord blood, despite high numbers of ureaplasmas being present within the chorioamnion. Finally, we utilised an *ex vivo* Ussing chamber system to model host immune responses to *Ureaplasma* spp. infection of the chorioamnion and demonstrated tissue pathology within 30 hours of exposure to ureaplasmas, including altered membrane integrity, ureaplasma infiltration within the chorioamnion tissue and the presence of ureaplasmas within the fetal membrane reservoir, confirming that ureaplasmas exposed to the maternal membrane surface had invaded through the chorioamnion membrane. We also identified increased activity of matrix metalloproteinase (MMP)-9 and elevated levels of the anti-inflammatory cytokine IL-10 within perfusates that were exposed to live *U. parvum* infection, consistent with the early stages of chorioamnion membrane rupture. Taken together, the data presented in this thesis provide novel insight into the complex host-microbe interactions which take place during *Ureaplasma* spp. infections and suggest that immune responses of the host may be a key determinant in the development of adverse pregnancy outcomes.

The pathogenic role of *Ureaplasma* spp. has been strongly debated within the literature and ureaplasmas have been traditionally considered to be colonisers of the female lower genital tract (LGT) and were of low virulence (Volgmann *et al.* 2005). Additionally, only some studies identified causal links between *Ureaplasma* spp. infections and the development of adverse pregnancy outcomes (Cassell *et al.* 1983; Hillier *et al.* 1988; Cultrera *et al.* 2006; Egawa *et al.* 2007; DiGiulio 2012), while other studies identified no significant association between the presence of ureaplasmas and adverse sequelae (Gray *et al.* 1992; Gerber *et al.* 2003; Perni *et al.* 2004). However, the data presented within this thesis clearly demonstrates that *Ureaplasma* spp. are an aetiological agent of chorioamnion infection in LPT and term pregnancies and the presence of these microorganisms is associated with the development of adverse pregnancy outcomes. Within our large study of 535 placentae, we identified that chorioamnion infection was present in 10.6% of all LPT and term pregnancies and that the human *Ureaplasma* spp. and in particular *U. parvum* were the most prevalent microorganisms identified within this study, accounting for almost 70% of isolates.

Significantly, the presence of *Ureaplasma* spp. within the chorioamnion of LPT and term placentae was associated with the development of histological chorioamnionitis, and 68.4% of all placentae infected with ureaplasmas demonstrated evidence of chorioamnionitis. While previous studies have identified that the human *Ureaplasma* spp. were the most prevalent microorganisms isolated from the UGT of women who deliver prior to 32 weeks of gestation (Hillier *et al.* 1988; DiGiulio *et al.* 2008; Hecht *et al.* 2008; DiGiulio *et al.* 2010; Namba *et al.* 2010; Marconi *et al.* 2011), these infections were often polymicrobial and so a causal association with adverse pregnancy outcomes could not be attributed to a single bacterium. However, the findings of this current study demonstrate for the first time, that UGT chorioamnion infections at >32 weeks of gestation are most frequently caused by *Ureaplasma* spp. alone and that the presence of these microorganisms was independently associated with histological chorioamnionitis, regardless of gestation. The findings of this study also correlate with previous animal model studies, which identify *Ureaplasma* spp. as a sole pathogen of adverse pregnancy outcomes. Mouse model studies have demonstrated that a single intraamniotic injection of *Ureaplasma* spp. into the amniotic sac resulted in placental inflammation and fetal inflammatory response syndrome within 72 hours of infection (von Chamier *et al.* 2012; Allam *et al.* 2014). Sheep intraamniotically injected with *U. parvum* clinical isolates demonstrated histological chorioamnionitis and fetal lung pathology following acute (7 day) or chronic (69 day) infections (Moss *et al.* 2005; Knox *et al.* 2010; Dando *et al.* 2012; Robinson *et al.* 2013). Novy *et al.* (2009) utilised a Rhesus macaque model of intraamniotic *Ureaplasma* spp. infection and demonstrated that *Ureaplasma* spp., as a sole pathogen, caused chorioamnionitis and fetal lung injury. Taken together, the data presented within this thesis and the findings of others confirm a strong clinical link between chorioamnion infection with *Ureaplasma* spp. and the development of histological chorioamnionitis.

However, within this current study, not all women with ureaplasma chorioamnion infection developed histological chorioamnionitis and not all women with histological chorioamnionitis delivered preterm, which suggests that there may be other factors which are involved in the development and progression of disease. Therefore, we serotyped ureaplasma clinical isolates to determine if there was a 'virulent' spp. or serovar of ureaplasmas that was associated more frequently with adverse pregnancy outcomes. While we determined that *U. parvum* was the species isolated most frequently (accounting for 85.7% of all ureaplasma isolates within our study) and that *U. parvum* serovars 1, 3 and 6 were the ureaplasma serovars isolated most frequently (accounting for 23.8%, 21.4% and 28.6% of all ureaplasma clinical isolates, respectively), we were unable to demonstrate any association between particular ureaplasma species or serovars and the development of adverse pregnancy or neonatal outcomes. This finding is consistent with previous

reports. Zheng *et al.* (1992) serotyped ureaplasma clinical isolates obtained from neonatal cerebrospinal fluid or blood and demonstrated that there were no particular 'virulent' *Ureaplasma* serovars that were associated with invasive disease. Similarly, other studies have demonstrated that some *Ureaplasma* spp. and serovars are more prevalent in some patient populations. For example, *U. parvum* serovars 3 and 6 were most frequently isolated from the UGT of women who delivered preterm in an American population (Naessens *et al.* 1988). *U. parvum* serovars 3 and 6 were among the most frequently isolated ureaplasmas from the UGT of women who delivered preterm in Australia (Knox *et al.* 1997) and *U. parvum* serovar 6 was shown to be the most adherent ureaplasma serovar identified within washed semen samples (Knox *et al.* 2003) within an Australian population. Sung *et al.* (2010) identified that *U. parvum* serovars 3 and 6 and *U. urealyticum* serovar 11 were the most prevalent ureaplasma isolates obtained from nasopharyngeal and endotracheal aspirates of American neonates, while Eun *et al.* (2013) identified that *U. urealyticum* serovar 9 was the most common ureaplasma isolate identified within tracheal and gastric aspirates of Korean neonates. Similar to our current study, these researchers reported prevalent *Ureaplasma* spp. and serovars; however, these studies were unable to provide convincing data to support the idea of 'virulent' *Ureaplasma* spp. or serovars. The difference in prevalent species or serovars reported within these studies may simply be due to differences in the serovars endemic within these geographical locations. Taken together, the results of this study and others demonstrate that it is unlikely that 'virulent' ureaplasma species or serovars exist and so there may be other factors that are critical in the development of adverse pregnancy outcomes.

Also within this PhD program of study, we investigated the role of antigenic variation in *Ureaplasma* spp. Antigenic variation is hypothesised to enable pathogens to avoid recognition by the host immune response and is a common trait among the *Mycoplasmataceae* family, including *Mycoplasma hominis, Mycoplasma pulmonis, Mycoplasma agalactiae, Mycoplasma genitalium, Mycoplasma penetrans* and the human *Ureaplasma* spp. A seminal study published by Watson *et al.* (1993) investigated the major differences between *M. pulmonis* isolates obtained from animals with severe sequelae ('virulent' isolates) and those isolated from animals with little evidence of disease ('avirulent' isolates) and identified that the only discernible differences in these isolates was the variation of their variable antigen (V)-1 surface-exposed lipoprotein. Similarly, the variable protein of *M. agalactiae* (Vpma) antigen has been shown to vary in size *in vivo*. While researchers determined that size variation of the Vpma was not involved in the establishment of infection, it was suggested that variation of the Vpma may critically influence the survival of these microorganisms *in vivo* and

may be responsible for invasive/systemic infections of *M. agalactiae* in ovine mastitis (Chopra-Dewasthaly *et al.* 2012).

Antigenic variation has been described previously within the human *Ureaplasma* spp. In a sheep model of infection, different MBA size variants were generated *in vivo* within amniotic fluid and the chorioamnion tissue of pregnant ewes (Knox *et al.* 2010; Dando *et al.* 2012; Robinson *et al.* 2013). Additionally, ureaplasma clinical isolates with different MBA size variants have also been isolated from humans (Watson *et al.* 1990; Zheng *et al.* 1994; Zheng *et al.* 1995). It has also been shown that ureaplasmas can undergo phase variation (on/off "switching") and this has been demonstrated *in vitro* (Monecke *et al.* 2003; Zimmerman *et al.* 2009; Zimmerman *et al.* 2011; Dando *et al.* 2012). However, there are currently no studies which have investigated ureaplasma MBA size variation in clinical isolates from the UGT during human pregnancies. Importantly, the effect of MBA size variation on the outcomes of pregnancies is currently unknown.

