Antigenic *Ureaplasma* Variation and Adaptation to Ovine Complement Response Following Experimental Intrauterine Infection

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Abstract

Ureaplasma parvum is one of the smallest (genome <0.8 Mb), free living, self-replicating organisms identified. There are seven species of *Ureaplasma* but only two infect humans: *Ureaplasma parvum* (serovars 1, 3, 6 and 14) and *Ureaplasma urealyticum* (serovars 2, 4, 5, 7–13). While frequently thought to be a commensal, *Ureaplasma spp*. has been found to be the most frequently identified infectious organism associated with preterm delivery. The mechanisms it uses to escape recognition by the host immune system and establish long term chronic infections in the host are unknown. However, association of infection with pregnancy and preterm birth, which have lower immune response are well known and its infection of and induction of preterm birth leads to short and long term sequel.

Objectives of the study

Using a model of experimental infection, this thesis sought to investigate differences between systemic *Ureaplasma* infection in mid-second trimester equivalent and early third trimester equivalent, and relate these differences to the stage of development of the complement system in preterm lambs. These studies required the development of a new assay to measure complement function in sheep. The ability of the infecting strain of *Ureaplasma* to adapt to the sheep immune system was also investigated, examined at varying lengths of infection. These studies required the use of a new *in vivo* catheterisation method to establish the kinetics of early experimental intrauterine *Ureaplasma* infection and to recover *Ureaplasma* from the amniotic fluid by amniocentesis at various times during chronic infection. Emerging resistance to sheep complement was investigated in these strains and related to coincident alterations in the major surface antigen (multiple banded antigen; MBA) and alterations in the whole genome sequence. These studies required the characterisation of new monoclonal antibodies that recognise the MBA and establishment of a synthetic *MBA* expressed in *E.coli* to aid in reagent characterisation.

Materials and Methods:

Different erythrocyte sources and sensitising antibodies were utilised to establish the guinea pig erythrocyte as the best target for measuring the 50% haemolytic activity in fetal and adult sheep sera. Flow cytometry methods were also used to establish endogenous anti-erythrocyte

antibodies in adult, but not fetal sheep, revealing a failure of maternal IgG transfer across the placenta in contrast to humans. A bactericidal assay for the decrease in surviving *Ureaplasma* following incubation with sheep sera was utilised and modified conditions revealed the rate of killing and calcium-dependence of *Ureaplasma* killing for both fetal and adult sera. Experimental infection of singleton pregnant ewes by catheter or ultrasound guided needle, followed by caesarean delivery at either 94 or 125 days gestation (term=150 days) was central to all of the studies. Over 3 years, different infection duration (from 5 days to 6 weeks) and gestational age at infection (from 70 days to 116 days gestational age) were investigated in this model. Western blot analysis was also a central method for both examining the antibody response of foetus and ewe to *Ureaplasma* as well as examination of the size and number of MBA isoforms in evolving strains during chronic infection. The use of genomic analysis software Geneious (Biomatters ltd., New Zealand) was also utilised to compare the genomic sequences of *Ureaplasma* strains recovered by amniocentesis during chronic infection relative to the original infecting strain to determine number and site of genetic alterations with evolving serum resistance. Only the repeat region of the *MBA* gene required investigation by PCR and traditional Sanger sequencing, due to limitations of Illumina sequencing methods and repeats greater than 150 bp.

Results:

The 50% complement haemolytic activity of preterm sheep foetuses was very poor at 95 day gestation and improved by 125 day gestation, but was still much lower than adult sera. This was reflected in both *in vivo* bacteraemia during experimental infection and *in vitro* examining *Ureaplasma*-cidal activity of sera. A direct correlation (R^2=0.30; $p<0.05$) was found between haemolytic activity of sera and capacity to kill *Ureaplasma in vitro*. The strain utilised for experimental infection (HPA5) has a single low mass isoform of MBA consisting of 17 PAGKEQ repeats in the C-terminus. Following 5-7 days infection two of six of the infected animals began to show emergence of a second larger MBA isoform. The number and size of MBA isoforms continued to increase at 3 and 5 weeks, until recovered strains appeared to solely consist of strains with much larger MBA isoforms (72->120 k Da). The underlying mechanisms of increased MBA mass was found to be increasing number of PAGKEQ repeats from 17 to >200 repeats. Genomic analysis of recovered strains identified conserved single nucleotide polymorphisms in the two genes encoding the ammonium ion transport, but otherwise very few alterations were observed and no obvious candidate to explain increased serum

resistance (other than increased MBA mass) were observed. For the most part, alterations occurred in polyA, polyT and polyAT intergenic regions. Despite the apparent correlation between increasing complement resistance of recovered strains and increasing MBA number of repeats, transposon delivery and expression of the *MBA* gene from a resistant recovered strain and the serum-sensitive original HPA5 strain did not transfer serum resistance in one experiment.

Conclusions: *Ureaplasma* can change its surface epitopes continuously however, the expected phase variation loss of MBA expression with development of anti-MBA antibodies was not observed. A strong selection for increased MBA mass was apparent with longer infections and this was coincident with evolution of serum resistance (which increased steadily after 1 week infection to high serum resistance after 5 weeks infection). The development of the complement system was directly related to the detection of *Ureaplasma* in the circulation of infected lambs, but even 6 weeks of chronic persistent *Ureaplasma* infection was found to induce an inconsistent and low antibody response, and only in the later gestational age foetuses. While increased MBA size appears to be coincident with evolved serum resistance, it must require either loss of the original MBA isoform (dominant serum susceptibility determinant) or other less obvious alterations in the genome of *Ureaplasma*.

Key words: *Ureaplasma*, preterm labour, animal models, amniotic fluid, MBA gene, antigenic variation.

Acknowledgments

In the name of ALLAH, the most compassionate, the most merciful

First and Foremost I do praise GOD (ALLAH) (SWT) without his love, grace, and mercy none of my achievements would have been possible.

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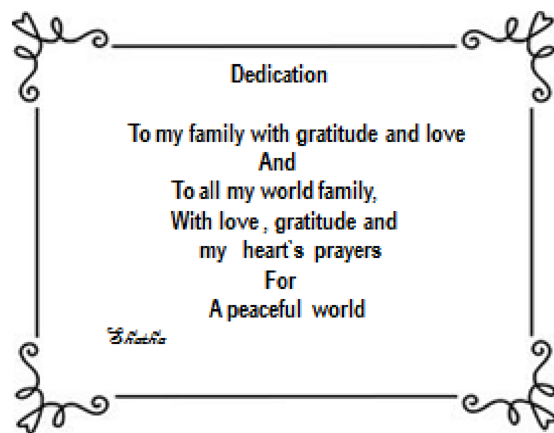
Dedication
To my family with gratitude and love
And
To all my world family,
With love , gratitude and
my heart`s prayers
For
A peaceful world

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List of abbreviations and symbols

5’	5 prime
3’	3 prime
µl	Microliter
ml	milliliter
ºC	Degrees Celsius
16S rRNA	16S ribosomal RNA
23S rRNA	23S ribosomal RNA
AP	Alternative pathway
Ab	Antibody
Ag	Antigen
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
bp	base pair
BLAST	Basic Local Alignment Search Tool
BPD	Broncho Pulmonary Dysplasia
BSA	Bovine Serum Albumin
BV	Bacterial Vaginosis
CCU	Colour Changing Units
CDS	Coding DNA Sequence
CFB	Complement Fixation Buffer
CFU	Colony forming unit
CH50	Complement haemolytic activity 50%
CLD	Chronic Lung Diseases

CP	Classical pathway
CR1	Complement receptor 1
DAB	DiAminoBenzidin
DAF	Decay accelerating factor
dNTP	deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
E.coli	Escherichia coli
FH	Factor H
FIGO	International Federation of Gynaecology & Obstetrics
FIRS	Fetal Inflammatory Response Syndrome
GA	Gestational age
G+C	Guanine and Cytocine
GIT	Gastrointestinal tract
HI	Heat Inactivated
HPA	Health Protection Agency
HRPO	Horse radish peroxidase
IA	Intra amniotic
IL-8	Interleukin-8
IL-6	Interleukin-6
IPA	isopropyl alcohol
I/R	Ischemia Reperfusion
IUGR	Intra-uterine growth retardation

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kDa	kilo Dalton
LDS	Lithium dodecyl sulphate
LPS	Lipopolysaccharides
LP	Lectin pathway
mAbs	monoclonal Antibodies
MAC	Membrane attack complex
MASP-2	MBL-associated serine protease -2
MASPs	MBL-associated serine proteases
MBA	Multiple banded antigen
MBL	Mannose binding lectin
Mb	Mega base
MIAC	Microbial invasion of the amniotic cavity
MIP-2	Macrophage Inflammatory Protein-2
MMP	Matrix metalloproteinase
M. hominis	*Mycoplasma hominis*
M. pneumoniae	*Mycoplasma pneumonia*
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
NGU	Non-gonococcal urethritis
NF-κB	Nuclear factor Kapa Beta
NHuS	Normal human serum
NShS	Normal sheep serum
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline

PBST	Phosphate buffered saline with 0.05% tween
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PHE	Public Health England
PL	Phospholipase
RFLP	Restriction Fragment Length Polymorphism
PRM	Pattern recognition molecules
PROM	Premature rupture of membrane
PRRs	Pattern recognition receptors
PTB	Preterm Birth
PTL	Preterm labour
RCA	Regulators of Complement Activation
RDS	Respiratory Distress Syndrome
RNA	Ribonucleic Acid
16S rRNA	16S Ribosomal RNA
sCR1	Soluble Complement regulator1
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SNPs	Single nucleotide polymorphism
SV	Serovar
TLR	Toll-like receptor
TNF-α	Tumour Necrosis Factor alpha
TX-114	Triton X-114

tRNA	transfer RNA
UHW	University hospital of Wales
UP	*Ureaplasma*
U. parvum	*Ureaplasma parvum*
Upvmp376	*Ureaplasma* phase-variable membrane protein 376
USM	Ureaplasma Selective Media
U. urealyticum	*Ureaplasma urealyticum*
Vsa	*Variable surface antigen*
WHO	World Health Organisation
W.B.	Western Blot

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CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Preterm labour (PTL)

1.1.1 Definition of preterm labour (PTL):

Preterm labour is defined according to the World Health Organisation and International Federation of Gynaecology and Obstetrics (WHO/FIGO) as delivery of a baby before completing 37 weeks of gestation. Those born before 32 weeks of gestation are called “very preterm” infants and those born before 28 weeks of gestation are called “extremely preterm” infants (Steer, 2005; Tucker and McGuire, 2004) (WHO 1977). In developed countries the rate of preterm birth is 5-10% compared to 25% in developing countries. It occurs in nearly a half million pregnancies each year in the United States alone especially in African-American women (Goldenberg *et al.;* 2008, Peltier, 2012) and accounts for ∼70% of all neonatal morbidity with long term sequelae on respiratory function and neurological consequences and the percentage of mortality and morbidity are inversely proportional to the gestational age (Steer, 2005; Wen *et al*., 2004; Tucker and McGuire; 2009, Goldenberg *et al.,* 2008).

Figure 1.1 Preterm baby; source: March of Dimes(Dezen *et al.,* 2012**)**

1.1.2 Categories of preterm labour (PTL):

PTL can be classified into the followings,

1. Spontaneous (idiopathic), about 50% of cases.

2. Premature rupture of membranes (PROM) 25%.

3. Induced (Iatrogenic) 25% for maternal or foetal indications.

(Moutquin,2003;dundleyand Papiernik, 1993; Ananth and Vintzileos*,* 2006; Allen, 1991; Goldenberg *et al*., 2008).

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1.1.3 Aetiology of preterm labour (PTL)

Preterm labour is multifactorial and in 30% of cases preceded by premature rupture of membranes (PROM), other common associations are low socioeconomic status, poor nutrition, smoking, alcoholism, uterine distension, abruption placentae, immunological disorders, however, the greatest risk is associated with infection/inflammation at the maternal–fetal interface triggered by improper production of pro inflammatory cytokines (Peltier, 2003). This project will mainly address the most common cause of prematurity, namely, intrauterine bacterial infection and compare it to other studies which confirmed the relationship between infection and PTL (Andrews *et al.,* 2000; Klein and Gibbs, 2005; Goldenberg *et al.,* 2008) although the rate of infection is not the same throughout pregnancy; as it is inversely proportional to the gestational age (Agrawal and Hirsch, 2012; Di Renzo, 2006).

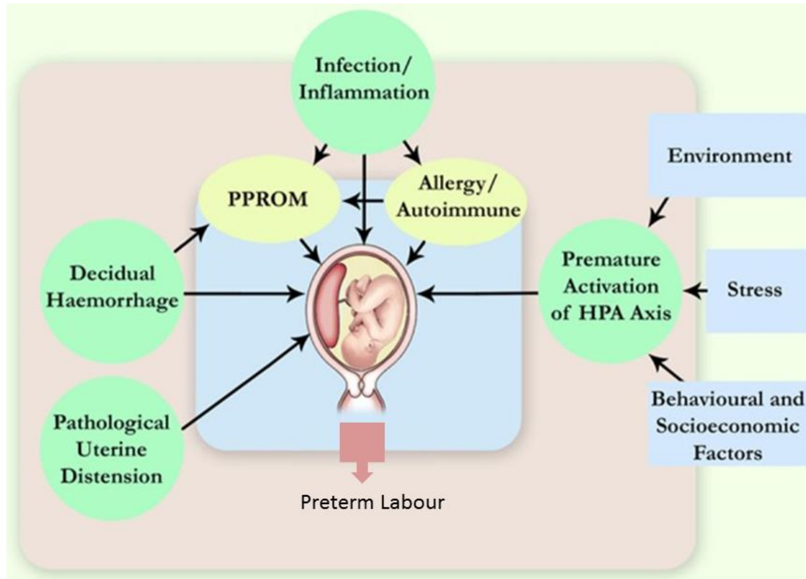

Figure 1.2 Factors contributing to PTL, adapted from (Mendz1*et al*.,2013)

1.1.4 Infection-driven PTB

During pregnancy there is a complex interaction of inflammatory events controlled by both the innate and acquired immune systems. Likewise, parturition can be initiated by activation of "pro-labour" inflammatory pathways, which lead to cervical ripening and myometrial contraction. Premature activation of these pathways, for example, by infection can lead to preterm labour mostly by improper activation of inflammatory pathways (Lappas and Rice, 2007; Peltier, 2003). At present, infection induced chorioamnionitis is the only factor for which a casual association has been made with preterm birth. Besides elevated levels of pro-inflammatory cytokines, experiments have also shown that infections stimulate the production and release of both PGE2 and PGF2 by the amnion, chorion and myometrium. Such prostaglandin molecules are the direct mediators of uterine contractions through their stimulation of prostaglandin receptors (Blanco, 1995; Larsen and Hwang 2010). Many studies have highlighted a cause-effect relationship between infection and preterm labour (PTL) or premature rupture of membranes (PROM). (Gibbs, 2001; Gravett *et al.,* 1986; Macintyre *et al.,* 2012; Romero and Mazor,1988; Romero et al., 1991).

Many clinical studies have shown that a pre-existing intra-uterine immune mediated inflammation can act as a predisposing factor in premature labour like pre-existing anti-sperm IgG antibodies signifying infection from male partner (Witkin and MCgregor, 1991; Toth et al., 1988), as well as a pre-existing history of pelvic inflammatory diseases (Epstein *et al.,* 2000; Gibbs *et al.,* 1992; Toth *et al.,* 1988; Abele-Horn *et al.,* 1997). Women who are smokers, and have multiple sexual partners (Cram *et al.,* 2002; De Francesco *et al.,* 2009), and/or use an intrauterine contraceptive device

(IUCD) have increased vulnerability to premature rupture of the membranes and subsequent preterm birth (Donders *et al.,* 1991; Steven and Fraser, 1974). However the frequency of pathohistological results and positive infection cultures was inversely proportional to gestational age (Watts, 1992; Horvath *et al.,* 2014; Goncalves *et al.,* 2002; Kundsin *et al.,* 1984).