In this PhD project, using western blot and polymerase chain reaction (PCR) analysis we identified that *in vivo* size variation in both the *mba* gene and its expressed protein (MBA) was associated with a lower incidence of histological chorioamnionitis, despite the presence of high numbers of *Ureaplasma* spp. infecting/colonising chorioamnion tissue. These findings are consistent with previous studies which demonstrated that MBA size variants were associated with only mild histological chorioamnionitis or the absence of inflammation within the chorioamnion or other fetal tissues (Knox *et al.* 2010; Robinson *et al.* 2013). The results of these studies suggest that MBA size variation plays an integral role in host-microbe interactions. We further investigated the role of MBA variation on the host innate immune responses and identified a novel association between the presence of MBA variation and concentrations of key cord blood cytokines. When *Ureaplasma* spp. demonstrated either a single or multiple MBA size variant(s), significantly lower concentrations of the cytokines G-CSF and IL-8 were present in cord blood. By contrast, placentae infected with ureaplasmas that did not alter the size of their MBA (i.e. the size of their MBA was the same as ATCC strain serovars) had significantly higher concentrations of both G-CSF and IL-8 detected within cord blood. These results strongly support the finding that MBA size variation plays an integral role in host-microbe interactions (Shimizu *et al.* 2008; Triantafilou *et al.* 2013); further studies into the pathogenesis of these microorganisms is warranted.

IL-8 and G-CSF are both inflammatory markers that are often produced by the host during infections. The production of these key cytokines triggers the recruitment of neutrophils towards the site of

infection. Our finding that both IL-8 and G-CSF concentrations are significantly lower within cord blood in association with ureaplasma infections which display MBA size variants is of great importance as this may be a mechanism by which ureaplasmas avoid eradication by the host immune system. Shimizu *et al.* (2008) demonstrated that the *Ureaplasma* spp. MBA is the immunodominant antigen recognised by the host and is capable of activating nuclear factor (NF)-kB *via* Toll-like receptors (TLRs) 1, 2 and 6. Alterations of the MBA protein may in turn affect TLR binding, activation and subsequent signalling of inflammatory pathways and this may result in reduction in the levels of G-CSF and IL-8, despite the presence of ureaplasmas within the chorioamnion.

This may also mean that there is a reduced likelihood of ureaplasma infections being eradicated by the host and chronic infections may develop. MBA size variation is also predicted to affect the adaptive immune response, as each MBA size variant is consistent with a single B-cell epitope. The ability of these microorganisms may also be associated with the concept of 'original antigenic sin'. Antigenic sin refers to the ability of the host to preferentially utilise immunological memory based on a previous infection with the same microorganism (Stromberg and Carlson 2013). When a secondary infection occurs, there may be slightly different antigens encountered by the host (e.g. *Ureaplasma* spp. MBA size or phase variation); however, the host preferentially utilises immunological memory to produce high-affinity memory B-cells in order to produce antibodies against the original antigens encountered during the original infection. The production of large numbers of memory B-cells also inhibits the activation of naïve B-cells, which would be able to mount an effective immune response against the current infection which displays slightly different surface antigens. This preference then leaves the host 'trapped' and often unable to respond to the different surface antigens produced by *Ureaplasma* spp., thereby enabling prolonged infections to ensue. This is supported by the findings of Dando *et al.* (2012) who demonstrated that maternal serum of sheep exposed to intraamniotic *U. parvum* for 69 days demonstrated serum reactivity to MBA proteins, which were different in size to the MBA antigens expressed by ureaplasmas within amniotic fluid of the same ewe and this supports the idea that infections with ureaplasmas are often chronic.

During these chronic infections, the fetus is also exposed to *Ureaplasma* spp. *in utero* and the inability of the host immune response to neutralise these infections may lead to serious infant morbidity, including bronchopulmonary dysplasia, sepsis, meningitis, and in severe cases, cerebral palsy (Cassell *et al.* 1993b; Abele-Horn *et al.* 1997a; Katz *et al.* 2005; Berger *et al.* 2009). Given this,

the identification of UGT ureaplasma infections during pregnancy is of great importance; however, these microorganisms are currently not screened for during pregnancy and there is no available assay that can easily detect, speciate or serotype ureaplasmas.

During this PhD, a real-time PCR and HRM assay for the simultaneous identification, speciation and serotyping of *U. parvum* clinical isolates was developed and validated. Given that *U. parvum* are the most prevalent *Ureaplasma* spp. (accounting for up to ~95% of all clinical isolates (Knox *et al.* 1997; Knox *et al.* 2003; De Francesco *et al.* 2009; Sung *et al.* 2010)), we sought to develop an improved assay to detect and characterise these microorganisms. The current "gold-standard" method for the detection of *Ureaplasma* spp. in clinical specimens is culture, which is often time-consuming, laborious and ureaplasma broth culture media is not available commercially, so media must be made "in house", all of which greatly increases the cost and time taken to diagnose these infections. The assay developed for this research project targets the multiple banded antigen gene (*mba*), which encodes the major surface-exposed, immunodominant antigen of *Ureaplasma* spp. This gene is present in all 14 ureaplasma serovars and the upstream portion of this gene has a relatively high A+T content and is conserved (Glass *et al.* 2000), making it an ideal candidate for the identification and serotyping of *U. parvum*. The presence of numerous single nucleotide polymorphisms (SNPs) within the upstream region of this gene in all *U. parvum* serovars allowed us to utilise fragments of the *mba* gene for the simultaneous identification, speciation and serotyping of the four *U. parvum* serovars in a single closed-tube assay. This novel assay successfully speciated and serotyped 64.5% of all clinical isolates tested; however, a further 11 isolates were unable to be typed using this assay. Those isolates which were successfully typed using this assay demonstrated high resolution melt curves that were very similar to those of the American Type Culture Collection (ATCC) strain serovars, so with additional improvements, this assay has the potential to be incorporated into routine clinical and diagnostic laboratory testing for *Ureaplasma* spp. and this is a major contribution to this field of research. However, the problem largely remains that *Ureaplasma* spp. infections are predominantly clinically asymptomatic (Gerber *et al.* 2003; Zdrodowska-Stefanow *et al.* 2006) and in our study, we identified no clinical signs or symptoms that were associated with UGT infections. Additionally, LGT colonisation with ureaplasmas is not predictive of UGT infections with *Ureaplasma* spp. or the development of adverse pregnancy outcomes (Cassell *et al.* 1993b; Eschenbach 1993; Knox *et al.* 1997) and identifying women with UGT ureaplamsa infections during pregnancy is extremely difficult.

Because of these difficulties in identifying women with UGT infection, many studies have sought to identify biomarkers for the prediction of UGT infections, PTB and other adverse pregnancy outcomes. Biomarkers are a valuable clinical tool for the identification of women at-risk of PTB who are clinically asymptomatic. Early detection of risk factors or signs and symptoms of infection may potentially inform treatment options and lead to a reduction in the high rates of PTB. Indicators of PTB (biomarkers) may include maternal pregnancy history and demographic characteristics, patient presentation during routine examination or the measurement of substances within bodily fluids. The preterm prediction studies are a series of publications, which attempt to identify a range of biomarkers to assist in the identification of women at-risk of delivering preterm. These studies identified a wide range of maternal risk factors, behavioural characteristics and bodily fluid markers which were predictive of PTB and other adverse pregnancy outcomes. These included maternal stress, cervical length, bacterial vaginosis or genital infection with *C. trachomatis* at < 24 weeks of gestation as well as other biomarkers such as elevated levels of cervical IL-6 or fetal ferritin and vaginal fetal fibronectin. These factors were all correlated with spontaneous PTB and pPROM (Andrews *et al.* 2000; Goepfert *et al.* 2000; Meis *et al.* 2000; Mercer *et al.* 2000; Goepfert *et al.* 2001; Newman *et al.* 2001; Moawad *et al.* 2002; Ramsey *et al.* 2002; Goldenberg *et al.* 2005; Hendler *et al.* 2005; Newman *et al.* 2008).