1.1.5 Bacterial vaginosis (BV) and preterm labour:

Under normal conditions the vagina is populated by the facultative lactobacilli which safeguard the vagina from pathogens by maintaining an acidic pH through the secretion of lactic acid, and by production of H_2O_2 which is toxic to other bacteria (Hillier *et al.,* 1993; Hellberg *et al.,* 2001), but sometimes alterations occur that modify the vaginal flora like after menstruation, hormonal therapy, prolonged antibiotic treatment, vaginal medication, spermicidal creams, after sexual intercourse, or use of vaginal douches. These conditions lower the acidity (i.e. Increase the PH) of the vagina and diminish production of H_2O_2 and this will give other bacteria a chance to establish a larger occurrence leading to "bacterial vaginosis" (BV) (Hillier *et al.,* 1993). In particular increased presence of resident gram-negative and anaerobic bacteria including *Gardnerella vaginalis*, *Mobiluncus*, *Bacteroides*, *Prevotella*, and *Mycoplasma* species have been found. *Ureaplasma* which belong to the class *Mollicutes* and the family of *Mycoplasmatales* are found to be one of the most common bacteria recovered from patients with BV and frequently associated with adverse pregnancy outcome and neonatal mortality and morbidity (Donders *et al*., 2009; Goldenberg and Rouse, 1998; Gray *et al.,* 1992; Hillier *et al.,* 1995; Kurki *et al.,* 1992; Romero and Mazor, 1988; Martius and Eschenbach, 1990; Nicola and Maxwell, 2006).

1.1.6 Pathophysiology of infection-induced PTL:

Pathophysiology of premature labour is still only partly understood. Experiments on animal models showed that preterm labour can be induced by bacteria, bacterial cell wall products, and also by inflammatory cytokines such as IL-1 and tumour necrosis factor (TNFα) (Dudley, 1997; Casey *et al.,* 1989; Mattsby-Baltzer *et al.,* 1998; Adams *et al.,* 2011; Farina and Winkelman, 2005).

The keystone of infection- induced labour are believed to involve signal transduction cascades that begin with recognition of invading pathogens by cell-surface pathogen recognition receptors (PRR) like toll-like receptors (TLRs). TLR are the first line of defence at the amniotic fluid epithelium layer which is in direct contact with the amniotic fluid leading to a host response causing an `intra-uterine inflammatory response syndrome' and elevated transcription of genes encoding proinflammatory cytokines leading to increased levels of nuclear factor kappa Beta (NF-κB) particularly those associated with labour (Shimizu *et al.,* 2008).

The inappropriate release of these pro inflammatory cytokines (which can be either clinically evident or subclinical), including TNF-α, IL-6, IL-1, and IL-8, into the amniotic fluid and gestational tissues, trigger leukocyte recruitment, activate prostaglandin synthesis and release of matrix metaloproteinases (MMPs)(Larsen and Hwang, 2010). These mediators in turn, lead to myometrial contractions, cervical dilatation, and rupture of fetal membranes, changing the state of the uterus from quiescence to active, ending in premature initiation of labour and expulsion of a preterm foetus. These cytokines have been detected in high concentration in the amniotic fluid and plasma of women with signs of PTL (Fig.1.3) (Bryant and Thornton, 2012; Shimizu *et al.,* 2008; Kawai and Akira, 2011; Koga and Mor, 2010; Triantafilou *et al.,* 2013).

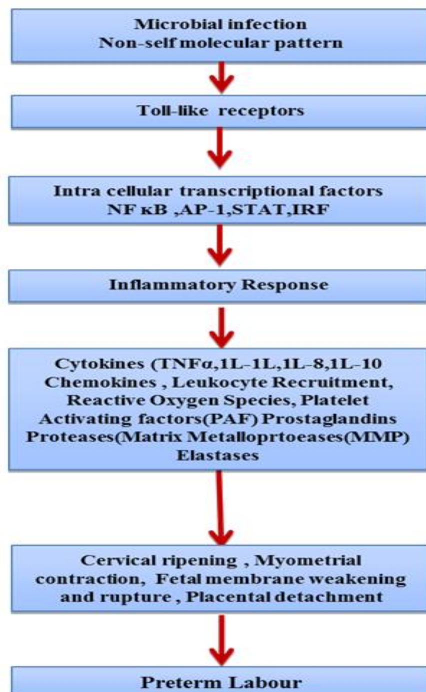

Figure 1.3 Pathogenesis of infection/inflammation induced PTL

1.1.7 Routes of *in utero* infection

The primary mechanism of transmission of *Ureaplasma* is by sexual contact then by vertical transmission i.e. infection ascends from the colonized lower genital tract to the uterine cavity and the foetus or infection of the neonate during passage through birth canal (Behnam *et al*., 2014; Garland *et al.,* 2002; Sanchez and Regan, 1987; Waites, 2006; Hill, 1998).

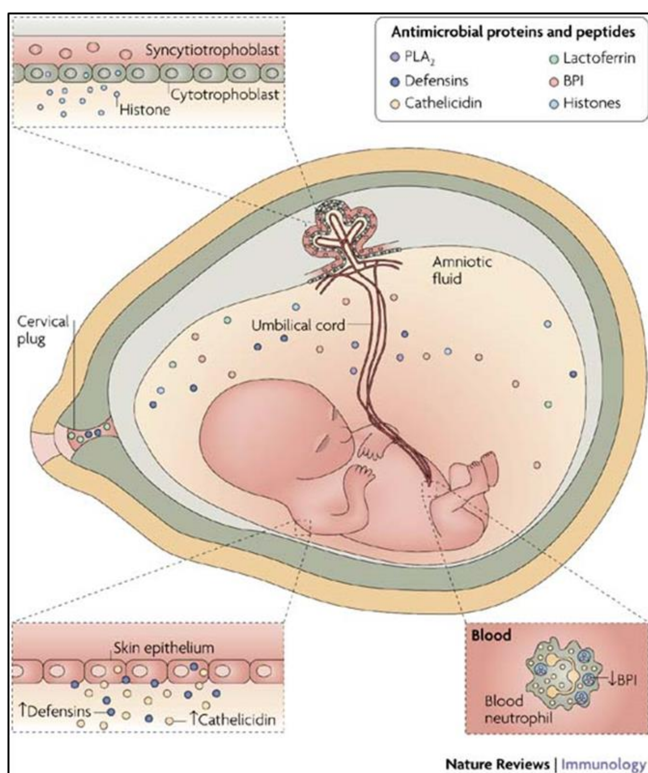

Figure 1.4 Routes of in utero infection ,cited from (Levy, 2007)

Infection can gain access to the uterine cavity and settle in the uterus even at the time of conception (Kasprzykowska *et al.,* 2014). Alternatively infection can spread by haematogenous route through the placenta from the infected mother's blood (Waites, 2006; Viscardi, 2010). It could also migrate through the fallopian tubes from the abdominal cavity, and in cases where medical intervention has occurred, iatrogenic infection by contaminated needle during amniocenteses, or chorionic villous sampling (CVS) cannot be ruled out (Romero and Mazor, 1988; Garland *et al.,* 2002). In case of ascending infection, *Ureaplasma* would ascend from the cervix, pass to the choriodecidual space, then cross the chorioamniotic membrane to colonise the fetal tissues, decidua and membranes (Hallman and Kaukola, 2012; Khare and Khare, 2007) (Fig.1.4). It is also possible to acquire *Ureaplasma* infection by vertical transmission during labour (due to premature rupture of membranes or passing through the colonised vagina) where it establishes an infection of the neonatal airway resulting in post-birth respiratory disease and related neonatal morbidity (Romero *et al*., 2007; Romero *et al.,* 1992; Sampson *et al*., 1997; Taylor-Robinson, 1999). The rate of vertical transmission ranges from 18- 55% among full-term infants and from 29- 55% among preterm infants (Abele-Horn *et al*., 1997; Sánchez, 1993). In a report by Sanchez (1987) fifty-nine term infants (45%) had at least one culture site positive for *Ureaplasma spp.* (eye 4%; nasopharynx 24%; throat 16%; vagina, 53%; and rectum 9%). Also Sung *et al* (2011) found that in 65% of preterm infants less than 26 weeks respiratory infection by *Ureaplasma spp*. could be detected (Sung *et al.,* 2011).

1.1.8 Outcome following preterm labour

PTL is a medical, social, and economic problem, especially in developed countries (Hannah *et al.,* 2013; Tricia *et al.,* 2013); it can contribute to the following:

Mortality: Globally, PTL is considered as the commonest cause of neonatal mortality it accounts for about 70% of neonatal deaths (Wen *et al*., 2004). Survival rate is inversely proportional to the gestational age. Infant death is death of an infant before his or her first birthday; it can be neonatal or post-neonatal. Preterm birth has a significant influence on the U.S. infant mortality rate. In England and Wales data from the office for national statistics, the mortality rate for preterm babies (24-36wks) was reported to be 25.4/1000 live births which is 11% less compared to the rate reported in 2006 (28.6/1000 live births) which could be attributed to the new strategies in the intensive care for preterm babies (MacDorman, 2014; Moser, 2008; Wen *et al.,* 2004). Statistics separated for different preterm gestational age groups reported decreases in infant mortality rates of 14% for preterm infants of 24–25 weeks' gestation and 40% at 28–31 weeks' gestation relative to previous rates, with an approximately equal reduction in neonatal and post neonatal mortality (Wen *et al.,* 2004).

Morbidity: Preterm birth is a predisposing factor for about 75% of neonatal morbidity (Wen *et al*., 2004). With increasing gestational age, the risk of adverse consequences of PTL declines. Preterm infants are at risk of infectious and non-infectious respiratory complications after delivery, with up to 40% of preterm survivors having bronchopulmonary dysplasia (BPD; also known as chronic lung disease of prematurity or CLD), neurodevelopmental disabilities, intraventricular haemorrhage, recurrent infections, retrolental fibroplasia and necrotizing enterocolitis (Saigal and Doyle, 2008; Gomez *et al.,* 1998) and even psychological, educational and behavioural problems (Moster *et al.,* 2008; Ira *et al.,* 2013).

1.2 Ureaplasma

1.2.1 Characterisation of Ureaplasma

1.2.1.1 Cell Structure and Metabolism:

Ureaplasma is one of the smallest free living, self-replicating organisms and was first discovered in primary agar cultures of urethral exudates from male non-gonococcal urethritis patients by Shepard *et al*. in 1954 (Shepard, 1954). Initially defined as a tiny" colony belonging to the pleuropneumonia-like organisms due to their minute agar colony size (15±5µm) which needed stereomicroscopy to be seen. It was reclassified as "*Ureaplasma*" in 1974 as part of the class *Mollicutes*. This class includes the *Mycoplasmas* and related organisms, all of which are characterised by a lack of a cell wall. Instead electron microscopy showed that their cell membrane as a 10nm thick, triple-layered membrane with 5-8nm pilus-like projections on its surface. All early *Ureaplasma* reports therefore are listed as T-strain *Mycoplasma* due to its tiny colonies. (Tully and Taylor-Robinson, 1986; Shepard *et al*., 1974). *Ureaplasma* has a restricted biosynthetic activity due to the limited genetic information in the small genome, so they are obliged to live a parasitic life in cooperation with eukaryotic cells (Robertson *et al.,* 2002). For culture *in vitro*, they need cholesterol-rich media to supply them with fatty acids and cholesterol necessary for the assembly of their membrane, but they are non-glycolytic and lack the arginine dihydrolase pathway that is found in *M. hominis* (Freundt, 1983). Being wall-less could explain the pleomorphic shape of *Ureaplasma*, it has been reported as spherical or ovoid coccus bacilli cells (Boatman, 1979; Miyata and Ogaki, 2006; Kahane and Horowitz, 1993; Smith *et al.,* 1994). It also cannot be classified by Gram stain. In the Gram stain test, gram-positive bacteria retain the crystal violet dye, while a counterstain (commonly safranin or fuchsine) added after the crystal violet gives all gram-negative bacteria a red or pink colouring. Therefore, *Ureaplasma* fail to stain with crystal violet, because they have no cell wall and are unlikely to be visualised by a light

microscope as they are 200-500 nm in sizes. Also absence of cell wall make *Ureaplasma* extremely sensitive to dehydration so it can be physically inactivated by heating it at 100◦C for 10 minutes. Also lack of cell wall renders *Ureaplasma* resistant to antibiotics which target the bacterial cell wall such as ampicillin, but susceptible to antibiotics that interfere with protein synthesis like tetracyclines, macrolides, spectinomycin and quinolones (Taylor-Robinson and Ronald,1997; Beeton *et al*., 2009; Taylor-Robinson and Furr, 1986). *Ureaplasma* also grows better at low pH and most media have a pH of 6.0-6.5 (Shepard and Lunceford, 1978; Romano *et al.,* 1980). *Ureaplasma* differ from other *Mollicutes* by their ability to hydrolyse urea as their primary carbon source (Shepard and Lunceford, 1976; Blanchard, 1990; Takebe *et al.,* 1984); they cannot ferment glucose or other carbohydrates (Robertson and Howard, 1987) or get energy by degrading arginine (Fiacco *et al.,* 1984). Urease activity was shown to be necessary for the generation of ATP by a chemosmotic mechanism (Willoughby *et al*., 1991; Romano, 1980). *Ureaplasma* also express a human immuoglobulin A1 protease that is thought to facilitate its adherence to mucosal surfaces by removing adherence-blocking antibodies (Felix and Nana, 1999; Robertson *et al.,* 1984).

1.2.2 Classification of *Ureaplasma*

Mycoplasmas and *Ureaplasma* are eubacteria belonging to class *Mollicutes*, which is comprised of 4 orders, 5 families, 8 genera, and about 200 known species, but only five species are recognized as human pathogens *(M.pneumoniae, M.hominis, Mycoplasma genitalium, Ureaplasma parvum* and *Ureaplasma urealyticum*) (Razin *et al.,*1998;Waites *et al.,*2005). Originally it is thought to have evolved from clostridium-like ancestors through the process of gene deletion (Fanrong *et al.,* 1999; Freundt, 1983; Waites, 2006). *Ureaplasma* was first discovered in 1954 samples collected from human urogenital tract and classified in 1974 as a

new genus and species. The name *Ureaplasma* was suggested for this new genus due to its ability to hydrolyse urea (Shepard *et al.,* 1974). Later it was subcategorized into 14 serovars and into two genetically related biovars, biovar 1 and biovar 2; however, these were subsequently renamed parvobiovar and T960 biovar (Yoshida *et al.,* 2005; Lin and Kass, 1980) on the bases of differences in a major *Ureaplasma* surface antigen called the multiple banded antigen (MBA) (Knox *et al.,* 1998). In 1999, a systematic reclassification was undertaken separating two species depending on phenotypic and genotypic evidence. The fourteen previously identified serovars of *Ureaplasma* from different isolates split between these two distinct species: *U. parvum* which contain four serovars 1, 3, 6 and 14 and *U. urealyticum* which contains serovars 2, 4, 5 and 7-13 (Robertson *et al*., 1993; Kong *et al*., 1999; Robertson *et al.,* 2002; Knox *et al.,* 1998; Ren and Zhu, 2003; Harasawa and Kanamoto, 1999). The parameters for separation included genome size, 16S rRNA genes, 16S-23S rRNA intergenic regions, urease enzyme variations, different patterns arising from DNA-DNA hybridization, differential growth requirements for manganese and differences in the major surface antigen, the multiple-banded antigen gene (MBA) (Shepard and Lunceford, 1978; Teng *et al.,* 1994; Robertson *et al.,* 1994; Razin and Yogev, 1986).

The arrangement of the C-terminal domain of the MBA is responsible for the serovar specificity and the sequence heterogeneity of the 5' region of the *MBA* gene assist in dividing these 14 serovars into subgroups. Teng, *et al*, (1994) and others succeeded in determining that a gene with high homology to the *MBA* gene of SV3 existed in the remaining 13 reference serovars as well as in all clinical isolates (Fanrong *et al.,* 1999; Pitcher *et al.,* 2001; Teng, 1994).