The data presented within this thesis has further identified a history risk factor and two potential biomarkers that were associated with UGT infection during pregnancy. In this large study, we identified for the first time women with UGT infections within the current pregnancy (reported in Chapter 4) had a history of chorioamnionitis in a prior pregnancy. This further suggests that the microorganisms causing infection in this current pregnancy may be the cause of chorioamnionitis within a prior pregnancy. While previous studies have identified that the best indicator of PTB within a current pregnancy is a history of prior PTBs in other gestations (Mercer *et al.* 1999); to the best of our knowledge, this is the first study to identify that a prior history of chorioamnionitis may be used as a marker to predict the likelihood of UGT infections within a current pregnancy. This finding appears to be regardless of gestational age, as women within our study with chorioamnionitis delivered LPT or at term (> 36 weeks of gestation). These findings are a major contribution to this field of research and should be of great importance to clinicians and aid in the identification of 'at-risk' women during pregnancy.

In addition, we identified two cord blood cytokines that were elevated in pregnancy in association with UGT infection and histological chorioamnionitis. Concentrations of both IL-8 and G-CSF were

shown to be increased in pregnancies complicated by infection; while levels of G-CSF were elevated within the cord blood of pregnancies affected by histological chorioamnionitis. Our findings are in agreement with previous studies which have shown that concentrations of IL-8 were significantly increased in pregnancies complicated by UGT infection (including infection with *Ureaplasma* spp.) (Marconi *et al.* 2011). Amniotic fluid G-CSF was found to be elevated within the amniotic fluid of pregnancies complicated by intraamniotic infection (Calhoun *et al.* 2001) or histological chorioamnionitis (Raynor *et al.* 1995; Hoskins *et al.* 1997). More recently, Payne *et al.* (2014) sought to identify amniotic fluid biomarkers specific for *Ureaplasma* spp. infections. Amniotic fluid samples obtained at the time of amniocentesis (15 – 20 weeks of gestation) were collected from 480 Chinese and 492 Australian women and analysed for the presence of inflammatory markers (IL-1β, IL-6, IL-10, TNF-α and MCP-1). *Ureaplasma* spp. were identified in only two (0.2%) women within this study and there were no significant correlations between the infection with ureaplasmas and elevated levels of amniotic fluid cytokines. While this study did not test for the presence of IL-8 and G-CSF (markers which we have identified as elevated during UGT infections), the presence of *Ureaplasma* spp. within this study was also extremely low, suggesting that the PCR assays used for identification of these microorganisms did not identify low-level ureaplasma infection. These findings also suggest that amniotic fluid is not the most appropriate specimen for identifying biomarkers of UGT infection.

Amniotic fluid is collected by an invasive procedure (amniocentesis) that is most frequently during pregnancies when there is an increased risk of neonatal genetic abnormalities. Amniocentesis can only be performed by clinicians and this procedure is associated with an increased risk of miscarriage, with approximately 1/200 women experiencing miscarriages after undergoing this procedure (Tabor and Alfirevic 2010). Given the increased risks and specialised training required, it is unlikely that pregnant women would routinely undergo amniocentesis to obtain amniotic fluid for biomarker analysis. Therefore, it is likely that non-invasive methods of screening would be more acceptable to pregnant women and so specimens such as serum or vaginal fluids are likely to be a superior specimen of choice. Additionally, women routinely undergo blood tests and most women will often undergo vaginal screening (specifically for *S. agalactiae* or GBS) throughout pregnancy and so testing for additional factors during these tests would likely be deemed acceptable by most pregnant women (Goldenberg *et al.* 2005). Currently, we are still yet to identify the most appropriate biomarker(s) for identification of UGT infections within these specimens.

Previous studies reported that maternal serum levels of IL-8 were elevated in women experiencing PTB with UGT infections (Bogavac and Brkic 2009). Additionally, maternal serum levels of G-CSF were

shown to be significantly increased in pregnancies complicated by histological chorioamnionitis (Boggess *et al.* 1997), pPROM (Murtha *et al.* 2007) and spontaneous PTB (< 28 weeks of gestation) (Goldenberg *et al.* 2000a). In our current study, we identified elevated levels of IL-8 and G-CSF within cord blood but unfortunately we were unable to test maternal serum for this marker. Cord blood is a derivative of maternal blood (maternal blood is passed through and filtered by the placenta) (Meyer *et al.* 1978) and it would be worthwhile to test for these key cytokines as they may also be increased within the maternal circulation.

G-CSF is often found in extremely low levels in maternal circulation, unless infection or inflammation is present; making this a particularly attractive candidate for a pregnancy biomarker. Goldenberg *et al.* (2000) previously investigated the utility of G-CSF as a serum biomarker and identified that it was very useful in the prediction of PTB at < 32 weeks of gestation. However, our study has provided novel evidence that G-CSF may also be a useful biomarker at > 32 weeks of gestation for the prediction of *Ureaplasma* spp. infection and histological chorioamnionitis. Gravett *et al.* (2007) also investigated proteomic markers of UGT infection with *Ureaplasma* spp. in a non-human primate model and identified 205 unique proteins produced in amniotic fluid and cervicovaginal fluid of Rhesus monkeys following intraamniotic infection with *Ureaplasma* spp. Additionally, 27 other proteins were found to be differentially expressed (up- or down-regulated) within these fluids following infection with ureaplasmas and so with further investigation; it may be possible to identify a panel of biomarkers which can be used for the prediction or identification of women with UGT infections and/or histological chorioamnionitis.

Within this PhD program of study, we also provided preliminary evidence which suggests that *Ureaplasma* spp. can infiltrate the placenta and chorioamnion. Using an *ex vivo* chorioamnion tissue model, we exposed the maternal surface of the membranes to live *U. parvum* (replicating) or UV-inactivated (non-replicative, but still with their major surface-exposed antigens intact) *U. parvum* in order to study the host immune responses to these microorganisms. The numbers of *U. parvum* CFU within the Ussing chambers did not differ significantly over time; however, we did see differences in the immune responses to these pathogens. In this pilot study, we identified that concentrations of IL-10 were significantly increased in Ussing chambers in which the maternal membrane was stimulated with live *U. parvum*. It was interesting that we identified increased concentrations of IL-10, and anti-inflammatory cytokine, within maternal perfusates but not in fetal perfusates in response to live *U. parvum*. Increased production of IL-10 can result in an immunosuppressive response on the chorioamnion tissues, 'dampening' or reducing the effects of any pro-inflammatory

immune responses. This finding is supported by the findings of Chapters Four and Five within this thesis; which showed that not all women whose chorioamnion was infected with *Ureaplasma* spp. demonstrate an inflammatory immune response (and some women with high numbers of ureaplasmas within their chorioamnion had little or no evidence of chorioamnionitis) and also that variation of the *Ureaplasma* spp. MBA may be associated with modulation of the host immune response. Significantly lower levels of cord blood pro-inflammatory cytokines IL-8 and G-CSF were detected in cord blood when placentae were infected with ureaplasmas that displayed variation of their surface-exposed MBA. The signalling of an immunosuppressive immune response in response to ureaplasma infections is interesting, and supports the hypothesis that these organisms may cause chronic, asymptomatic *in utero* infections for prolonged periods and the increase in immunosuppressive cytokines (IL-10) and the reduction of immunostimulatory cytokines (IL-8 and G-CSF) may allow for the establishment of chronic UGT infections with ureaplasmas.

Within this pilot study, we were also able to demonstrate that stimulation of the chorioamnion membranes with live *U. parvum* resulted in the significant loss of chorioamnion tissue integrity and the invasion of *U. parvum* through the chorioamnion membranes to the fetal compartment by 30 hours of infection. This finding is novel, as no previous studies have investigated whether *Ureaplasma* spp. are able to cross the chorioamnion membrane; despite ascending invasive infections being the most commonly accepted origin/route of UGT infection. At the conclusion of these experiments, approximately 2 x 10^4 CFU of *Ureaplamsa* spp. was detected within the fetal compartment of all Ussing chambers that were stimulated with live ureaplasmas; and while it is currently not clear if these microorganisms were present simply due to membrane degradation, this is still a unique finding that warrants further investigation. A previous study has identified that *S. agalactiae* (Group B Streptococcus or GBS) was able to penetrate the chorioamnion membrane within a similar *ex vivo* model (using Trans-well assay plates) (Winram *et al.* 1998) and this supports the hypothesis that ureaplasmas and other microorganisms can infiltrate the placenta and chorioamnion during pregnancy. These findings have serious implications for the health and wellbeing of mothers and neonates. If *Ureaplasma* spp. are able to degrade the chorioamnion membrane *in vivo,* this may allow ureaplasmas and other microorganisms to cross the chorioamnion and infect the fetus and amniotic cavity. Previous studies of very early PTBs have shown that *Ureaplasma* spp. are commonly isolated in the presence of other microorganisms, including *Fusobacterium* spp., *Streptococcus* spp., *Mycoplasma* spp. and *Bacteroides* spp. (DiGiulio *et al.* 2010). The ability of these microorganisms to pass into the amniotic cavity at the time of pregnancy are associated with adverse pregnancy outcomes.