Later on, Kong *et al,* (2000) managed to further separate U. *parvum* into three subtypes, represented by serovars 1, 3/14, and 6 using amplicon size differences with PCR. *Ureaplasma*

urealyticum was also separated into three subtypes by subtype-specific PCR primer pairs targeting the *MBA* genes and could distinguish subtypes within each species. Genotype A consisted of serovars 2, 5, 8, and 9; genotype B contained serovars 4, 10, 12, and 13; and Genotype C contained serovars 7 and 11 (Kong *et al.,* 2000).Later on, the same team subdivided *U.urealyticum* into five genotypes depending on sequencing of the whole 5`ends and partial repetitive regions of the *MBA* genes of the ten *U.urealyticum* serovars (Kong *et al*., 2000). as in the following table,

Table 1.1 Amino acid sequences of repetitive units of the MBA genes of 14 serovars of *U. parvum* and *U. urealyticum.*

Serovars / subtype	Repeat amino acid sequence
UP Serovar 1	PGKEQQ
UP Serovar 3	PAGKEQ
UP Serovar 6	PGKE
UP Serovar 14	PAGKEQQ
UU MBA genotype A Include serovars 2, 5,8	TKPGSGET
UU MBA genotype B include serovar10	TQPGSGST
UU MBA genotype C include serovars 4, 12 ,13	TSPEKPGNGT
UU MBA genotype D serovars 9	KKPETGSTEGGS1TEG
UU MBA genotype E serovars 7 and 11	GAQSGNGSGSASGS

1.2.2.1 Ureaplasma genome

Mycoplasma are thought to have evolved from more classical bacteria of the *firmicutes* by massive genome reduction through a process called regressive evolution and hypotheticaly, this is attributed to its parasitic life which renders most of metabolic processes unnecessary (Weisburg *et al*., 1989; Gundersen *et al.,* 1994; Moran, 2002; Snel *et al*., 2002; Silva and Latorre, 2008). The genome size of the various strains of *Ureaplasma* appears to differ extensively and matches the two serovar clusters. The genome size of *U. parvum* is about 760 kb, whereas *U. urealyticum* ranges from 880 to 1,140 kb. It has an circular DNA which encodes for a predicted 614 open reading frames and 38 RNA genes, making it the second smallest genome ever sequenced for a free-living prokaryote (smallest being *M. genitalium* at 580,040 bp)(Robertson *et al.,* 1990; Robertson *et al.,* 2002; Paralanov *et al.,* 2012). The genome of the prototype *U.parvum* SV3 contains 613 predicted protein encoding genes and 39 RNA genes, including two identical rRNA operons (consisting of an rRNA-16s, rRNA-23s and rRNA-5s) (Paralanov *et al*. 2012). *Ureaplasma* thought to descend from low G+C content gram positive bacteria and use a non-standard genetic code (Razin *et al*., 1983). Moreover, all *Mycoplasma* use the codon UGA=tryptophan (trp) as a genetic code rather than being a stop codon (Blanchard, 1990). Serovar identification can be accomplished by serology (Pitcher et al., 2001; Watson et al., 1990), immunofluorescence (Bowie *et al*., 1977; Muenster *et al*., 1979) and ELISA (Brown *et al.,* 1983; Von Chamier *et al*., 2012). The latter is the least sensitive and has been used to predict patients at risk of PTL (Horowitz *et al.,* 1995). Sometimes it is difficult to detect all serovars because of variable growth rates, and multiple serovars per specimen (Yi *et al.,* 2005). Surprisingly, *U. urealyticum* has a relatively unique respiration system with six closely related iron transporters, which apparently arose through gene duplication (Glass *et al.,* 2000). *Mycoplasma* genomes also contain numerous repeated sequences, which

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has an important role in their evolution (Rocha and Blanchard, 2002). Moreover, *Mycoplasma* can gain or delete genes from its chromosome through a process called horizontal gene transfer, which renders all *Mycoplasmas* continuously dynamic and increases their pathogenicity (Ochman *et al.,* 2000; Xiao *et al.,* 2011).

1.2.3 Culture Characteristics

Ureaplasma are characterised by their rapid growth in broth media, the rapid pH increase caused by production of high levels of ammonium ions being detected by phenol red (clinical samples can range from about 10-10^7 colour changing units/ml) in less than 24 hrs. Colour change occurs only at the end of the log phase of growth as the bacteria are entering the static phase, but culture viability drops sharply in these wells (bright red-fushia) as the increasing PH by liberation of ammonium ions slow their growth and division. For this reason *Ureaplasma* never reach a high enough culture density to cause turbidity and turbidity is a sure sign of culture contamination by another bacteria. *In vivo*, of course the toxic intermediates and fresh urea substrate would be exchanged by normal biological processes.

Ureaplasma growth can also be shown by colony formation on A8 agar plates or *Ureaplasma* selective agar in the presence of $CaCl_2$ indicator. Colonies of *Ureaplasma* can be seen after 48 hours of incubation with a stereomicroscope. Colonies are typically 15–30 µm in diameter and have a brownish granular appearance caused by urease activity in the presence of $CaCl_2$ indicator in the agar and can be differentiated from the large *Mycoplasma* colonies, which have a "fried egg" appearance.

More recently in clinical diagnostic laboratories, more rapid and specific methods to detect *Ureaplasma* by Real-Time PCR using primers designed against the *MBA* or *urease* genes (or even species and serovar specific targets) are predominant. However, it is interesting to note

that many reference labs (including Public Health England) still run plate culture in parallel for diagnostics.

1.2.4 Potential virulence factors of *Ureaplasma* species.

Although *Ureaplasma* is considered a low virulence organism and live as commensals in the urogenital tract of 40-67 % of sexually active women, it is potentially pathogenic and had been described as an "under estimated enemy of human reproduction" as it can invade extra genital sites particularly in immunocompromised patients were it can cause septic arthritis. During pregnancy the altered immune status of the mother and the foetus will provide the conditions for these organisms to cause invasive and destructive disease (Waites, 2006). *Ureaplasma* spp. can be transmitted from a colonized woman to her new-born infant and it can colonise a wide range of human tissues resulting in multiple clinical manifestations such as infertility, histologic chorioamnionitis, stillbirth, preterm delivery, congenital pneumonia, bronchopulmonary dysplasia, meningitis and perinatal death. In men it can cause non-gono-coccus urethritis prostatitis and renal stones (Yu *et al.,* 2008*;* Juhász *et al.,* 2011), but still no direct link has been found between these pathological conditions and a specific serovar or species (Dando *et al.,* 2012). Therefore, the invasive strains of *Ureaplasma* have some as yet unidentified potential pathogenic virulence factors (Paralanov *et al.,* 2012).

The speculated *Ureaplasma* virulence factors in the literature include

1.2.4.1 Urease enzyme:

Ureaplasma spp. differ from *Mycoplasma* by uniquely expressing urease activity which hy-drolyses urea to generate metabolic energy through creating a chemical gradient across the membrane (Romano *et al.,* 1980; Willoughby *et al.,* 1991), but at the same time liberating

toxic secretory products such as ammonia, which may cause a local injuries to mucosal surfaces and precipitate stone formation by increasing alkaline pH of urine (Takebe *et al.,* 1984). Urease by-products may also induce generation of reactive oxygen radicals secreted in close vicinity to host cell membranes (Glass *et al.,* 2000).

1.2.4.2 Phosphatase A1, A2 and/or C:

It has been hypothesised that degradation of placental phospholipids by *Ureaplasma* phospholipase A1, A2, and/or C and hydrolysing phospholipids of amniotic membranes would liberate arachidonic acid facilitating membrane rupture and initiating the onset of premature labour (Cassell *et al.,* 1986). In particular, phospholipase C shows maximum activity during the exponential phase of *Ureaplasma* growth (De Silva and Quinn, 1986). *Ureaplasma* phospholipase is also thought to be a potent inducer of fibroblast cytokine release *in vitro* and may lead to the development of neonatal BPD (Stancombe *et al.,* 1993). However, Paralanov *et al.,* 2012 disputed the presence of phospholipase activity through functional assays utilising the prototypic strains, raising questions as to whether this activity is real or not (Paralanov *et al.,* 2012).

1.2.4.3 IgA Protease:

This membrane-attached enzyme is necessary for pathogenicity of *Ureaplasma* as it facilitates its colonisation, gaining access to mucosal surfaces and causing diseases (Soeharso, 2005; Glass *et al.,* 2000). In spite of the presence of IgA1 antibodies on mucosal surfaces, the IgA hinge region of the heavy chain between proline and threonine can be cleaved by the *Ureaplasma* IgA1 protease (Spooner *et al.,* 1992). It could potentially additionally facilitate

colonisation of other microorganisms on the mucosal surface due to local functional deficiency of IgA which act as the principle component of the mucosal immunity (Kapatais-Zoumbos *et al.,* 1985; Glass *et al.,* 2000; Kilian *et al.,* 1996).

1.2.4.4 Haemolysins:

Shepard *et al.* (1974) found a soluble haemolysin that can be produced from the T-*Mycoplasma* with haemolytic action against guinea erythrocytes but less against human and sheep erythrocytes. These authors speculated that it was caused by a weak peroxide reaction (Shepard *et al.,* 1974). Glass *et al.*(2000) reported two types of haemolysin in *Ureaplasma* encoded by two genes in SV3, *hyl*A which possibly functions as a virulence factor for *Ureaplasma* spp. The other is hlyC, and has an orthologous gene in *M. pneumoniae*, in which haemolysis is mediated by H_2O_2 activity (Glass *et al.,* 2000). Supporting evidence for this concept lies in the fact that orthologous of the haemolysin hlyA mediate the haemolytic and cytotoxic activity in other microbes and some *Mycoplasma* that lack this gene are non-pathogenic (Robertson and Sherburne, 1991; Gardella and Del Giudice, 1983).

1.2.4.5 The Multiple Banded Antigen (MBA):

This species-specific surface expressed lipoprotein first discovered in 1990 by Watson is predicted to play a major role in antigenic recognition of both *Ureaplasma* species by serum antibodies in humans during infection (Watson *et al.,* 1990; Zheng *et al.,* 1996).

The MBA contains both serovar-specific and cross-reactive epitopes. Nucleotide sequence analysis of the MBA antigen of SV3 predict that this antigen consist of 2 regions, the C-terminal domain which is immunogenic, contains the serovar specific repeats (the number of

these a. a. repeats define the size variation). The other is the N-terminal domain, which consists of a conserved signal peptide and a membrane anchor (Teng *et al.,* 1994). The MBA gene consists of a largely conserved 5' non-coding region; however it does contain biovar- and serovar-specific polymorphisms. The 3' region, usually making up the majority of the gene which contains the codons for the repeats: For example, an 18-nucleotide tandem repeat encoding the PAGKEQ for SV3, but 12-nucleotide tandem repeat encoding PGKE for SV6. Phenotypic diversification of its surface antigenicity resulting from variation in the number of these repeating units by additions or deletions of units via a Xer-like recombinase located downstream of the MBA gene although there is no diversity in the repetitive sequence (Zheng *et al.,* 1995). Variation in MBA is considered an important mechanism for this wall-less pathogen to adapt and evade the host immune response and explain the role of antigen size variation in disease production and their ability for persistent infection in a wide range of hosts (Dybvig, 1990).

1.2.4.6 Pathogenicity islands and horizontal gene transfer

Horizontal gene transfer (HGT) is transfer of genetic material between bacterial cells uncoupled with cell division, e.g. *Ureaplasma* may acquire antibiotic resistance through this way. On the other hand, vertical gene transfer occurs during cell division where genetic material is transferred from the mother to the daughter cells (Karlin, 2001). Horizontal gene transfer plays a significant role in the adaptation and evolution of many microbial species. Although the average *Ureaplasma* genome size is unusually small for the bacterial kingdom, and such a feature generally does not favour DNA acquisition, nonetheless, *Ureaplasma* species have nonetheless been shown to engage in extensive horizontal gene transfer (Xiao, *et al.* 2011; Paralanov, *et al.* 2012). This transfer of genetic information has been documented among cells

of the same serovar, and between cells of different serovars. In fact, comparative genomic analysis has indicated the possible exchange of genetic material between *U.Parvum* and the closely related *Mollicute Mycoplasma hominis* (Sabine *et al.,* 2009). In the largest *Ureaplasma* sequencing study to date, it was found that nearly 40% of all isolates were genetically mosaic in nature due to horizontal gene transfer, and this included the exchange of serovar specific markers, including the MBA. This high frequency of genetic transfer may indeed explain the weak association between individual serovars and specific disease outcomes. Moreover, it questions the very existence of distinct serovar populations *in vivo* since specific markers maybe lost, gained, or changed by horizontal gene transfer. The genomes of all 14 *Ureaplasma* serovars have now been found to contain multiple mobile genetic elements, including transposases, recombinases and integrative and conjugative elements. The presences of these elements provide a means for *Ureaplasma* species to engage in genetic transfer, although the exact underlying mechanisms have yet to be established.

1.2.4.7 Adherence of Ureaplasma to cell surfaces

Lack of cell wall enable *Ureaplasma* to adhere to host cell-surface and guarantee the exchange of compounds which facilitate its growth, on the other hand it may damage the host cell by delivering toxic metabolic compounds to it. *Ureaplasma* pathogenicity may be attributed to its adherence to respiratory, genitourinary, and intestinal mucosa. Certain adhesins could be antigenic and act as an essential characteristic for their adhesion and some of which are species- and serotype-specific (Kahane and Horowitz, 1993; Smith *et al.,* 1994). Depending on the ligand (bacterium)-receptor (host-cell) complex, the outcome of this interaction will be determined: if the bacterium can attach itself to the uroepithelium of human (or other species) it has a greater likelihood of causing non-gonococcal urethritis, encrusted cystitis, chronic

prostatitis, or urolithiasis (Shimada *et al.,* 2014). It may also interfere with male fertility by adhering to the surface of spermatozoa, interfering with the sperm motility and sperm/egg recognition (Xu *et al.,* 1997; Potts *et al.,* 2000; Zhang *et al.,* 2011; Diaz-Garcia *et al.,* 2006; Knox *et al.,* 2003). Also adherence of *Ureaplasmas* to the surface of spermatozoa and endometrium even before implantation could be the mechanism whereby intrauterine infection is established in the amniotic fluid persistently throughout pregnancy (Volgmann *et al.,* 2005; Foulon *et al.,* 1985; Knox *et al.,* 2003). *Ureaplasma* can also adhere to sulfoglycolipid receptor of male germ cell membrane interfering with human fertility (Lingwood *et al.,* 1990). In addition, *Ureaplasma* can attach itself to the sialyl residues and/or sulphated compounds on the surface of erythrocytes which enable scientists to use erythrocytes as a model system to study adherence *Ureaplasma* to cell surfaces (Sachse *et al.,* 1996; Saada *et al.,* 1991). Chronic lung diseases associated with *Ureaplasma* infection may result from direct *Ureaplasma* adhesion and damage to the epithelial cells of human lungs or indirectly through inducing inappropriate pro-inflammatory response in the lungs that increases the risk of evolving bronchopulmonary dysplasia (BPD) (Schelonka *et al.,*2005; Torres-Morquecho *et al.,* 2010).

1.2.4.8 Antigenic variation in Mycoplasmas

In spite of reduced genomic size of *Mycoplasma*s and their lack of protective cell wall, it still shows a striking ability to vary its expression of surface proteins. This is as an effective means of facing challenges when infecting a wide range of hosts and evading the immune competent host response successfully to cause diseases that are often chronic. This can be attributed partly to the highly dynamic lipoproteins in the membrane which can be genetically changed by a number of highly sophisticated mechanisms (Zimmerman, 2014; Monecke *et al.,* 2003). A lot of genetic events are responsible for the genetic modulation affecting a number of gene

families or "hot spots". It can alter the expression, the size and the antigenic profile, generating a highly dynamic surface within a cloned population (Citti *et al.,* 2010; Zheng *et al.,* 1996).