Furthermore, prolonged exposure to *Ureaplasma* spp. *in utero* has been associated with altered lung structure and altered gut structure in sheep (Collins *et al.* 2010; Robinson *et al.* 2013; Wolfs *et al.* 2013); while in humans, exposure to ureaplasmas has been correlated with adverse neurological disorders, cerebral palsy and adverse respiratory outcomes (Viscardi *et al.* 2002; Berger *et al.* 2009; Payne *et al.* 2010).

Additionally, our study identified significant increases in the presence and activity of MMP-9 following exposure to *U. parvum*. This is perhaps not surprising, as MMP-9 has been shown previously to play a key role in maintenance in chorioamnion tissue. MMPs regulate the degradation and renewal of extracellular matrix proteins within the chorioamnion and any disturbance to this balance may result in chorioamnion degradation and membrane rupture. MMP-9 has been shown to be elevated in pregnancies that end prematurely (Tency *et al.* 2012) and combined, these findings further demonstrate that ureaplasmas have a negative effect on chorioamnion tissues in an *ex vivo* model.

Numerous studies have identified associations between UGT infection with *Ureaplasma* spp. and the development of pPROM. A review by DiGiulio *et al.* (2012) identified that the human *Ureaplasma* spp. are the bacteria isolated most frequently from the amniotic fluid of women experiencing pPROM; however, many of the studies reported in this review article identified multiple microorganisms within the amniotic fluid. The presence of multiple microorganisms often makes it more difficult to identify the true causative agent of pPROM; but in this pilot study, we have demonstrated for the first time that infection of the chorioamnion with *Ureaplasma* spp. causes alterations that are consistent with the early stages of pPROM.

Conclusions

The data presented within this thesis has provided novel information on the role of *Ureaplasma* spp. infections within human pregnancies. Our findings have shown that the host immune responses during *Ureaplasma* spp. infections are complex, with both immunostimulatory and immunosuppressive pathways activated following infection with *Ureaplasma* spp. This is further complicated by the fact that these microorganisms can vary their surface-exposed lipoprotein, the MBA; and in this thesis we provided the first evidence that *Ureaplasma* spp. clinical isolates obtained from the chorioamnion of human pregnancies demonstrate differences in the size of their MBA.

We also identified that variation of the ureaplasma MBA was associated with a lower incidence of histological chorioamnionitis and lower levels of cord blood cytokines IL-8 and G-CSF; suggesting that this protein may be involved in host-microbe interactions and modulation of the host immune response. While we were unable to show that MBA variation prevented the recognition of these pathogens *in utero*, the constant varying of this major immunodominant antigen may aid in preventing the eradication of *Ureaplasma* spp. and assist in the establishment of chronic *in utero* infections.

Also of importance, we have demonstrated that live *Ureaplasma* spp. infections can negatively affect chorioamnion tissue and are able to infiltrate and cross the chorioamnion membrane in an *ex vivo* Ussing chamber model of ureaplasma infection. Within this small pilot study, we provided the first evidence that live ureaplasmas can alter the integrity and structure of the chorioamnion membrane, and also elicit immune responses in the form of increased MMP-9 and IL-10 production.

Given the new and novel information presented within this thesis, a potential model of UGT infection with *Ureaplasma* spp. based on these new findings has been proposed (Figure 7.1). Ureaplasmas may ascend from the LGT, through the cervix and reach the choriodecidual space, where they may then infiltrate the placenta and cross the chorioamnion membranes. Once inside the amniotic cavity, the host can recognise and mount an immune response to eradicate the ureaplasmas. However, ureaplasmas can vary the size of their immunodominant antigen, the MBA, and the *Ureaplasma* spp. are not eradicated. MBA size variation leads to a reduced inflammatory response within the placenta and amniotic cavity, resulting in mild or no histological chorioamnionitis and subclinical (asymptomatic), chronic ureaplasma infections may be established. Due to the reduced inflammatory responses, PTB is not triggered and chronic infections continue; until normal term delivery. Prolonged exposure to *Ureaplasma* spp. *in utero* may result in an increased risk of neonatal and childhood diseases, due to the constant exposure of the fetus to these microorganisms even in the absence of histological chorioamnionitis. In cases where *Ureaplasma* spp. do not vary their surface-exposed MBA, strong pro-inflammatory responses may be seen and these microorganisms may be eradicated, thus 'healing' the UGT infection and leaving evidence of prior infection in the form of histological chorioamnionitis. However, in most cases, ureaplasmas may not be eradicated and these strong pro-inflammatory responses (such as increased IL-8 and G-CSF) result in recruitment of inflammatory cells, production of MMPs and other immune factors which weaken the chorioamnion membrane. This membrane weakening may result in pPROM and subsequent PTB. Those infants who are exposed to these severe pro-inflammatory responses are at

greater risk of developing severe sequelae, such as respiratory distress syndrome (RDS) or neurological sequelae, such as cerebral palsy.

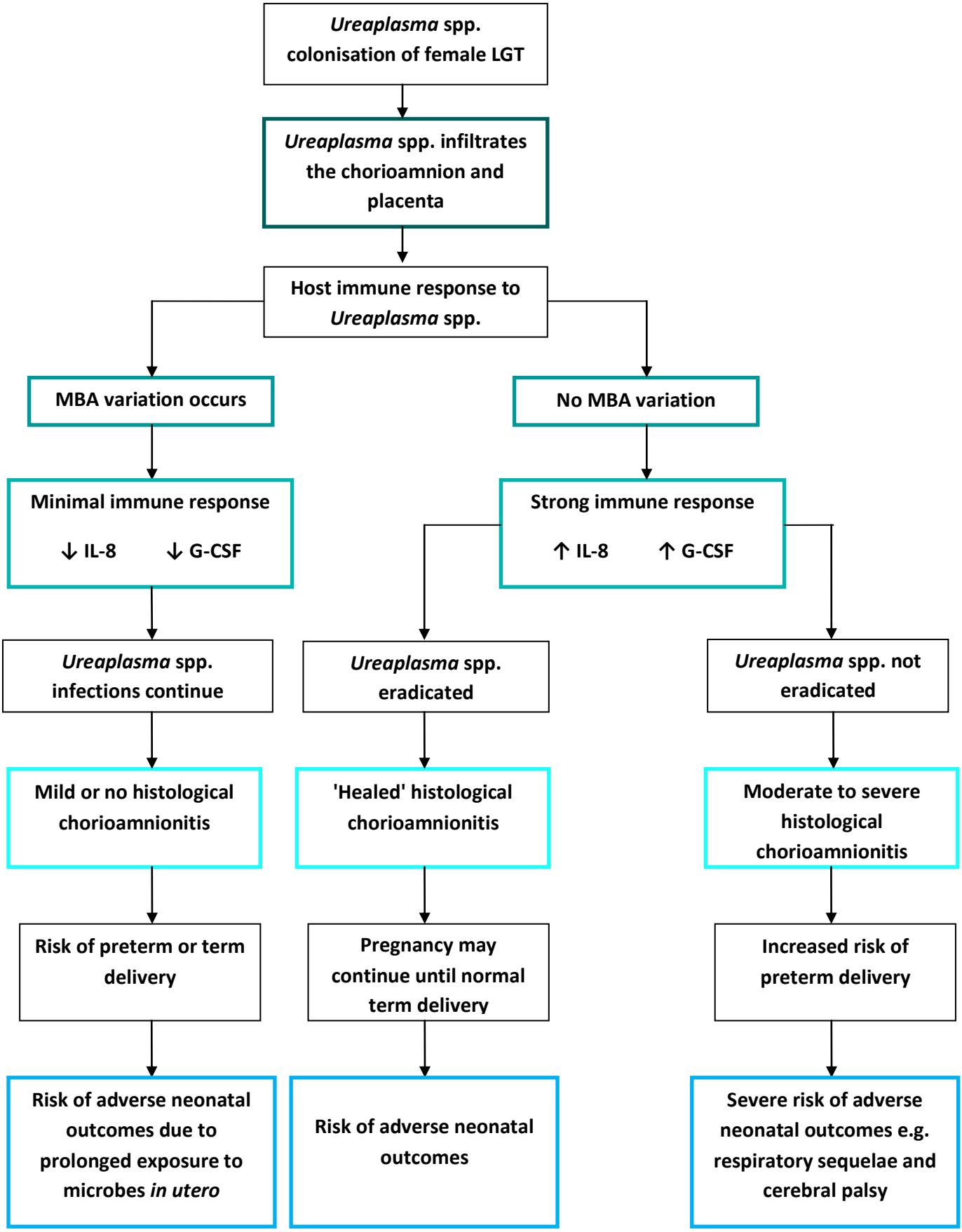


Figure 7.1. Proposed model of UGT infections with *Ureaplasma* spp.

The *Ureaplasma* spp. are important pathogens of the female UGT during pregnancy and are associated with severe adverse pregnancy outcomes. While the results generated from this PhD project suggest that there are no 'virulent' *Ureaplasma* spp. or serovars, they do strongly suggest that host-microbe interactions play a crucial role in the development of pregnancy sequelae. The ability of ureaplasmas to vary their surface-exposed MBA also appears to contribute to the ability of these microorganisms to avoid eradication by the host immune system, facilitating chronic infections. As the host immune response alone is often unable to eliminate these pathogens, more effective treatment options or therapeutic strategies are required in order to eradicate these pathogens from the UGT, or to prevent *in utero* infections from occurring.

Future Directions

Based on the findings of this PhD project, we propose that *Ureaplasma* spp. are an underestimated pathogen during pregnancy and their role in adverse outcomes requires further investigation. The initiation of screening and identification of women at-risk of developing UGT infections with ureaplasmas is of great clinical importance. Additional studies which focus on the identification of clinical risk factors and other maternal serum biomarkers or improved detection techniques (such as the real-time PCR and HRM assay developed within this PhD project) appear to be the most appropriate strategies for identifying these microorganisms during gestation. Collaborative studies, undertaken by researchers and clinicians are required to trial antimicrobial treatments or therapeutic strategies in order to improve pregnancy outcomes for women infected with *Ureaplasma* spp. *in utero*.

Furthermore, the role of the MBA should be further investigated in order to determine the mechanisms by which variation of the surface-exposed lipoprotein modulates chronic UGT infections and the host immune system. Because *mba/*MBA variation has been shown to be associated with a reduction of inflammation, studies which investigate the outcomes of infants exposed to these organisms *in utero* are warranted to determine if the presence of these microorganisms in the absence of inflammation are associated with any immediate adverse neonatal outcomes and also with long-term outcomes of infants exposed to ureaplasmas. Additionally, further studies which investigate other host immune factors, such as TLR activation and signalling may improve our knowledge and understanding of host immune responses to these pathogens during such an immunologically unique period, where the host immune response is predominantly TH2-mediated. Further studies should include the investigation of macrophage polarisation (M1/M2 phenotypes) on the host immune response to *Ureaplasma* spp. infections in order to determine if a TH2-mediated

response to ureaplasmas may in fact prolong infection, due to the initiation of an immediate TH2 response which may not eradicate the pathogen. Additionally, it would also be of great interest to determine if ureaplasmas are able to stimulate the polarisation of macrophages by exposing unpolarised macrophages to ureaplasmas and determining the responses generated, to better understand if macrophages play an important role in the host immune responses during pregnancy.

As the ureaplasma MBA is known to be the major immunodominant antigen recognised by the host immune system, an MBA vaccine may be developed. However, problems such as the size and phase variation of this protein inherently diminish the efficacy of this approach. Furthermore, it has been shown that the presence of *Ureaplasma* spp. antibodies present within maternal serum may also be associated with adverse pregnancy outcomes, such as spontaneous abortions (Naessens *et al.* 1988) and so therefore an ureaplasma vaccine should be carefully considered. While there are currently no ureaplasma-specific vaccines which have been trialled, issues in the feasibility of these lie in the fact that these microorganisms are also commensals of the female LGT, and the eradication of these organisms at this site may result in significant alterations of female vaginal microflora, which could have serious consequences.

Because there is such an interesting association between the presence of circulating maternal ureaplasma-specific antibodies and an association with adverse pregnancy outcomes; the concept of immune 'tolerance' should be investigated. Exposure to *Ureaplasma* spp. at a young age (e.g. infants exposed to *Ureaplasma* spp. at the time of birth) may develop an immune 'tolerance' to these microorganisms and this may potentially reduce their host immune response to ureaplasma infections later in life. Long-term animal studies which investigate immune tolerance to ureaplasmas, the presence of maternal ureaplasma antibodies and the outcome of pregnancies of these same subjects may further elucidate the role of the host immune response to ureaplasmas.

Overall, it is likely that the best way forward in the identification, treatment and eradication of ureaplasmas involve the immediate improvement of the detection of these organisms or of biomarkers of infection during gestation and improvement in the management and treatment of these women. By further investigating the host immune response to ureaplasmas, it may also be possible to identify targeted therapies for the eradication of these *Ureaplasma* spp. from the UGT during pregnancy.

Chapter Eight:

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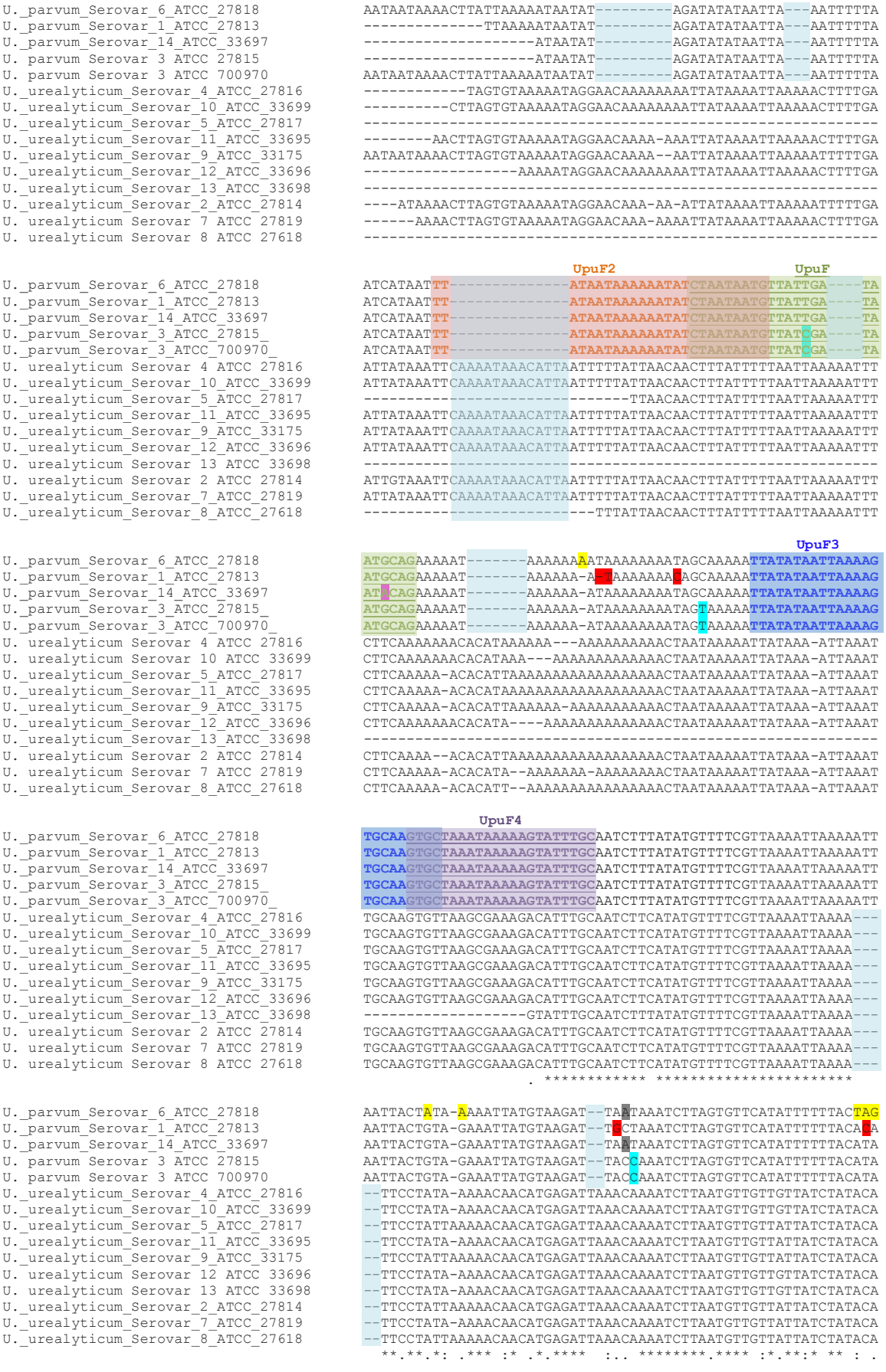
Chapter Nine:

Supplementary Figures and Table

Supplementary Table 9.1. Comparison of culture- and PCR-positive placental specimens. Culture data was obtained from cultivation of organisms from swabs and chorioamnion tissue. PCR data was obtained from DNA extracted from chorioamnion tissues.