At least four major processes have been shown to modulate *Mycoplasma* gene expression. These complex systems are based on arbitrary actions that affect the expression and the structure of a number of copious surface lipoproteins, so that modified clones arise at a rate of 10^{-2} to 10^{-5}events/cell/generation (Zimmerman, 2014). This *Mycoplasma's* striking antigenic variation explains why most protective control methods and vaccine trials against *Mycoplasmas* have been unsatisfactory (Zimmerman *et al.*, 2009; Zimmerman, 2014).

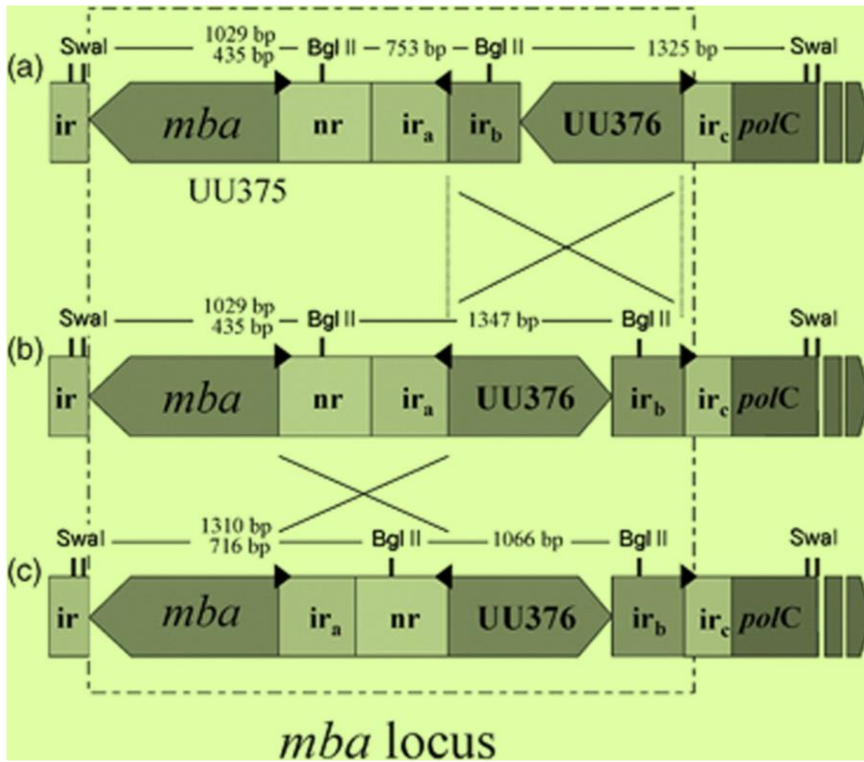

Figure 1.5 Schematic illustration of phase variation in Mycroplasma, and the possible DNA inversion events, within the *MBA* locus copied from (Zimmerman *et al.,* 2009).

This flexibility of their surface architecture might be due to lack of some regulatory genes found in more classical bacteria and used as a mean to avoid the immune response and allow host adaptation in spite of its minute genome (Yogev *et al.,* 2002; Silva and Latorre, 2008). Other mechanisms by which these unusual prokaryotes evolve to evade host immune response is by masking and unmasking constantly expressed molecules, some of which might be vital for *Mycoplasma* persistence and their adhesion and penetration of non–phagocytic cells (Rottem, 2003). Changes in protein length at the *Ureaplasma* surface due to modulation of C-terminal domains in lipoproteins is a common mechanism of antigenic size variation in *Mycoplasmas*. ON/OFF molecular switching (called phase variation) and size variation of surface lipoproteins are of two types; either based on spontaneous mutations in regions susceptible to DNA slippage by nucleotide insertion/deletion in a simple sequence repeats leading to stop codons, or on DNA rearrangements involving site-specific recombinase activity via a cut-and-paste mechanism. ON/OFF switch mechanisms modulate surface accessibility by masking and unmasking stably expressed components that are essential in host interaction and survival (Citti *et al.,* 2010; Wisniewski-Dyé and Vial, 2008; Yogev *et al.,* 2002). In *Ureaplasma*, the switching OFF of certain genes is matched with the switching ON of other genes within a gene family or locus. For example, a series of reports have shown site-specific DNA inversion events that change the direction of the *MBA* (UU375) gene promoter so that it drives the expression of the adjacent UU376 gene instead. Interaction of the putative tyrosine recombinases RipX (UU145), XerC (UU222), and CodV (UU529) of *Ureaplasma parvum* serovar 3 with specific DNA (Zimmerman *et al.,* 2013) is another example suggesting the MBA N-terminal paralogue UU172 being switched off leading to the alternating expression of the neighbouring conserved hypothetical ORF UU171.

1.2.5 The role of *Ureaplasma* species in human diseases

Ureaplasma are regarded as host-adapted and often as a commensal that mainly colonises the mucosal surfaces of the genitourinary and respiratory tract. However, their pathogenic potential is often underestimated. For example, *Ureaplasma* and *Mycoplasma* have been found to cause septic arthritis particularly in hypogammaglobuliaemic individuals, and the role for contributing to a poor clinical prognosis in ventilated preterm babies is well known. These patients are immunocompromised and *Ureaplasma* pathology in patients with AIDS also needs to be considered (Cordova and Cunha, 2000; Richard *et al.,* 1993, Izraeli *et al.,* 1991). Many physicians and microbiologists think of *Mycoplasmas* and *Ureaplasmas* as the last possible resort because the fastidious bacteriological media required cultivating them, left them undetected in clinical situations (DiGiulio *et al.,* 2008).

Ureaplasma spp. can be isolated from cervices or vagina of 40 -80% of sexually active asymptomatic women as a normal mucosal parasite and during pregnancy it increases to as high as 95% and decreases to 25% in menopausal women (Viscardi, 2010; Volgmann *et al.*, 2005), suggesting an interaction with the normal female cycle and hormone levels.

Healthy males harbour the infection with lower frequency (about 20-29%) in their lower genitourinary tract and these infections are associated with non-specific urethritis, infertility, prostatitis, pyelonephritis, epididymitis and renal stones (Andrade-Roch,2003; Taylor-R, 1986). In adolescents, the incidence is about 5% (Foy *et al.,* 1975). In women, *Ureaplasma* species can cause adverse pregnancy outcomes like spontaneous miscarriage, stillbirth, postpartum infection, chorioamnionitis, preterm labour, preterm rupture of membranes, and neonatal/perinatal infections (Witt *et al.,* 2005; Sanchez and Regan, 1987; Gibbs, 2001). Outside of pregnancy, *Ureaplasma* can cause gynaecological conditions including: pelvic inflammatory disease, infertility, and vaginal discharge (Taylor-R and Ronald, 1997; Elias *et al.,* 2005).

1.2.5.1 *Ureaplasma* spp. infection in neonates

Ureaplasma intrauterine infection is commonly associated with neonatal diseases and morbidity during the course of delivery including: congenital pneumonia, chronic lung diseases of prematurity (or bronchopulmonary dysplasia; BPD), otitis media, meningitis, opthalmia neonatorum, and even perinatal mortality (Viscardi, 2010; Waites, 2006; Prentice *et al.,* 1977). The rate of colonization was inversely correlated with gestational age; one study found that in infants younger than 28 weeks gestation, 80% were found to be colonized with *Ureaplasma*, in contrast to 17.9% of those who were 28–36 weeks of gestation (Izraeli *et al.,* 1991). Thirty percent of the infants colonized with *Ureaplasma* spp. developed chronic lung disease, compared to 8% of those not colonized who developed chronic lung disease (Sanchez and Regan, 1988).

1.2.5.2 Association of *Ureaplasma* respiratory tract colonization with chronic lung disease of prematurity

Chronic lung diseases of prematurity also recognised as bronchopulmonary dysplasia (BPD) is a mixed obstructive/restrictive respiratory disease of preterm infants. Multiple aspects contribute to BPD, and probably act together to stimulate lung injury (Viscardi *et al.,* 2002). The influence of neonatal *Ureaplasma* pulmonary colonisation on the development of BPD had been argued and its causal-association with BPD has changed since the introduction of mechanical ventilation, antepartum steroid, oxygen therapy and exogenous surfactant (Colaizy *et al*., 2007; Hannaford *et al.,* 1999). On the other hand, intrauterine infection, colonisation/ infection with *Ureaplasma* have been reported to protect premature infants against the development of respiratory distress syndrome (RDS) by inducing corticosteroid production. Maternal antibiotic treatment for intrauterine infections will not prevent BPD and not improve

the outcome. Diagnosis of *Ureaplasma* infection by culture alone may not be adequate as infants found negative by culture methods are sometimes found positive by PCR methods (Viscardi *et al.,* 2002).

1.2.5.3 Mechanisms and pathology of lung injury

Bronchopulmonary dysplasia is multifactorial and was first described as a lung injury in preterm infants resulting from oxygen toxicity and mechanical ventilation by Northway and colleagues in 1967 (Northway *et al.,* 1967) and has been characterised in new born animal models and in human stillbirth and full term human births (Johanson, *et al.,* 1982; Broström, 2010). The traditional diagnosis revealed that BPD was triggered primarily by oxidant- and ventilation-mediated injury, prior to the era of surfactant treatment, by inducing pro-inflammatory responses triggered by the injurious effect of mechanical ventilation (barotrauma) (Donn and Sinha,2006). Administration of high oxygen levels alone can arrest alveolar septation of lungs in the saccular stage of development. The higher the level of supplementary oxygen the preterm neonate is exposed to, the more persistent lung disease they will develop postnatally (Alan and Kallapur, 2010; Jobe and Bancalari, 2001). A review of studies showed that culturing of *Ureaplasma* from endotracheal aspirates of mechanically-ventilated infants was significantly associated with BPD development even after improvement of postnatal treatment with surfactant, lower oxygen levels and improvement of ventilators to remove barotrauma; however this still remains an area of contention (Abele-Horn *et al.,* 1998).

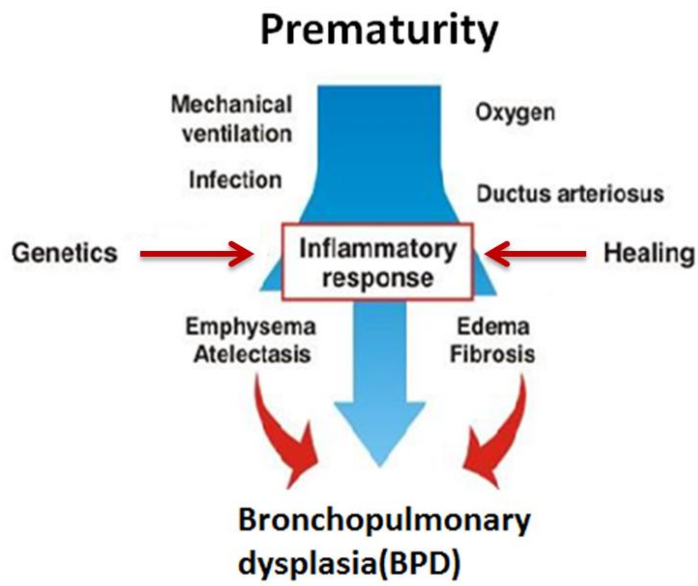

Figure 1.6 Pathogenesis of bronchopulmonary dysplasia, adapted from(Luciana *et al.,* 2005)

1.2.5.4 *Ureaplasma* infection and adverse pregnancy outcome

It is difficult to establish the role of *Ureaplasma* in diseases of the genital tract for many reasons; its high isolation rate in healthy asymptomatic individuals its fastidious requirement for transport and cultivation and the risky outcome associated with sample taking from the upper genital tract, nevertheless, during pregnancy ascending infection of this microorganism to the upper genital tract by unknown mechanisms may lead to adverse pregnancy outcomes including: chorioamnionitis, abortion, preterm labour, still birth, intrauterine growth restriction (IUGR),or resulting in low birth weight infants, premature rupture of membranes (PROM) (Gray *et al.,* 1992; Horowitz *et al.,* 1995; Kwak *et al.,* 2014), adverse assisted reproductive technology (Zhang *et al.,* 2014; Mändar *et al.,* 2005; Knox *et al.,* 2003) and post-partum and post-caesarean section wound infection (Maccato *et al.,* 1990; Newton, 2005) .

1.2.5.5 Experimental Evidence for Causative Role of *Ureaplasma* spp. in PTL from the Study of Non-Human Primates (NHP)

Chorioamnionitis or inflammation of the fetal membranes plays an important role as a precursor in the pathogenesis of premature rupture of the fetal membranes (PROM), or preterm labour (Gibbs *et al.,* 1992). The association of *Ureaplasma* with chorioamnionitis in preterm birth in humans is strongly manifested and is best outlined by the review by Viscardi (2010) (Viscardi, 2010). To prove this correlation, many experimental *in utero Ureaplasma* infections in animals have been carried out in recent years; animal models have been thoroughly developed and used in the study of intrauterine *Ureaplasma* infections, particularly in relation to infection associated inflammation and preterm birth. These models have been instrumental in the determination of the underlying relationship which exists between intrauterine infec-

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Ureaplasma species: role in diseases of prematurity.
Viscardi RM.

tions and preterm birth, and also the cellular mechanisms which drive such pathology. Utilising animal models in these studies allows for the control of a number of experimental variables which would simply be impossible to regulate in human studies, including maternal age and time of pregnancy. As a result, animal studies can be designed to address causal relationships between these exposures and outcomes Novy *et al*. (2009)inoculated pregnant rhesus monkey with *ureaplasma parvum* and demonstrated for the very first time that a causal relationship exists between chorioamnionitis and premature labour with *Ureaplasma* as a sole pathogen(Figure 1.7) (Novy *et al*., 2009).

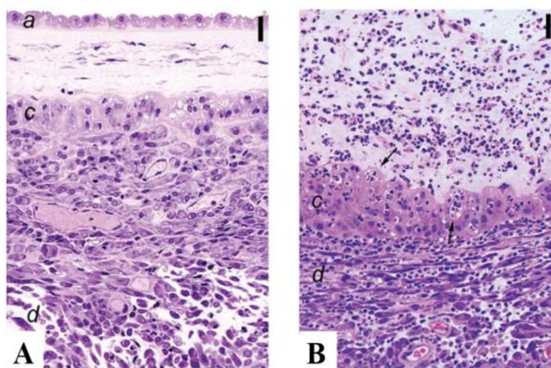

Figure 1.7 Microscopy displaying inflammation of the amnion, chorion and parietal decidua membranes of a pregnant Rhesus monkey following inoculation with *Ureaplasma parvum* (250x magnification, haematoxylin and eosin straining, scale bar = 25µm). a = Amnion, c = chorion, d = decidua (maternal membrane).A – Shows the membranes from a control primate, and demonstrates an intact foetal amnion epithelium with no leukocyte invasion. B – Shows the membranes at day 143 of gestation, and 7 days post-inoculation with *Ureaplasma parvum*. Demonstrates oedematous thickening of the membranes and leukocyte infiltration throughout the chorion, decidua and the neighbouring connective tissue. Arrows point to chorion trophoblast cells which display signs of necrosis and micro-abscess formation. Source: Novy *et al*. 2009.

Other examples of the experimental animal models used for intrauterine *Ureaplasma* infection are mice (Allam *et al*., 2014; Uchida *et al.,* 2013; Von Chamier *et al.,* 2012). *In utero* infection of CD-1 strain of mice at 13.5 days gestation resulted in established *Ureaplasma* infection, chorioamnionitis and elevated IL-1beta and MIP-2 at 17.5 days gestation. Of interest, age and species has an impact on *Ureaplasma* infection in mice: attempts to inoculate 8 week old CD-1 mice intratracheally failed, but succeeded in C3H/HeJ mice. Furthermore, while inoculation of *Ureaplasma* into the uterine horn of C57BL/6 and BALB/c mice resulted in equivalent placental infection and colonization rates, but greater numbers of BALB/c foetuses were infected and only BALB/c showed severe protracted chorioamnionitis with cellular necrosis with greatly elevated cytokines. The significant difference in response to intrauterine *Ureaplasma* infection was subsequently determined to correlate to increased syncytiotrophoblast expression of CD14/TLR2 (Allam *et al.,* 2014; Von Chamier *et al.,* 2012). Further studies using mice found that *in utero* injection at 15 days gestation of enriched fractions of the major surface protein of *Ureaplasma* (the multiple-banded antigen or MBA), or a synthesized diacylated lipopeptide derived from the 21 amino acids of MBA, into C3H/HeN mice showed that MBA alone was capable of causing increased fetal mortality or induction of preterm birth depending on the dose or purity of the MBA (Uchida *et al.,* 2013). Due to ethical considerations and the notorious behavioural difficulties of non-human primates, sheep models have gathered much interest in recent years. These have been used to demonstrate the link between intrauterine infection and preterm labour and neonatal consequences (Dando *et al.,* 2014; Knox, 2010; Moss *et al.,* 2005).