Patient Number	*Ureaplasma* spp.			Other microorganisms		
	Culture	PCR	Identity	Culture	PCR	Identity
1A	Positive	Positive	*U. parvum*	Negative	Negative	-
1B	Positive	Positive	*U. parvum*	Negative	Negative	-
8	Positive	Positive	*U. urealyticum*	Negative	Negative	-
13A	Negative	Negative	-	Positive	Positive	*Bifidobacterium* spp.
13B	Negative	Negative	-	Positive	Positive	*Bifidobacterium* spp.
27	Positive	Positive	*U. parvum*	Negative	Negative	-
33A	Positive	Positive	*U. parvum*	Negative	Negative	-
33B	Positive	Positive	*U. parvum*	Negative	Negative	-
43	Positive	Positive	*U. parvum*	Negative	Positive	Uncultured bacterium
44A	Positive	Positive	*U. parvum*	Negative	Negative	-
44B	Positive	Positive	*U. parvum*	Negative	Negative	-
46	Negative	Negative		Positive	Positive	*Gardnerella vaginalis*
50	Positive	Positive	*U. parvum*	Negative	Negative	-
55B	Positive	Positive	*U. parvum*	Negative	Negative	-
79	Negative	Negative	-	Positive	Positive	*Propionibacterium* spp.
122	Positive	Positive	*U. parvum*	Negative	Negative	-
175	Negative	Positive	*U. parvum*	Negative	Negative	-
176	Negative	Positive	*U. parvum*	Negative	Negative	-
182	Positive	Positive	*U. parvum*	Negative	Negative	-
205T	Negative	Negative	-	Positive	Positive	*Streptococcus agalactiae*
206T	Negative	Negative	-	Positive	Positive	*Streptococcus agalactiae*
242A	Negative	Negative	-	Positive	Positive	*Bacteroides fragilis*
242B	Negative	Negative	-	Positive	Positive	*Bacteroides fragilis*
251	Negative	Positive	*U. parvum*	Negative	Negative	-
258	Positive	Positive	*U. urealyticum*	Negative	Negative	-
262T	Positive	Positive	*U. parvum*	Negative	Negative	-
290T	Positive	Positive	*U. parvum*	Negative	Negative	-
297	Positive	Positive	*U. urealyticum*	Negative	Negative	-
300	Positive	Positive	*U. urealyticum*	Negative	Negative	-
301	Positive	Positive	*U. parvum*	Negative	Negative	-
310	Positive	Positive	*U. parvum*	Negative	Negative	-
314T	Positive	Positive	*U. parvum*	Positive	Positive	Uncultured bacterium
317	Negative	Negative	-	Positive	Positive	*Streptococcus agalactiae*
320T	Negative	Negative	-	Positive	Positive	Uncultured bacterium
322T	Positive	Positive	*U. parvum*	Negative	Negative	-
325	Positive	Positive	*U. parvum*	Negative	Negative	-
334A	Positive	Positive	*U. parvum*	Negative	Negative	-
334B	Positive	Positive	*U. parvum*	Negative	Negative	-
337	Negative	Negative	-	Positive	Positive	*E. coli*
340T	Negative	Negative	-	Negative	Positive	Uncultured bacterium
351	Positive	Positive	*U. urealyticum*	Negative	Negative	-
364A	Positive	Positive	*U. parvum*	Negative	Negative	-
365	Positive	Positive	*U. parvum*	Negative	Negative	-