Table 1.2 Differences in pregnancy and parturition in animal models from human.

Species	Gestational Length (days)	Placentation	Fetus	Reproductive Biology	Advantages	Disadvantages
Mouse	18	Nonvillous hemochorial	Multiple fetuses; delayed myelination of cerebral white matter	Corpus luteum is source of progesterone; progesterone withdrawal precedes parturition	Inexpensive and readily available; genetic manipulationis possible (knockout variants are available)	Progesterone dependence; delayed cerebral maturation; short gestation
Rat	21	Nonvillous hemochorial	Multiple fetuses; delayed myelination of cerebral white matter	Corpus luteum is source of progesterone; progesterone withdrawal precedes parturition	Inexpensive and readily available	Progesterone dependence; delayed cerebral maturation; short gestation
Rabbit	31	Discoid hemoendothelial	Multiple fetuses	Corpus luteum is source of progesterone; progesterone withdrawal precedes parturition	Transcervical inoculation of infectious stimuli possible	Intermediate expense; progesterone dependence
Sheep	150	Cotyledonary syndesmochorial	Single fetus; myelination of cerebral white matter similar to that in humans; fetal growth similar to that in humans	Cortisol surge precedes parturition	Continuous access to intrauterine environment and myometrial contractility by chronic instrumentation	Expense; cortisol and progesterone dependence
Nonhuman primate	167	Discoid hemochorial	Most similar to humans; single fetus; myelination of cerebral white matter similar to that in humans; fetal growth similar to that in humans	Fetal HPA activation	Most similar to human parturition; continuous access to intrauterine environment and myometrial contractility by chronic instrumentation	Expense; limited availability
human	280 days	discoid hemochorial placentat	single fetus	fetal hypothalamic adrenal axis activation.		

Because sheep have a relatively long gestational period relative to mice (150 days versus 20 days), sheep models uniquely allow the study of chronic intrauterine infections. Furthermore, unlike the rodent placenta, both sheep and humans maintain pregnancy through the corpus luteal production of progesterone, further supporting the validity of using sheep to model human preterm birth. The much larger size of sheep also allows for longitudinal sampling of the amniotic fluid during pregnancy, providing a way to precisely determine the physiological changes which take place during infection. Beside these advantages, sheep can also be selectively bred for single pregnancies, can be catheterised long term, and the physiology underlying their parturition is well understood. Vitally, even after the intra amniotic injection of *Ureaplasma* species, pregnant sheep appear resistant to the induction of preterm birth through the production of oxytocin, allowing the maintenance of pregnancy to term despite significant amounts of uterine inflammation. Overall, sheep models are very well suited to the study of intrauterine infections and allow for the valid extrapolation of results to the human population.

1.3 Host response to intrauterine *Ureaplasma* infection (innate and adaptive)

The sterile amniotic fluid plays an important role in protecting the mother and the foetus. It has a wide range of anti-inflammatory innate and adaptive factors like immunoglobulins, lysozyme, cytokines, and antimicrobial peptides (like defensins, lactoferrin, and LPS-binding protein) (Underwood *et al.,* 2005). The fetal immunological responses vary from patient to patient and are dependent on timing, intensity, duration, and virulence of infection. An intact immune system is necessary for limiting the infection and prevents spreading of *Ureaplasma* beyond the mucosal surfaces. Normally the immune response to any immunological stimuli is mediated by both innate and adaptive arms of the immune system. Innate immunity is at the

forefront to fight microbial infections by stimulating trans membrane pathogen-recognising receptors like Toll-like receptor (TLR), mainly TLR1, 2, and 6 in case of *Ureaplasma* (Triantafilou *et al.,* 2013). These act as immune modulators and are expressed on epithelial and immune cells, both in the trophoblast and decidual cells. Following receptor engagement, signalling occurs through the nuclear factor NF-kB and results in up regulation of antimicrobial proteins and peptides, as well as stimulates production of immune-modulating cytokines and chemokines. These soluble mediators in turn recruit immune cells to the site of infection and alter their activation state and cellular function. Another key aspect of the innate immune system, the complement system plays a vital role in protecting the host against invading organisms including *Ureaplasma* by several mechanisms. Complement activation results in attachment of opsonins to the activating pathogen surface that facilitate engulfment by phagocytes, as well as enhancing the humoral response to the proteins bound to the opsonins (adjuvant effect) mediated through CR2 on B-cells. Complement activation also releases anaphylotoxins (C3a and C5a) which recruit innate and adaptive immune cells, as well as altering the immune cells activation state. Complement can also directly cause lysis of pathogens through formation of the terminal membrane complex. In this way, complement initiates inflammation and acts as an important link between the innate and adaptive immune systems through its receptors on B and T cells (Lynch *et al.,* 2008; Soto *et al.,* 2009; Sung, 2010). Many studies have shown higher concentrations of complement products C5a and C3a in the plasma of mothers with spontaneous preterm labour with evidence of microbial invasion of the amniotic cavity (MIAC) compared to those without MIAC (Soto *et al.,* 2009; Benstein *et al.,* 2004; Swierzko *et al.,* 2009). Complement activation can occur through three separate pathways, most commonly through binding of a specific IgG or IgM antibody to the pathogen (Beeton *et al.,* 2012; Webster *et al.,* 1988). Bredt *et al*., in 1977 proved that some components of *Mycoplasma pneumoniae* can activate the first component (C1) of the complement system

directly leading to complement system activation, rounding and killing of mycoplasma cells by guinea pig serum (GPS) and by a purified C1 after subsequent addition of C2, C4 and C-EDTA. In addition, *Ureaplasma* can bind to mannose-binding lectin (MBL) through terminal mannose residues (Benstein, 2004). Furthermore, addition of complement is known to enhance the growth inhibitory effect of specific antibodies to *Mycoplasma* (Webster *et al.,* 1988). The resultant inflammatory response is essential to overcome the pathogen evasion, to decrease tissue injury and to enhance regeneration. However, sometimes the immune response is a double-edged sword and over-activation of the innate and adaptive immune response is regarded as a negative predictor of fetal well-being. A number of emerging studies have showed a strong causal- association between *in utero* inflammation and adverse pregnancy outcome. The inappropriate and vigorous production of the inflammatory cytokines and chemokines was associated with apoptosis at maternal-fetal interface, resulting in tissue injury and fetal morbidity and mortality (Kemp *et al.,* 2010; Abrahams, 2008; Witkin *et al.,* 2011). Furthermore, excessive stimulation or inadequate regulation of complement can lead to leukocytes recruitment and increased release of potent inflammatory and anti-angiogenic mediators this in turn could lead to placental insufficiency, maternal endothelial dysfunction and pregnancy complications. Some reports indicate that phagocyte engulfed *Mycoplasma* can remain viable inside the neutrophil, which is inaccessible to circulating antibodies and disseminate the infection to different parts of the body (e.g. septic arthritis, septic osteomyelitis), however, this is most commonly associated with hypogammaglobulinaemic patients, suggesting that the presence of specific antibodies is capable of stopping systemic infection (Jorup-Rönström *et al.,* 1989). Several reports indicate that antibody alone can inhibit the growth of *Mycoplasmas in vitro* possibly by direct interference with their metabolism. This emphasizes the protective effect of mucosal antibodies against invasion (Webster *et al.,* 1988), and the importance of the *Ureaplasma* IgA proteinase in immune evasion. Activated NK cells have

also been reported to directly inhibit growth of *Mycoplasmas* by secreting IFN-γ through an unknown mechanism (Lai *et al.,* 1990).

1.3.1 Immunity in pregnancy

Pregnant women are an immunologically unique population; due to the presence of a paternal allograft (the foetus) requiring suppression of maternal immunity to tolerate the foetus and maintain pregnancy (Mor *et al.,* 2012; Zenclussen *et al.,* 2005).

During normal human pregnancy, the fetal/placental unit represent an additional immunological organ. The foetus has poor immunogenicity; the decidua on the other hand has no major histocompatibility complex (MHC) antigens which are responsible for variability within the same species and this will enable the trophoblast to invade the maternal territory (Manyonda, 2006). The placenta contains a high number of immune leukocytes, of which 70% are natural killer (NK) cells, 20–25% are macrophages and regulatory T cells (T reg) and 1.7% are dendritic cells. Regarding the adaptive immune system, B cells are absent, but T lymphocytes constitute about 3–10% of the decidua immune cells and its percentage rises during pregnancy in response to the developing fetal allograft (Moffett-King, 2002; Mor *et al.,* 2012). Therefore, the immune system in pregnancy is functional and reinforced by fetal/placental unit and not suppressed as it was thought before (Genbacev, 2011). Furthermore, it is a common misunderstanding that the newborn is immunologically naïve. The foetus can evolve an active immune response to invading pathogens which lead to a pathological condition known as fetal inflammatory response syndrome (FIRS). FIRS is characterised as in increase in fetal plasma IL-6 concentration > 11 ng/L. IL-6 is an acute phase cytokine and increased in response to infection and often associated with tissue damage. It also stimulates production of C-reactive

protein (CRP) by the liver and activates T and NK cells (Galinsky *et al.,* 2013; Romero *et al.,* 2007).

Additionally, neonatal human T cells differentiate early during human embryogenesis and flourish in response to antigens, including allergens, autoantigens and parasites. By 14 weeks of gestation, single positive thymocytes can be detected in the thymus, then CD8+ and CD4+ T lymphocytes migrate into the fetal liver and spleen (Helen, 2002). Many reports showed that there is significant level of IgM antibody in the serum of foetuses whose mothers received tetanus toxoid vaccine during pregnancy and excess IgE in serum of those born from mothers with *Ascaris* which indicate a specific fetal immune response as only IgG antibodies are actively transported across the placenta in humans (Holt and Jones, 2000; Mutwiri *et al*., 1999; Silverstein *et al*., 1963; Boggess *et al.,* 2005; Kane and Acquah, 2009).

1.3.2 Host defences in the preterm neonate

The mucosal barriers of preterm infants are known to be relatively deficient in function owing to the limited production of secretory IgA in response to invading pathogens, beside the immature innate and adaptive immune responses (Miller *et al*., 2003; Brandtzaeg *et al.,* 1991), deficiencies in complement components, defensins, fibronectin and cytokine response decreases their capacity to kill microbes. Furthermore, preterm infants have less chance to receive maternally derived immunoglobulin which is transported actively through the placenta reaching near maternal levels by 28 weeks gestation (Pearay, 2010; Jauniaux *et al.,* 1995). This contributes further to the relative immunodeficiency resulting in greater vulnerability of preterm neonates to even low virulent organisms including viral, fungal and some bacterial infections (Millar *et al.,* 2003; Eibl *et al.,* 1988). Moreover deficiencies in cellular phagocytosis decreases their capability for microbial killing rendering them more liable for systemic

spread of bacteria especially after colonisation of their respiratory tract mucosa (Miller, 1969; Maródi, 2006).

1.3.3 The Complement System

Complement acts immediately and lacks immunological memory. The complement cascade is regarded as the first line of defence particularly in neonates who have never been exposed to an antigen before. It involves a complex sequence of more than 30 soluble and membrane attached proteins distributed in the circulation, body fluids and on the surface of the cells. Once triggered, it acts sequentially to mediate an inflammatory response to protect the host against infection. The function of the complement system is not confined to the innate immunity only as it was thought before, in fact there is an interaction between the adaptive and innate wings of immunity .Complement acts as an adjuvant for the stimulation of adaptive T- and B-cell response and eventually in the generation of antibodies, by which, bridging innate and adaptive immunity basically through complement receptor type 2, CR2 (CD21), expressed by B cells. Deposition of complement opsonin fragments on the surface of pathogens assists in clearance of immune complexes and mediate phagocytosis by neutrophils and monocyte which express complement receptors (CR1,CR3, CR4) (Nielsen *et al.,*2000; Dunkelberger and Song,2010).Thus complement maintains a normal physiologic homeostasis by clearing apoptotic bodies, toxic cell debris, immune complexes and inflammatory products which can be otherwise directed against self-tissues thus preventing autoimmune and immune complex diseases (John *et al.*2002; Gasque *et al.*, 2000).

Experimental studies have shown that excessive complement activation at inappropriate sites or inadequate regulation of complement recruits leukocytes and releases potent inflammatory and anti-angiogenic mediators. These can contribute to placental insufficiency which is the

basis of many pathological conditions complicating pregnancy, such as pre-eclamptic toxaemia (PET) and intra uterine growth restriction (IUGR) (Gasque *et al.,* 2000; Carroll, 2004; Sahu and Lambris, 2001; Lynch *et al.,*2011).

1.3.3.1 Pathways of complement activation

Three main pathways; the classical lectin and alternative pathway. They differ from each other by their triggering factors for activation. The three pathways converge at the generation of a C3 convertase which cleaves C3 (the most abundant complement component) into C3a and C3b. The latter covalently binds to the activating surface and acts as an opsonin by facilitating engulfment by phagocytic cells. A second molecule of C3b binds to the C3 convertase to produce the C5 convertase which cleaves C5 into C5a and C5b. C5b is the first component of the membrane attack complex (MAC) and associates with membranes at the site of activation. Sequential additional binding of components C6, C7 and C8 allow insertion of the complex into the membrane. Additional binding of multiple molecules of C9 then completes a lytic pore in the membrane called the membrane attack complex (MAC). Formation of large numbers of MAC on a membranous surface disrupts the osmotic balance of cells and leads to cell lysis or death (Cosmin *et al.*, 2011; Janeway *et al*., x2001).

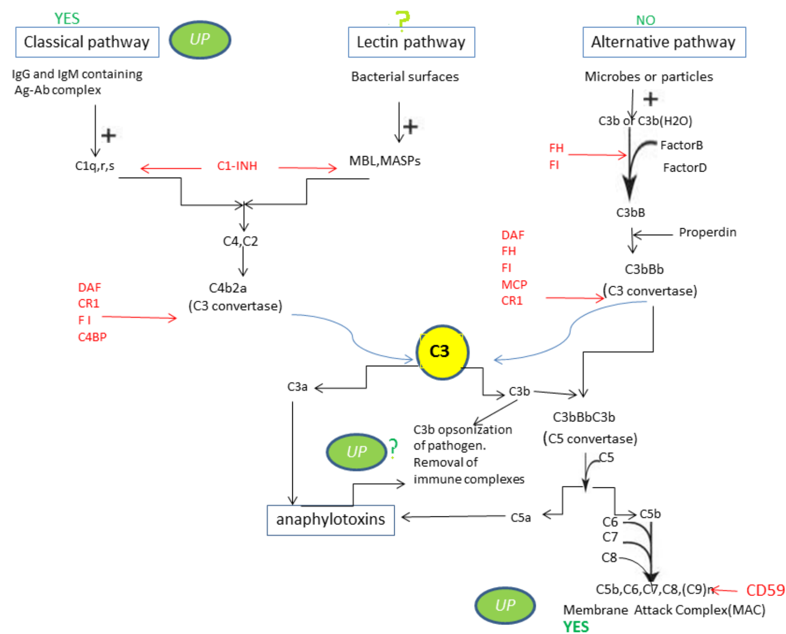

Figure 1.8 Activation of the complement system via three different pathways (the classical, lectin and alternative pathways), which are activated differently; the classical pathway via Ab/Ag complex; the MBL of the lectin pathway is activated by binding to pathogen specific carbohydrates; the alternative pathway activated by spontaneous hydrolysis of C3. However, they all converge at the cleavage of a central C3 component by C3 convertases to generate the same set of complement effector molecules, which play roles in opsonization, recruitment of host effector cells and direct killing of invading pathogens via MAC .Complement regulators are indicated by red lettering. Predicted method of Ureaplasma (UP) killing by the complement also shown on the diagram indicated in green.

1.3.3.1.1 The classical pathway

The complement protein C1q binds to the Fc portion of IgM or IgG deposited on the surface of the pathogen. Interaction with a single bound IgM or multiple bound IgG in close proximity of the pathogen leads to conformational changes in C1 which cleave C1r and C1s into their active form so they can cleave C4 and C2. C4a and C2b are small fragments that are released while freshly activated C4b binds covalently to the activating surface and binds to C2a (likely as un cleaved C2 which is then further processed by C1 to generate the C4b2a complex which is called C3 convertase (named because it cleaves C3 into C3a and C3b). This complex is inherently unstable and requires calcium to retain a short lived enzymatic complex. The physiological degradation of this complex forms part of the control for activation of complement.

1.3.3.1.2 The Alternative Pathway

This arm of the complement system is activated by the spontaneous low-level hydrophilic attack of C3. $C3_{H2O}$ can initiate a fluid phase C3 convertase by forming a complex with factor B that is subsequently cleaved by factor D into Ba and Bb (Fig.1.8). Properdin (P) binds to C3 convertases to stabilize it and increases its half-life (Müller-Eberhard and Schreiber, 1980). Freshly cleaved C3b, like C4b forms covalent bonds with proteins or carbohydrates in the near vicinity and generate surface bound, more stable alternative C3 convertases (C3bBb). However, host cells rapidly degrade C3b into inactive fragments utilising surface bound factor H, therefore, only non-host surfaces without regulators can establish an active alternative C3 convertase. This pathway is also used by classical and lectin pathways to create a positive amplification loop as C3b generated by other pathways can bind factor B (Fig.1.8). C3bBb

can bind an additional C3b molecule and create a C5 convertase leading to the formation of the MAC (Sarma and Ward, 2011).

1.3.3.1.3 The mannose-binding (lectin) pathway

Mannan-binding lectins (MBL) or ficolins in the serum bind to non-host conformations of complex polysaccharide common to bacteria. Similar to C1q, the bound lectin alters conformation and binds to an MBL-associated serine protease (MASP-1, MASP-2 and MASP-3 have been described), which can then cleave C4 and C2, identically to the classical pathway. Therefore, the lectin C3 convertase (C4b2a) is indistinguishable from the classical C3 convertase (Fujita, 2002; Teizo *et al.,* 2004).

1.3.3.1.4 The terminal complement complex (TCC)

The C5 convertases in both the classical and alternative pathways cleave C5 to produce C5a and C5b. Thereafter, C5b sequentially binds to C6, C7, and C8 to form C5b-8 that catalyses the polymerization of up to 12 C9 molecules to form the MAC that inserts into target membranes and causes Ca2+-dependent (and hypotonic) acute cell death (Podack and Tschopp 1982). While, erythrocytes lyse quickly by even one MAC in the cell membrane, in nucleated cells, deposition of limited C5b-9 complexes channels may be sub-lytic due to ability of some mammalian cells to repair their membrane rapidly (Telga *et al*., 2011).

The biological functions of complement are mediated through the production of activation fragments which initiate as well as amplify inflammation (Lynch *et al.,* 2008). Concentrations of the smaller complement cleavage products or anaphylatoxins (C3a, C4a, and C5a) in biological fluids reflect the magnitude of complement activation. A study by Soto *et al.* showed

supporting evidence that preterm labour is associated with complement activation in the amniotic fluid determined by ELIZA measurement of plasma concentrations of fragment Bb (fBb) (Soto *et al.,* 2009).

However, humans with acquired or inherited complement proteins deficiencies are more susceptible infections and PTL (Ram et al., 2012). Many studies reported a Link between immunological disorders and increased risk of in utero infections and recurrent spontaneous abortion. Kilpatrick et al. (1995) reported that 16% of women with low MBL levels had preterm labour (Kilpatrick et al., 1995). Swierzkoa et al., in 2009 found an association between relative deficiency in l-ficolin with prematurity, low birth weight and infections (Swierzkoa, et al., 2009). Mycoplasma pneumoniae is an important and sometimes overlooked pathogen causing infections of the respiratory tract in patient with X-linked agammaglobulinaemia and Common variable immunodeficiency (CVID) (Beeton et al., 2012; Franz et al., 1997).

1.3.4 Host tissue protection against complement lysis

As the process of C3b and C4b covalent attachment is essentially non-specific (proximity being the only influencing factor), the host cells need to be able to eliminate or dampen bystander damage at sites of inflammation. Human cells continuously express a series of complement regulatory molecules which protect all cells exposed to plasma or other biological fluids to resist the continuous low-level complement attack and to direct complement deposition to the surface of pathogen (Gasque, 2004). There are four important cell-membrane complement regulatory glycoproteins: membrane cofactor protein (MCP; CD46), decay-accelerating factor (DAF: CD55), CD59 and complement receptor type 1 (CR1; CD35). DAF and CR1 act by accelerating the decay of the C3 convertases, MCP and CR1 bind to C3b and recruit serum protease factor I to cleave it to inactive fragments, CR1 can additionally induce

factor I cleavage of C4b. CD59 on the other hand binds to the incomplete complex C5b678 and blocks the binding of C9 and the formation of the lytic pore. Complement regulators vary in their relative importance, CD59 and DAF appear to be the most widely and highly expressed regulators on cells allowing them to survive complement-induced lysis, and their expression noticeably increases in inflammation and cancer (Varsano *et al.,* 1995). MCP is expressed on many tissues except erythrocytes, while CR1 is mainly expressed on erythrocytes and thought to aid processing of circulating immune complexes. In order to achieve maximum protection, control is required both at the level of the C3/C5 convertase and at the level of the MAC. Another circulating complement regulator is C1 inhibitor which covalently binds to activated C1r and C1s to neutralise them. Factor I, as previously described, can cleave surface bound C3b into its inactive forms iC3b and C3dg but it has very low activity unless C3b is bound by cell surface regulators CR1 or MCP, or fluid phase factor H. Similarly, it can cleave C4b into its inactive fragment C4d in the presence of cell surface bound CR1 or fluid phase C4b-binding protein (C4bp). These regulatory proteins are lacking on the surface of pathogens, but have been reported to be recruited by some (Funabashi *et al.,* 1994; Jones *et al.,* 1994).

1.3.4.1 The therapeutic role of complement regulators

Complement regulatory proteins or complement inhibitors are normally expressed on cells to protect them from bystander attack by activated complement. Any imbalance in the mechanism that regulates cell-surface complement or damage to these protective membrane proteins, such as after ischemia reperfusion injuries I/R, may allow direct complement–induced host tissue damage or precipitate inappropriate immune cell recruitment by liberating biologically active anaphylatoxins (Arumugam *et al.,* 2004; Mollnes *et al.,* 2002). Hypoxic/reoxygenated human endothelial cells in particular can be injured by a marked recruitment of neutrophils to

the site of inflammation with superoxide production (Monsinjon *et al.,* 2001; Collard *et al.,* 2000). Abnormal expression of these complement-regulatory proteins like CD55, CD46 and CD59 may result in complement-mediated and autoimmune diseases. Exogenous administration of recombinant soluble therapeutic forms of complement regulators have been examined *in vitro* as well as in animal models. In particular addition of these synthesized soluble forms have been found to prevent complement –induced damage to host cells following reperfusion injury and increase tolerance of transplanted organs (Makrides *et al.,* 1998; Kalli *et al.,* 1994). Many experiments in molecular biology, physiology and biochemistry have been tried to find powerful synthetic complement inhibitors or modulators to use as therapeutic tool for complement-associated diseases. Some of these therapies are available in for treatment, some are in clinical trials, and others are still in pre-clinical development. In contrast to the novel role of these proteins in protecting normal tissues from accidental injury by activated complement components, it can provide cancer cells with some resistance. For example, CD59 appears to be the most effective mCRP protecting tumour cells from complement-mediated lysis (Fishelson *et .al.,* 2003), trials to use neutralising antibodies targeted specifically the membrane complement regulators (mCRP) on cancer cells in combination with anticancer complement-fixing antibodies treatment hopefully will improve the therapeutic efficacy by increasing tumour cell susceptibility to *in situ* complement deposition . DAF, MCP, and CD59 expressed on the glomeruli protect them against immune-mediated renal injury, hence, any change in their expression levels can result various glomerular disorders. Promising results have been published for several animal experiments administering soluble recombinant complement regulatory proteins and trials for overexpressing complement regulatory proteins locally on the glomeruli are hoped to be future therapeutic approaches (Nangaku, 1998). Similarly, in ovarian cancer, experiments showed that the use of antibody neutralization of CD59 expressed by the ovarian

tumour significantly increased the susceptibility of the cells to complement-mediated lysis (BJØRGE, *et al.,* 1997).

In 1974, Kavanagh *et al.,* submitted evidence for the pathogenic role of the alternative pathway of complement following mutations in the complement regulatory proteins factor H, MCP protein (CD46), and factor I which predispose to development of Haemolytic Uremic Syndrome (HUS) (Kavanagh *et al.,* 1974). Some clinical inhibitors are well known like Eculizumab (trade name Soliris) which is a humanized monoclonal antibody that blocks the cleavage of C5 and blocks the terminal pathway, approved to be effective in treating atypical HUS and paroxysmal nocturnal haemoglobinuria (PNH) (Hellman *et al.,*2006; Rother *et.al.,*2007) and in other diseases such as after kidney transplantation to decrease the chance of rejection (as reviewed on Alexion Pharmaceuticals website: http://alxn.com/research-development/alexion-research/soliri). Another pharmaceutical monoclonal antibody is Pexelizumab, which also blocks C5 cleavage and has been found to reduces the risk of death in patients undergoing coronary artery bypass grafting (Testa et al., 2008).

Commented [Brad Spil3]: Pexelizumab in ischemic heart disease: a systematic review and meta-analysis on 15,196 patients.
Testa L, Van Gaal WJ, Bhindi R, Biondi-Zoccai GG, Abbate A, Agostoni P, Porto I, Andreotti F, Crea F, Banning AP.
J Thorac Cardiovasc Surg. 2008 Oct;136(4):884-93. doi: 10.1016/j.jtcvs.2007.12.062. Epub 2008 Jun 18. Review.
PMID:
18954626

Also a small, natural, broad-acting complement inhibitory protein (termed OmCI) was extracted from the soft tick *Ornithodoros moubata*. This native 17-kDa non glycosylated protein inhibits classical and alternative complement pathways activation of both human and guinea pig targeting C5 itself and complement terminal pathway (Nunn *et al.,* 2005).

However, in my model I used a recombinant form of a native blocker of complement components, that is the soluble Complement Receptor type 1(sCR1) which has the ability to block both the classical and the alternative pathway by binding to C3b, enhancing cleavage of C3b dimers by factor I, it also inactivates C3 and C5 convertases assembled on pathogen membrane. In 1997, a study by Klickstein showed that in humans, C1q can bind specifically to CR1, therefore all three complement opsonins C3a,C5a and C1q could be recognised by sCR1

(Klickstein *et.al.,* 1997). Nevertheless, sCR1 has a disadvantage as it inhibits important anti-bacterial complement products like C3b and C3 opsonic product which limits its therapeutic application (Ross and Densen, 1984). Some mutant versions have been created to be used as an alternative to sCR1 with fewer side effects (Scesney *et al.,* 1996). Wild type CR1 is composed of 35 extracellular repeat domains and a trans-membrane domain. Soluble versions containing the entire 35 extracellular repeat domains (TP10 or CDX-1135), or versions only containing the 3 N-terminal repeats (APT070) have been examined. It was used by Weisman *et al*. in 1990 in rats to decrease the ischemic /reperfusion injury by 44%. They noted a significant decrease in both C5a levels and MAC deposition, indicating that it reduced both direct and indirect routes of tissue damage. Most of the early work has focused on a cardio protective effect in animal models of ischemia/reperfusion injuries (Homeister and Lucchesi, 1994; Weisman *et al.,* 1990); however, in theory it should be able to diminish any complement-mediated injury.

Aims

1- To characterise complement activity in preterm lambs sera compared to matched maternal sera by creation of a novel haemolytic assay.

2- To characterise complement killing by preterm fetal sera compared to the matched maternal sera using an established *Ureaplasma* bactericidal assay.

3- To manipulate the *Ureaplasma* bactericidal assay to determine the activation pathway and rate of killing, comparing different gestational age lamb and adult sera.

4- To examine systemic *Ureaplasma* infection in lambs of different gestational ages.

5- To evaluate the physiological and physical alterations in *Ureaplasma* with increasing length of *in vivo* infection.

6- To investigate genomic changes in *Ureaplasma* after chronic intrauterine infection.

Hypothesis

The ability of the infecting strain of *Ureaplasma* to adapt to the ovine immune system for extended durations in spite of its extreme sensitivity to fetal and adult serum is related to co-incident alterations in the major surface antigen (multiple banded antigen; MBA) and alterations in the whole genome sequence in order to enable *Ureaplasma* to escape the evolving fetal immune system.

CHAPTER 2

MATERIALS AND METHODS

2 Materials and Methods

2.1 Ureaplasma

2.1.1 Source of Ureaplasma isolation

Labouratory samples of *Ureaplasma parvum (Achilias, Roupakias et al.)* serovar 3 (HPA5), SV1 (DFK1), SV6 (HPA2), SV14 (HPA32) were kindly provided by Dr. Victoria Chalker from the Health Protection Agency (HPA; Colindale, London, UK) currently known as Public Health England (PHE). Measurement of *Ureaplasma* isolated from experimentally HPA5-strain infected animals were from sheep samples (serum, amniotic fluid and tissue) collected from date-mated singleton pregnant ewes housed and acclimatised in a purpose built facility at the University of Western Australia. Samples were given ID numbering and sent in dry ice boxes to Cardiff. All prototype serovar reference strains available from the American Type Culture Collection (ATCC) were received in my institution from original sources: the Institute of Medical Microbiology, University of Arthus, Denmark or from Prof. Janet Robertson (University of Calgary, Canada). The complete genome sequence of *U. parvum* serovar 3 (ATCC strain 700790) was published in 2000, which is available in GenBank under accession no. NC_002162, (Glass, 2000). Likewise, the full genome sequence of *U.urealyticum* serovar 10 (ATCC 33699 strain) is also accessible in the GenBank (accession no. NC_011374). All strains were aliquoted and stored as stocks at -80 ˚C freezers until used for analysis.

Commented [Brad Spil4]: This has nothing to do with Ureaplasma isolation why is it here?

2.1.2 Culture media of *Ureaplasma*

Trials to find the best media for growth of *Ureaplasma* was found to be the commercial *Ureaplasma* selective medium (USM; Mycoplasma experience ltd., Surrey, UK), using standard techniques as previously published (Beeton, Daha *et al.* 2012). This medium is made up of a simple broth base medium complemented with yeast extract 2.5 g/L, urea 1 g/L, and 10% heat-inactivated porcine serum, with a pH indicator (phenol red), medium pH= 6.65, Amphotericin B 2.5 µg/ml and Ampicillin 0.25 mg/ml. One hundred and eighty microlitres of USM was loaded into each well of the first column of 96 well flat- bottom plates then 20 µl of stock growth or fresh clinical samples were inoculated into the first well in the column to give an initial dilution of 1:10 from which 20 µl was titrated down to a dilution of 10^{-7} leaving the last well as a negative control. Plates were covered with clear air tight adhesive sealing tape (Elkay, Basingstoke). For Western blot analysis the last positive well was scaled up in 7 ml bijoux tubes (Elkay, Basingstoke). All incubations were performed in a humidified tissue culture incubator set at 37°C, with ambient CO_2 concentration overnight. Non-turbid colour changes of the broth from orange-yellow (negative) to reddish-pink (positive) indicated *Ureaplasma* growth (Shepard and Lunceford, 1976; Shepard and Lunceford, 1978; Stemke and Robertson, 1982). Sub culturing was performed at 24 hours; whereas titrations were allowed to proceed for 48 hrs to reach completion as colour changing unit /ml are the quantification measurement of *Ureaplasmas.* Change in colour of the medium from yellow to red is due to liberation of ammonium ions and production of $NH4^+$ causes a pH rise from 6.5 to > 9, detected by the pH indicator in the medium *Ureaplasma* cultures never reach turbidity, unless the culture get contaminated, due to self-toxicity caused by ammonium ion accumulation.