368T	Positive	Positive	*U. parvum*	Negative	Positive	Uncultured bacterium
374B	Negative	Negative	-	Negative	Positive	Uncultured bacterium
375	Negative	Negative	-	Negative	Positive	Uncultured bacterium
429	Positive	Positive	*U. parvum*	Negative	Negative	-
435	Positive	Positive	*U. parvum*	Negative	Negative	-
437	Negative	Negative	-	Positive	Positive	*Streptococcus agalactiae*
438	Positive	Positive	*U. urealyticum*	Negative	Negative	-
473T	Positive	Positive	*U. parvum*	Positive	Positive	*Streptococcus agalactiae*
480T	Positive	Positive	*U. parvum*	Negative	Negative	-
483T	Positive	Positive	*U. parvum*	Negative	Negative	-
498A	Positive	Positive	*U. parvum*	Negative	Negative	-
498B	Positive	Positive	*U. parvum*	Negative	Negative	-
507	Positive	Positive	*U. parvum*	Negative	Negative	-
510T	Positive	Positive	*U. parvum*	Negative	Negative	-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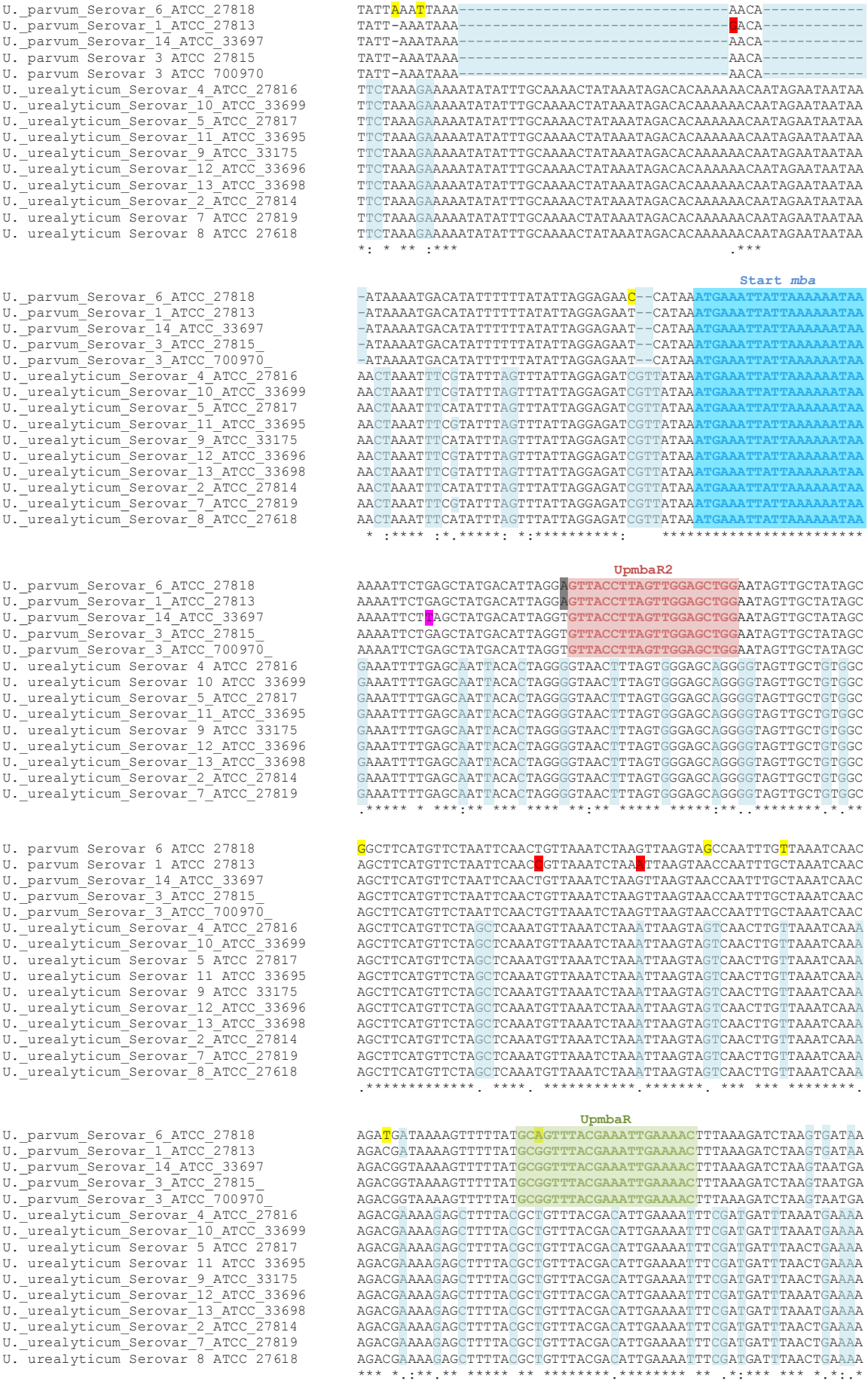
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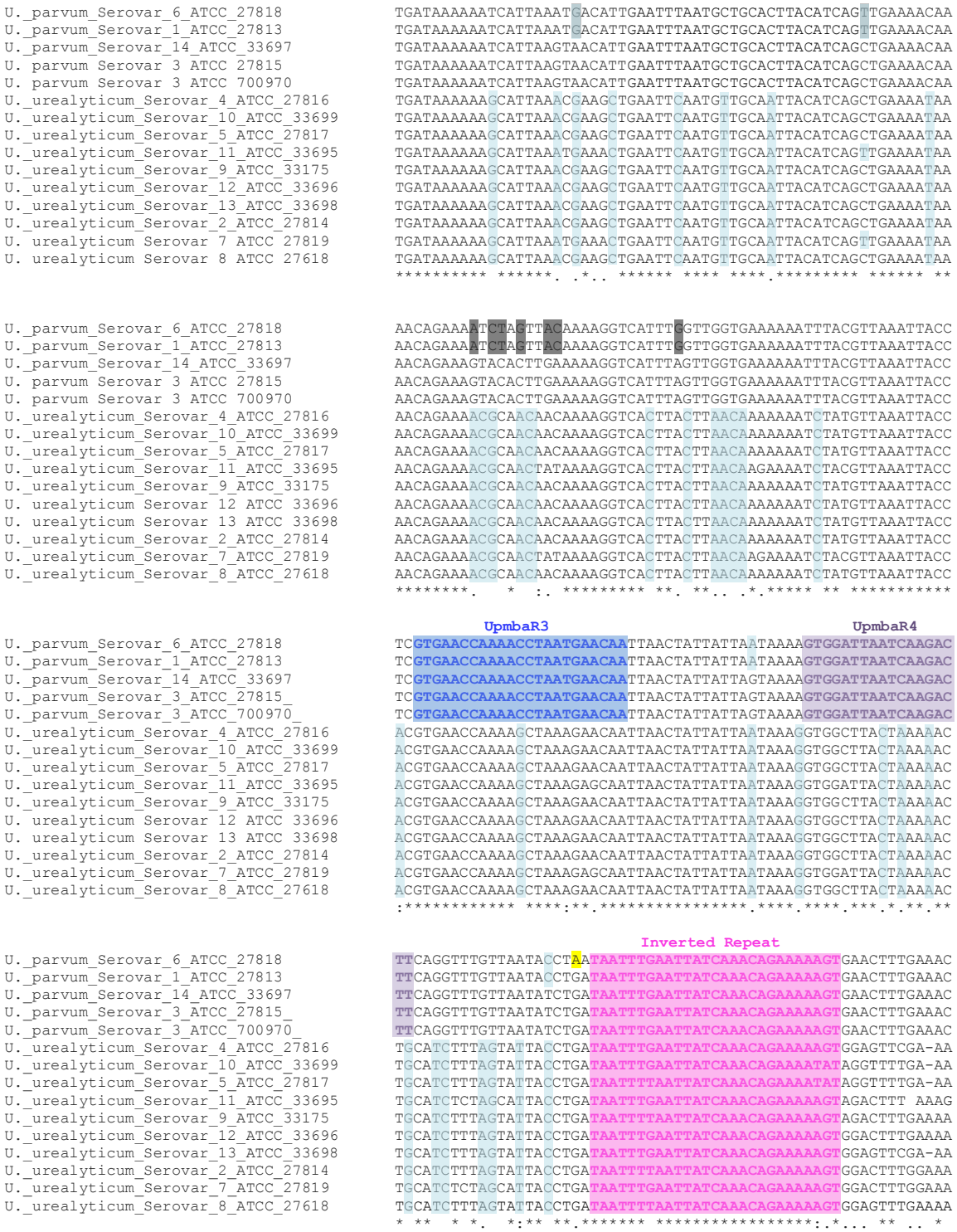

Figure 9.1. *U. parvum* serovars 1, 3, 6 and 14 gene alignment of intergenic upstream region of the *mba* and the upstream conserved portion of the *mba*. Gene alignments were performed using Geneious software and Clustal Omega. Designed primers are shown in shaded boxes and individual single nucleotide polymorphisms (SNPs) are highlighted in each of the *U. parvum* serovars.

Supplementary Table 9.2. Summary of serotyping based on the designed real-time PCR and HRM assay. Real-time PCR serotyping was confirmed by comparing *U. parvum* clinical isolates to ATCC strain serovars of *U. parvum.* Specimens had also been previously serotyped using sequencing and/or western blot using serovar-specific antisera. A total of 64.5% of clinical isolates were able to be successfully typed using the current real-time PCR and HRM assay.

Patient Sample Number	Previous serovar identified	DNA successfully amplified?	Serovar identified by novel real-time PCR and HRM assay	Correct identity confirmed by real-time PCR and HRM assay?
1A	1	Yes	1	Yes
1B	1	Yes	1	Yes
27	6	Yes	6	Yes
33A	3	Yes	3	Yes
33B	3	Yes	3	Yes
43	1	Yes	6	No
44A	3	Yes	3	Yes
44B	3	Yes	3	Yes
50	6	Yes		
55B	6	Yes	6	Yes
182	6	Yes	6	Yes
262T	1	Yes	6	No
290T	1	Yes	1	Yes
301	1	Yes	6	No
310	6	Yes	1	No
314T	3	Yes	14	No
322T	3	Yes	3	Yes
325	3	Yes	3	Yes
334A	6	Yes	6	Yes
334B	6	Yes	1	No
364A	6	Yes	1	No
365	3	Yes	1	No
429	6	Yes	6	Yes
435	3	Yes	3	Yes
473T	1	Yes	1	Yes
480T	6	Yes	Did not serotype	No
483T	1	Yes	1	Yes
498A	1	Yes	1	Yes
498B	1	Yes	1	Yes
507	1	No	-	-
510	6	No	3	No
912p	3	Late amplification	-	-
46e	6	No	-	-
402s	6	Late amplification	-	-
231e	3	Late amplification	-	-
291s	6	Late amplification	-	-
84e	6	No	-	-
33e	1 &3	Yes	6	No
1040e2	1	Late amplification	-	-
163w	6	No	-	-
924e	1	Late amplification	-	-
1043	6	No	-	-
928e2	3	Late amplification	-	-

1020e2	1	Late amplification	-	-
405e	1	No	-	-
196e1	3	Late amplification	-	-
268so	6	No	-	-
405eo	1	Late amplification	-	-
226so	3	Late amplification	-	-
1630eo	3	Yes	3	Yes
7.004 85 d	6	Late amplification	-	-
7.005 99d	6	Late amplification	-	-
7.002 99d	6	Late amplification	-	-
7.042 85 d	6	Late amplification	-	-
7.085 85 d	6	Late amplification	-	-
7.113 99d	6	Late amplification	-	-
TOTAL	-	**31/55 (56.4%)**	-	**20/31 (64.5%)**

Supplementary Table 9.3. Comparison of neonatal outcomes of infants exposed to *Ureaplasma parvum* and *Ureaplasma urealyticum*

	***U. parvum* (n = 36)**	***U. urealyticum* (n = 6)**	**Significance**
Gestational age	35.9 ± 0.4 (32 – 41)	34.7 ± 0.6 (32 – 36)	NS[1]
Apgar[2] - 1 minute	7.9 ± 0.3 (1 – 9)	7.8 ± 0.6 (5 – 9)	NS
Apgar - 5 minutes	8.8 ± 0.1 (8 – 9)	8.7 ± 0.2 (8 – 9)	NS
Birth weight	2550.0 ± 102.5 (1380 – 3873)	2674.2 ± 148.2 (2290 – 3330)	NS
Placental weight	456.0 ± 22.9 (260 – 710)	400.4 ± 14.3 (374 – 461)	NS
Continuous positive airway pressure (CPAP)	8/36 (22.2%)	2/6 (33.3%)	NS
Demonstrated features of RDS	8/36 (22.2%)	3/6 (50.0%)	NS
Required oxygen or positive pressure support > 6 hrs	5/36 (13.9%)	3/6 (50.0%)	NS
Diagnosed RDS[3]	5/36 (13.9%)	2/6 (33.3%)	NS
Length of stay	6.0 ± 1.2 (1 – 28)	5.5 ± 1.7 (2 – 13)	NS

[1] NS - not statistically significant

[2] Apgar – a measure of neonatal health scored at 1 min and 5 minutes post-delivery; measures appearance, pulse, reflex, activity and respiration of the newborn.

[3] RDS - respiratory distress syndrome.

Supplementary Table 9.4. Comparison of pregnancy outcomes of women who were infected with *U. parvum* serovars 1, 3 or 6

	Serovar 1 (n = 10)	Serovar 3 (n = 9)	Serovar 6 (n = 12)	Significance
Signs/symptoms of infection[1]	2/10 (20.0%)	1/9 (11.1%)	0/12 (0.0%)	NS[2]
Chorioamnionitis[3] **documented previously**	1/10 (10.0%)	0/9 (0.0%)	0/12 (0.0%)	NS
Chorioamnionitis in current pregnancy	7/10 (70.0%)	5/9 (55.5%)	7/12 (58.3%)	NS
- **Maternal Stage**	1.6 ± 0.2 (1 – 2)	1.2 ± 0.1 (1 – 2)	1.6 ± 0.3 (1 – 3)	NS
- **Fetal Stage**	2.5 ± 0.2 (2 – 3)	2.0 ± 0.3 (1 – 3)	1.7 ± 0.3 (1 – 3)	NS
Antibiotics administered < 3hrs prior to delivery	5/10 (50.0%)	3/9 (33.3%)	5/12 (41.7%)	NS
Cervical incompetence	4/10 (40.0%)	4/9 (44.4%)	5/12 (41.7%)	NS
Preterm premature rupture of membranes (pPROM)	3/10 (30.0%)	3/9 (33.3%)	3/12 (25.0%)	NS

[1] Signs and symptoms of infection included: maternal temperature > 38 °C, uterine or abdominal tenderness, foul-smelling vaginal discharge, maternal tachycardia (> 120 bpm) or fetal tachycardia (> 160 bpm)

[2] NS - not statistically significant

[3] Chorioamnionitis was determined by US pathologists (blinded to outcome) according to (Redline *et al.* 2003)

Supplementary Table 9.5. Comparison of fetal outcomes of those infants exposed to *U. parvum* serovars 1, 3 or 6 *in utero*.

	Serovar 1 (n = 10)	Serovar 3 (n = 9)	Serovar 6 (n = 12)	Significance
Gestational age	36.3 ± 0.9 (33 - 41)	35.9 ± 0.7 (33 - 39)	35.2 ± 0.7 (32-40)	NS[1]
Apgar[2] - 1 minute	8.1 0.7 (2 - 9)	7.5 ± 0.7 (3 - 9)	7.7 ± 0.7 (1 - 9)	NS
Apgar - 5 minutes	8.9 ± 0.1 (8 - 8)	8.7 ± 0.2 (8 - 9)	8.9 ± 0.1 (8 - 9)	NS
Birth weight	2643.9 ± 182.6 (1975 - 3873)	2650.7 ± 219.5 (1525 - 3855)	2370.8 ± 186.6 (1380 - 3825)	NS
Placental weight	541.9 ± 39.5 (270 - 635)	482.4 ± 48.5 (279 - 710)	395.8 ± 31.7 (260 - 655)	NS
Demonstrated features of RDS[3]	3/10 (30.0%)	2/9 (22.2%)	3/12 (25%)	NS
Required oxygen or positive pressure support > 6 hrs	1/10 (10.0%)	2/9 (22.2%)	2/12 (16.7%)	NS
Respiratory distress syndrome (RDS)	1/10 (10.0%)	2/9 (22.2%)	2/12 (16.7%)	NS
Length of stay	7.6 ± 2.7 (1 - 28)	5.0 ± 2.1 (1 - 16)	6.9 ± 1.8 (2 - 17)	NS

[1] NS - not statistically significant

[2] Apgar – a measure of neonatal health scored at 1 min and 5 minutes post-delivery; measures appearance, pulse, reflex, activity and respiration of the newborn.

[3] RDS - respiratory distress syndrome

Supplementary Table 9.6. Comparison of maternal demographic data from pregnancies exposed to *Ureaplasma* spp. which demonstrated no *mba*/MBA size variation, and those pregnancies which were exposed to ureaplasmas that varied the size of their *mba*/MBA

	No *mba*/MBA variants (n = 11)	**Single *mba*/MBA variant (n = 16)**	**Multiple *mba*/MBA variants (n =4)**	**Significance**
Maternal age	25.5 ± 1.2 (20 - 32)	23.9 ± 1.3 (17 - 32)	24.0 ± 2.3 (19 - 28)	NS[1]
Gravida[2]	1.9 ± 0.4 (1 - 4)	1.6 ± 0.2 (1 - 4)	3.2 ± 0.8 (1 - 5)	NS
Parity[3]	2.1 ± 0.3 (1 - 4)	1.4 ± 0.2 (1 - 2)	2.2 ± 0.7 (1 - 4)	NS
Marital status				
- **Married**	6/11 (54.5%)	5/16 (31.2%)	1/4 (25.0%)	NS
- **Single**	5/11 (45.5%)	11/16 (68.8%)	3/4 (75.0%)	NS
Medical Insurance				
- **Private**	7/11 (63.6%)	4/16 (25.0%)	2/4 (50.0%)	NS
- **Medicaid**	3/11 (27.3%)	8/16 (50.0%)	2/4(50.0%)	NS
- **Self pay/uninsured**	0/11 (0.0%)	4/16 (25.0%)	0/4 (0.0%)	NS
- **Unknown**	1/11 (9.1%)	0/16 (0.0%)	0/16 (0.0%)	NS
Evidence of prenatal care	10/11 (90.9%)	16/16 (100.0%)	4/4 (100.0%)	NS

[1] NS - not statistically significant

[2] Gravida - number of clinical pregnancies

[3] Parity - number of viable offspring resulting from all pregnancies

Supplementary Table 9.7. Raw data and standard error of the mean (SEM) relating to figure 6.1 – *Ureaplasma* spp. growth in M199 media. As replicate data were remarkably similar for this graph, error bars were not generated using statistical software.

Time	M199 Media + 10% Fetal Calf Serum		SEM	M199 media + 10% Horse Serum		SEM	10B broth		SEM
4	1×10^1	1.2×10^1	± 1.0	1.1×10^1	1.3×10^1	± 1	1×10^1	1×10^1	± 0
8	2.5×10^1	2×10^1	± 2.5	2.1×10^1	2.3×10^1	± 1	4.8×10^2	4.1×10^2	± 2.5
20	5×10^3	5×10^3	± 0.0	3.1×10^1	3.2×10^1	± 0.5	7.9×10^6	8×10^6	± 1
30	6×10^3	5.7×10^3	± 150	3.2×10^1	3.3×10^1	± 0.5	5.9×10^3	6×10^3	± 1

Supplementary Table 9.8. Raw data and standard error of the mean (SEM) relating to figure 6.3 – *Ureaplasma* spp. growth under different oxygen tensions. As replicate data were remarkably similar for this graph, error bars were not generated using statistical software.

Time	5% O_2		SEM	8% O_2		SEM	20% O_2		SEM
0	1×10^1	1.1×10^1	± 0.5	1×10^1	1.5×10^1	± 2.5	1×10^1	1.1×10^1	± 0.5
4	1.1×10^1	1.2×10^1	± 0.5	1×10^2	1.5×10^2	± 42.5	1.2×10^2	1.5×10^2	± 10
8	1.2×10^1	1.4×10^1	± 1	1.5×10^3	2×10^3	± 250.0	1.3×10^3	1.3×10^3	± 0
12	1.3×10^2	1.5×10^2	± 10	DNA[1]	DNA[1]	N/A[2]	1.5×10^3	1.7×10^3	± 100
24	1.5×10^2	1.8×10^2	± 15	2×10^4	2×10^4	± 0	1.9×10^3	2×10^3	± 850

[1] Data was not available for this sample at this time point

[2] Not applicable – no data was available, so therefore no standard error of the mean was able to be calculated