2.1.3 Quantification of *Ureaplasma* growth

To determine the number of colour changing units (CCU) of *Ureaplasma* per millilitre of sample fluid which is directly correlated to colony-forming units (CFUs) on agar plates, *Ureaplasma* titres were quantified by serial 10-fold dilution (or 2-fold dilution for killing assay) in USM, in triplicate using the methods outlined in the previous section. Colony-forming units were quantified for comparison by inoculating *Ureaplasma* selective agar plates (Mycoplasma experience) with 5-µl drops of the 1:10, 1:100, and 1:1000 dilutions the plates were then incubated in atmospheric CO_2 at 37°C for 48–72 hrs; subsequently, colonies were counted under x4 and x10 objective lenses using a stereo microscope (Leica Microsystems, North Ryde, NSW, Australia) to count colony forming units per ml of broth. Purification of single colonies was also performed on these plates by scraping a single colony by microscope guided sterile 10 µl tip and inoculating in 100 µl USM overnight. Strains for sequencing were colony purified three consecutive times (triple-cloned).

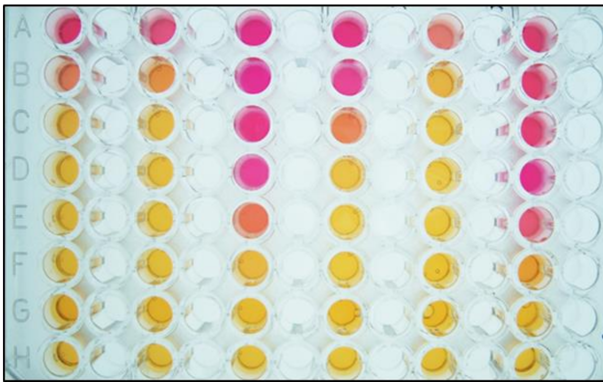

Figure 2.1 96 well plate displaying the colour change which occurs during *Ureaplasma* culturing. Following serial dilution and incubation for 24 hours, this plate shows the differential growth patterns exhibited by 6 different *U.Parvum* SV3 strains. A pink colour indicates shift toward alkaline PH. By 48 hour, all positive wells will be dark pink.

2.1.4 Scaling up of *Ureaplasma* growth

Following culturing of *Ureaplasma* isolates from frozen stock, the final well was added to 5 ml USM in a 7 ml bijou. If this were to be used for Western blot analysis, then it was allowed to incubate at 37°C for 48 hours. If it was to be scaled up for genome sequencing, it was incubated for 8 hours prior to addition to 250 ml of USM prior to 48 hour incubation at 37°C. Five ml cultures were spun in 4 sterile 1.5 ml Eppendorf tubes for 20 min at maximum speed (13,500 *xg*) in a bench top centrifuge, while 250 ml cultures were spun at 13,000 *xg* for 3 hour in sterile polycarbonate bottles. Pellets were washed with 1x sterile phosphate-buffered saline (PBS), and then pooled into a single 1.5ml centrifuge tube, centrifuged at 13,500 *xg* for 30 mins. For Western blot, pellets were washed 3 times prior to preparation for separation on polyacrylamide gel. Pellets for genomic sequencing were extracted using DNeasy Blood & Tissue Kit (Qiagen, UK) following manufacturer's instructions, which resulted in an elute of 400 µl in elution buffer. This was concentrated by speed vac in Central Biotechnology Services to 50 µl or less prior to submission to PHE for sequencing.

2.1.5 Quantification of proteins

Pellets for Western blot were solubilised in 50 µl of 1% SDS in PBS prior to using the Quanti-Pro™ BCA Assay kit (Sigma- Aldrich Company). A standard curve was obtained from a dilution series of standard bovine serum albumin (BSA; Sigma Aldrich). The assay was set up in a 96-well plate following manufacturer's instructions and colorimetric analysis performed using a Dynex plate-reader and protein concentration calculated using the quadratic curve fitting program and the Dynex MRX Revelation internal software. Sample and control dilutions were incubated for 2 hours at 37°C. Purple-blue colour is caused by the reduction of

CU^{+2} into Cu^{+1} mediated by the protein and buffers, measured by absorbance at 562 nm (Walker, 2009).

2.2 Sheep Experiments

2.2.1 Animal ethics

All experimental sheep procedures were performed in Perth, Australia following approval by the University of Western Australia Animal Ethics Committee (RA/3/100/1289) to use the animals for scientific purposes.

2.2.2 Animals and Sample Collection

In these experiments date-mated merino-cross ewes (Ovis aries) carrying singleton pregnancies were used as a model to replicate intrauterine infection and follow the pathological changes of our lab reference strain of *U.parvum* serovar 3 (HPA5).

Phase one experiments were started in July 2012. These experiments utilised 6 pregnant sheep with catheter implantation into the foetuses and 6 pregnant sheep where ultrasound guided needle injection were utilised to deliver the experimental infection. Strain HPA5 (SV3) was thawed on ice and titrated in USM media then (10^7) of fresh infectious units/ml were injected in all cases.

Catheterised lambs: 6 date-mated ewes with singleton foetuses at 120d gestational age (GA) had aseptic recovery surgery to install catheters into the fetal jugular vein, trachea and two catheters into the amniotic fluid as described previously (Kemp *et al.,* 2013). After a 24h recovery, animals were experimentally infected through amniotic fluid catheter "A". Using catheter "B", amniotic fluid was serially sampled immediately before and 2, 6, 12, 24 and 48h

Commented [Brad Spil5]: KEMP MW, MUSK GC, SAITO M. Chapter 35 - Animal Models for the Study of Infection-Associated Preterm Birth. In: Conn PM, ed. *Animal Models for the Study of Human Disease*. Boston: Academic Press, 2013.

after infection. Animals were euthanized with an intravenous bolus (100mg/kg) of pentobarbitone at 120 h. Fetal cord blood, maternal venous blood and amniotic fluid were collected at delivery and fetal tissues were snap frozen in liquid nitrogen for subsequent analysis.

Non-catheterised lambs: 6 date-mated ewes with singleton foetuses at 119d GA were injected by ultrasound guided needle and infection allowed proceeding for 1 week, when animals were euthanized as above. Fetal cord blood, maternal venous blood and amniotic fluid were collected at delivery and fetal tissues were snap frozen in liquid nitrogen for subsequent analysis.

Phase two experiments were started in July 2013. For these experiments, ultrasound guided needle injection into the amniotic fluid was used to experimentally infect the animals with the same strain (HPA5) at a much earlier GA. Eight date-mated merino-cross ewes carrying singleton pregnancies received a single intra amniotic (IA) injection of 10^7 CCU *Ureaplasma* at 83 d GA. Ultrasound guided amniocentesis samples were taken from these animals at 3 and 5 weeks post-infection. Animals were euthanized with an intravenous bolus (100mg/kg) of pentobarbitone at 6 weeks post-infection (125 d GA). Fetal cord blood, maternal venous blood and amniotic fluid were collected at delivery and fetal tissues were snap frozen in liquid nitrogen for subsequent analysis.

Phase three experiments were started in July 2014. For these experiments, ultrasound guided needle injection into the amniotic fluid was used to experimentally infect the animals with HPA5 for 10 days at 116 d GA or for 24 days at 70 d GA (N=8 per group), so that delivery was performed at 125 d GA (as for all experiments above) and 94 d GA (earlier than all previous experiments). Again animals were euthanized with an intravenous bolus (100mg/kg) of pentobarbitone at the appropriate GA for the foetus and delivered by Caesarean section. Fetal cord blood, maternal venous blood and amniotic fluid were collected at delivery and fetal tissues were snap frozen in liquid nitrogen for subsequent analysis.

Control animals: For each of the experiments above there were 4 to 6 singleton pregnancies run in parallel and instead of *Ureaplasma*, these animals received an injection of sterile saline. Samples were collected as for the infected animals and these are used as comparative controls. Sera were also obtained from 3 non-pregnant, non-infected sheep from TCS Bioscience by peripheral venepuncture of Merino cross to be used as non-immune sheep sera.

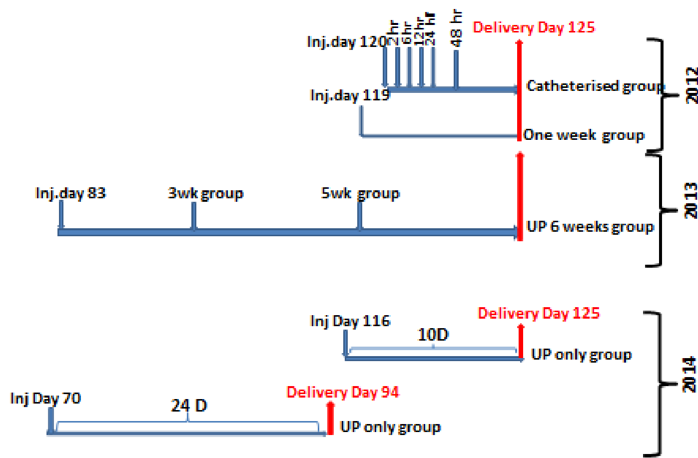

Figure 2.2 Diagram showing the Study design

2.3 Complement Assay

2.3.1 Preparing serum for complement studies

Sera to be tested had been collected into Becton Dickinson serum separator tubes and allowed to clot for 10 min at room temperature, then kept on ice until they could be centrifuged at 3000 *xg* for 10 min and the sera aliquoted into 400 microliter aliquots in sterile Eppendorf tubes, which were stored at -80°C prior to use. Sera was only thawed once and used immediately prior to each assay. The heat–inactivated serum (HI) was prepared by heating the serum aliquots at 56◦C for 30 min; this will inactivate C2 component of the complement system on which both the classical and the lectin pathway depend on for their activation. Heating also inactivate factor B which is necessary for alternative pathway activation.

2.3.2 Serum bactericidal assay

Serum bactericidal assay, or serum killing, was performed as described previously by Beeton *et al.* (Beeton *et al.,* 2012). For killing assays, log-phase growth *Ureaplasma* was diluted to 1:10 in USM and set up in v-shaped bottom 96 well plates in triplicate for each serum dilution or control and centrifuged at 3,600 *xg* for 10 min to pellet bacteria. All supernatant was removed and pellets re suspended in serum diluted in complement fixation buffer (CFB; Oxoid ltd., Basingstoke, UK) and incubated at 37°C. Following incubation, serum was removed by centrifugation and the bacterial pellet re suspended in USM prior to titration of each replicate individually to quantify the surviving *Ureaplasma*, as previously published (Beeton *et al*., 2012). For some experiments, serum concentration was varied, while for other experiments the length of incubation with serum was varied. For those experiments examining fixed incubation times, 10 mM EDTA (final concentration) was added to block further complement activation and the serum removed by centrifugation and re suspension in USM prior to titration of surviving bacteria. Serum killing was calculated as the fold decrease relative to the

titration of surviving bacteria following identical exposure to the same sera, except that all complement activity had been removed by heat-inactivation at 56◦C prior to the experiment. All killing assays were repeated three times to avoid bias.

To disable the calcium-dependent classical and lectin pathways (LP) but allow the alternative pathway (AP) to stay intact, the serum was diluted in EGTA buffer (CFB supplemented with 10mM EGTA and 10mM Mg^{+2}). Using this buffer the calcium ions were differentially chelated from serum, while the added Mg^{+2} enhanced the assembly of C3bBb keeping the activity of the alternative pathway intact.

Additionally, *Ureaplasma* populations were subjected to continuous immunological pressure by transferring the surviving bacterial growth from the last well serially through broth containing 1:100 dilution of rabbit polyclonal antibodies against serovar 3 (Gift from Janet Robertson, University of Calgary), to test if there is any possibility of phase variation of MBA *in vitro*. After each attack 20 µl of the surviving bacteria were collected and stored at -80 °C to be analysed by Western blot and/or PCR.

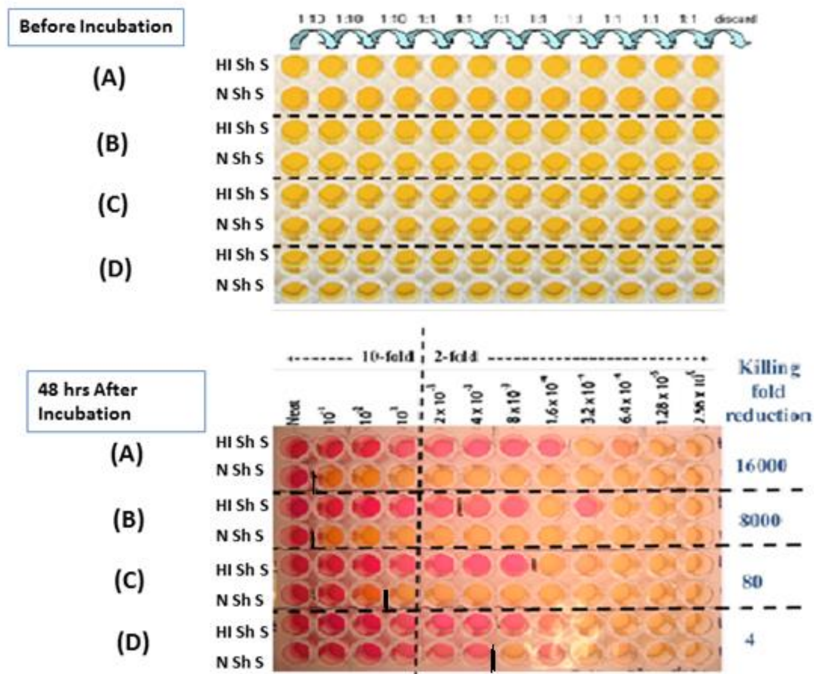

Figure 2.3 Schematic diagram of the 96-well plate set up for complement killing assay for four representative sheep sera (A - D) under two conditions: 50% heat inactivated sheep serum (HI-ShS) and 50% normal sheep serum (NShS). 20µl of *Ureaplasma* following incubation with sera was added to the first well of each column to a dilution of 1:10. This was repeated for the next 2 wells to give a final dilution of 1:1000, surviving *Ureaplasma* were then diluted by 2-fold dilution (equal volume for the rest of the plate. This was then allowed to incubate for 48 hr at 37°C, resulting in the plate at the bottom. Red wells represent growth and the comparative growth between N and HI allows calculation of killing as shown.

2.3.3 Pathways responsible for non-immune sheep serum killing

2.3.3.1 Classical pathway (CP), depletion of sheep serum Igs

One ml of ice cold sheep serum was pushed by a syringe through a HiTrap™ Protein A-sepharose column (GE Healthcare, Slough) and then flushed through with ice cold CFB into a collecting tube to elute all Igs from sheep serum. The depleted samples were collected in 1.5 ml Eppendorf tubes and an aliquot tested by Western blot to check for complete depletion from Igs and the rest was stored in -80◦ C for future use in serum killing assays.

2.3.3.2 MBL depletion

In order to deplete lectins from the sheep serum, one ml of ice cold sheep serum was circulated several times through a mannan-agarose column on ice (Sigma-Aldrich) and then collected in sterile Eppendorf tubes. There are no reagents available to test the depletion of MBL or other lectins from sheep sera, however, the samples were compared for their capacity to kill *Ureaplasma* relative to un-depleted sera.

2.3.3.3 Alternative pathway

Killing assays were performed following dilution of the sera in 10mM ethylene glycol tetra acetic acid (EGTA) and 10mM Mg^{+2}, so that calcium ions which is necessary for activation of the classical and lectin pathway were chelated allowing only the alternative pathway to function. This makes presumptions about the cation requirement of complement pathways based on other species; however, is standard practice for isolating the alternative pathway.

2.3.4 Measurement of complement activity

2.3.4.1 Antibodies and erythrocytes

Guinea pig, chicken, sheep and rabbit erythrocytes were purchased from TCS Biosciences (Oxford, UK). Human erythrocytes were obtained from consented healthy volunteers by phlebotomy (approved by the Cardiff University School of Medicine Research Ethics committee) and coagulation was inhibited by the addition of 100 U of heparin sulphate. Sheep serum from non-pregnant sheep was obtained from TCS Biosciences, whereas maternal serum from pregnant sheep was obtained by peripheral venepuncture of Merino cross ewes and fetal cord blood was obtained from their lambs during Caesarean delivery at 85, 95 and 125 days of gestational age.

Table 2.1 Type and source of reagents used in modified complement haemolysis test

Type of reagent	Source
Rabbit polyclonal anti-guinea pig and anti-chicken erythrocyte antibodies	purchased from Fitzgerald Industries International (North Acton, MA).
Rabbit polyclonal anti-human CD55 and anti-CD59	were provided by Prof. B. Paul Morgan (Cardiff University, UK)
Rabbit polyclonal anti-sheep erythrocyte antiserum (Amboceptor)	provided by Institut Virion\Serion GmbH (Würzburg, Germany)

2.3.4.2 Modified measurement of the CH_{50} assay

The CH_{50} is a screening assay for the total functional activity of the complement system by determining the dilution of serum required to lyse 50% of a one percent suspension of rabbit antibody-coated sheep erythrocytes (Whaley, 1985; Nilsson and Nilsson, 1984). However, erythrocytes are generally protected from complement-mediated lysis in a species-restricted manner (i.e. serum has poor ability to lyse erythrocytes from the same species) due to the expression of complement regulators on the erythrocyte surface (Ish et al. 1993; Kim *et al.,* 1995; Rushmere *et al.,* 1997; Yu *et al*., 1997). Hence, the standard CH_{50} test using sheep erythrocyte targets is suboptimal for measuring sheep complement activity so a range of different species erythrocytes (guinea pig, sheep, chicken, rabbit and human erythrocytes) were tested to find that guinea pig erythrocytes were the optimal target for this test. Herein, I describe an optimized, modified CH_{50} test that is more sensitive for sheep complement.

Two hundred microliters of pelleted erythrocytes of each species (guinea pig, rabbit, chicken and human erythrocytes) were washed three times in 1x phosphate buffered saline (PBS), by centrifugation at 1000 *xg* for 5 min and resuspension in PBS (1%). When pre-sensitised erythrocytes were investigated, following 2 washes, they were resuspended in 10 ml PBS containing 1mg purified rabbit anti-erythrocyte antibody (antibody matched for chicken or guinea pig erythrocytes) or 100 µl of anti-human CD55 and 100 µl of anti-human CD59 rabbit polyclonal antisera (human erythrocytes) or 50µl of rabbit anti-sheep erythrocyte antiserum (Table 2.1) and incubated at 225 rpm and 37°C in a shaking incubator for 30 min. Pre-sensitised erythrocytes were washed two further times with 1x PBS before being washed twice in complement fixation buffer (CFB; Oxoid Ltd. Basingstoke, UK).

A working stock of erythrocytes was diluted to approximately 1% in CFB (or in CFB containing 20 mM (EGTA) and 40 mM Magnesium chloride for alternative pathway studies) and the

dilution adjusted to yield a reading of 1.00 absorbance units at 595 nm in a 96 well flat bottomed plate. Unlike some other methods, % lysis for this method is calculated by measuring the increased transmission of light (at 595 nm), which is blocked by whole erythrocytes but not absorbed by haemoglobin released after lysis. This also allows repeated lysis measurements with increasing incubation at 37ºC (as outlined in the supplementary material for Ahmed *et al.,* 2014).

Percent remaining unlysed target cells was calculated = 100 x (A/B).

Where A= absorbance sera-absorbance background and

B = absorbance non-lysis control-absorbance background

Then %lysis = 100 - % remaining unlysed targets. (See supplementary methods appendix for detailed explanation and calculation using a representative data set). Serum for measurement by haemolysis assay (non-pregnant adult, maternal adult, or preterm fetal sera) were set up as doubling dilutions from 50% in CFB in triplicate to 0.78% in a 96 well plate (50 µL in rows B to G). No serum lysis controls (50 µL CFB only) were set up in row H, and 100% lysis controls (50 µL 0.1% sodium dodecylsulphate) were set up in row A. Fifty microliters of erythrocyte suspension were then added to all wells for each triplicate (rows A to H) and incubated at 37ºC. Absorption readings at 595 nm were taken at 5, 10, 20, 30, 60, 90 and 120 min using a Dynex MRX II micro plate reader (Worthing, UK). The integral Revelation ™ software subtracted the background absorption of the 100% lysis control (row A) from the rest of the plate. The corrected absorbance for all serum dilutions was then transformed as a ratio of the 0% lysis control (row H) to reflect the % lysis of target cells and all data transferred to Graphpad Prism for analysis (La Jolla, CA).

2.3.4.3 Inhibition by complement inhibitor CDX-1135

CDX-1135 is a recombinant form of sCR1, representing the N-terminal 30 short-consensus repeats (Weisman *et al.,* 1990) and was provided by Celldex Therapeutics, Inc (Needham, MA). Inhibition of haemolysis by CDX-1135 was determined by comparing haemolysis of antibody sensitised guinea pig erythrocytes as detailed above, except that the dilution series for each serum (in triplicate) was performed in CFB, compared to CFB containing 1 mg/mL or 0.25mg/mL CDX-1135.

2.3.5 Flow cytometry

The presence of endogenous cross-reacting antibodies binding to erythrocytes was determined by incubating unsensitised 1% rabbit or guinea pig erythrocyte suspension (diluted in flow cytometry buffer (FCB): PBS containing 15 mM EDTA, 1% fetal bovine serum albumin(BSA), and 30 mM sodium azide) with sheep serum (non-pregnant adult, maternal adult, or preterm fetal) pre diluted in FCB. Erythrocytes were incubated for 30 min at 4°C and then washed by centrifugation at 1000 *xg* for 2 min followed by resuspension in 200 µl CFB, repeated three times. Bound sheep antibody was detected by incubation with 10 µg/ml FITC-conjugated rabbit anti-sheep serum (Abcam plc, Cambridge UK) for 30 min at 4°C and unbound secondary removed by three washes in FCB. Cellular fluorescence was measured using a Becton Dickinson FACscalibur flow cytometer, collecting 10,000 events per sample and analysed using CellQuest software (Becton Dickinson; Oxford UK). Titration of individual sheep serum was performed in triplicate and the experiment repeated twice.

2.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot

Ureaplasma growth from samples or from stock was cultured overnight in 96 wells flat-bottom plate as detailed before in 2.2. The presence of anti-*Ureaplasma* antibodies in the fetal sera or amniotic fluid were determined by Western blot analysis. Gels made to a final concentration of 7.5%, 10%, 12.5% or 15% were utilised according to the expected size of the test protein (table 2.2 & 2.3). Using the first well to load 5 µl of the molecular mass marker (Sigma-Aldrich Ltd.) 3 µg of HPA5 (or other test strain as indicated) per lane was separated by non-reducing polyacrylamide electrophoresis prior to electrophoretic transfer to nitrocellulose membranes. In some blots a non-related *Ureaplasma* serovar 6 strain (HPA61) was also included to examine the presence of pan- *Ureaplasma* reacting antibodies. Proteins were separated for 60 minutes at 120 volt and approximately 120mA. Then by using a BioRad Mini-Protein 3 system, the proteins were transferred from the gel into 0.22 mm nitrocellulose membrane (Appleton Woods). Transfer apparatus were assembled as per manufacturer's instructions and immersed in 1x transfer buffer (14.4 g/L glycine, 3.03g/L Tris and 20 % methanol), and an ice block and a magnetic stir bar to circulate the transfer buffer and keep it cool. After one hour at 100 volts, the membranes were transferred into falcon tubes (EIkay, Basingstoke) and blocked with 25 ml blocking buffer (5% w/v non-fat milk in 1x PBS +0.05% Tween (PBST)) for 1 hour on a roller at room temperature. Membranes were then probed with primary antibodies or sheep serum diluted 1:1000 and 1:500 respectively in blocking buffer as shown in(table 2.4 and 2.5), incubated on a roller at 4◦C overnight. After repeated buffer exchange wash (PBST then PBS alone) bound sheep antibodies were detected with peroxidase-conjugated donkey anti-goat antibodies (minimum species cross-reaction, Jackson ImmunoResearch UK) or with species matched peroxidase-conjugated secondary antibodies for alternative primary antibodies (Sigma-Aldrich Ltd.) at a dilution of 1:10,000 in blocking

buffer (1% dry skimmed milk powder in PBST) for 1 hour prior to repeat buffer exchange washes. The membrane was then transferred to chemiluminescence reagent (Pierce® ECL Western Blotting Substrate; Thermo scientific, Loughborough) and the signal captured by exposing the blot to FUJI Super RX X-ray films (FUJIFILM, Tokyo, Japan) in a dark room. X-ray films were then scanned and images

were stored as TIFF files.

Table 2.2 Recipes for preparing 4% stacking gel

Stacking gel 4%	
dH2O	6.4ml
40% bis/acrylamide37.5:1	1.012ml
upper buffer(PH6.8)	2.4ml
10% APS w/v	100 µl
TEMED	40 µl

Table 2.3 Recipes for preparing different percentage resolving gel

Resolving gel				
Concentration of the gel	7.5%	10%	12.5%	15%
dH2O	5.47ml	4.53ml	4.3ml	3.66ml
40% bis/acrylamide37.5:1	1.93ml	2.5ml	3ml	3.73ml
Lower buffer(PH8.8)	2.5ml	2.5ml	2.5ml	2.5ml
10% APS w/v	100µl	100µl	100µl	100µl
TEMED	10 µl	10 µl	10 µl	10 µl

Table 2.4 Primary and secondary antibodies used in my experiments

Serum or Primary Antibody	Dilution	Source	Secondary Antibody	Dilution	Source& catalogue no.
Sheep serum	1:500	Experimental Sheep (University of Western Australia)	Peroxidase-conjugated Donkey anti goat	1:10000	Jackson Immuno-Research (705 035 147)
Anti-MBA mono-clonal antibodies	1:500	MonoTop™ Virostat, Portland,ME)	Peroxidase-conjugated Affinipure donkey Anti-Mouse	1:10000	Jackson Immuno-Research (715 035 150)
Human sera	1:500	Normal Human Volunteers (Beeton *et al.*,2012))	Peroxidase- AffiniPure conjugated Donkey Anti-HumanIgG (H+L)	1:10000	Jackson Immuno-Research (709 035 149)
Anti-His tag	1:5000	BioLegend	Peroxidase-conjugated AffiniPure donkey Anti-Mouse IgG (H+L)	1:10000	Jackson Immuno-Research (715 035 150)

Table 2.5 Anti-MBA monoclonal antibodies (mAbs) used in this study

MAb	Serotype to which mAb was designed	Source
6522	**both *U.parvum &urealyticum***	**Virostat Inc, Portland ME**
6523	**all *U. parvum***	**Virostat Inc, Portland ME**
6525	**only SV3 & SV14**	**Virostat Inc, Portland ME**
2G9	**SV1&6**	**Virostat Inc, Portland ME**
4H2	**SV1&6**	**Virostat Inc, Portland ME**
2B11	**all *U.parvum***	**Virostat Inc, Portland ME**
8B5.2	**SV3&14 &all other SVs but weakly**	**Watson *et al.*, 1990**
8A1.2	**SV10**	**Watson *et al.*, 1990**
10 C6.6	**SV3**	**Watson *et al.*, 1990**
5B1.1	**SV3 and 14**	**Watson *et al.*, 1990**

2.5 Molecular Biology Studies

2.5.1 Polymerase Chain Reaction (PCR) assay for *Ureaplasma* cultured isolates

PCR for differentiating the two groups of *Ureaplasma* spp. into *U. parvum* and *U. urealyticum* was utilised as described by Teng (1994) (Teng, 1994). Primers (UMS-125 and UMA226 (PCR UM-1) (Table 2.7) to amplify a DNA fragment within the MBA gene were used to verify the two strains of *Ureaplasma* where they give different sizes of PCR products, 403 bp for *U. parvum* and 448 bp *U. urealyticum. U. parvum* isolates were further differentiated into the four serovars (1, 3, 6, 14) by sequencing with the UM-1 primer set and aligning sequence results with the known sequences of MBA of the serovars (Kong, 2000). Differentiation of the four serovars of *U.Parvum* mainly based on variations within two regions (between -54 to -56 and -81 to -83) of the MBA gene (Table 2.6).

Table **2.6** Location of the nucleotide variations within MBA gene of *U. parvum*

Serovar	**Location of variation in nucleotides within *MBA* gene**					
	Region 1			**Region 2**		
	-54	-55	-56	-81	-82	-83
SV1	A	C	A	T	C	G
SV3	A	T	A	C	C	A
SV6	G	A	T	T	A	A
SV14	A	T	A	T	A	A

PCR for multiple-banded antigen gene (*MBA*) assay for *Ureaplasma* in sheep serum or amniotic fluid was prepared by centrifuging 300µl of overnight *Ureaplasma* growth for 10 min at 13,000*xg*, the pellet was dissolved in 50 µl deionized water, boiled for 10 minutes to lyse the cell proteins and release its DNA. The DNA was used immediately or stored at -20◦C to be used for future analysis. *Ureaplasma* PCR was performed using 2 X KAPA2G Hotstart PCR master mix (KAPA Biosystems, London, UK) and run on a Techne PCR thermal cycler. Reactions were run in 200 µl PCR tubes (Star lab, Milton Keynes), in accordance with the manufacturer's instructions to make a final PCR mixture of 50µL (including 2.5µL DNA template and 2 µL of each 10pmol primer pair working stock dilutions).Table 2.7 shows the primers used in this thesis are as follows (Primers are shown in Figure 6.9 for placement relative to the genome; sequences are provided from 5' to 3'):

Table 2.7 Primers used in this thesis (Teng *et al.,* 1994)

No	Primer Name	Sequence (5` to 3`)
	Multiple Banded Antigen *UM-1*	
1	UMS-125	GTATTTGCAATCTTTATATTTTCG
2	UMA-226	CAGCTGATGTAAGTGCAGCATTAAATTC
3	**SV3 end**	TCTAGATTATTTTCCAGTAGTTTCTTTAC-CTG
4	**SV3 start**	AAAAGCTTAACCAGCAGGTAAA GAACAACC
5	**PreMBA**(containingall intergenic sequence between UU375 and UU376)	GTGTCTAGAGAGATGGTTGTTGGCTTGG
6	**UU374 rev**(gene 3' to UU375)	CATCTTCTCTTAATTGTCTTCCCA

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All PCR reactions were subject to the cycling conditions of: initial denaturation at 95°C for 5 min, followed by 40 cycles (denaturation at 95°C for 30s, annealing at 54°C for 40s, extension at 72°C for 1min/1000 bp) with a final extension at 72°C for 5 min after which the products were cooled to 10 ºC until required. Following PCR completion, the presence and size of the amplified DNA fragment(s) were confirmed by running 5µL of the PCR product on a 1% agarose gel electrophoresis relative to the KAPA Universal DNA ladder to determine the DNA fragment size. Control reactions with *U. parvum* obtained from the American Type Culture collection (ATCC 700970, serotype 3) were used as positive controls while the negative control consisted of reactions run without the addition of boiled *Ureaplasma* as source of DNA to rule out any false positive results.

2.5.1.1 Agarose gel electrophoresis

Depending upon the desired agarose concentration, agarose gel was prepared by dissolving a specific amount (i.e. 1g per 100 mL for 1%) of molecular grade agarose powder into a 1x Tris-borate EDTA (TBE) buffer (composed of Tris base 8.9 mM, boric acid 8.9 mM, EDTA 0.2 mM, pH 8.3). Agarose was melted in a microwave oven for 2 min on high then ethidium bromide was added at a concentration of 4µg/ 100 mL. After the gel was set in the casting apparatus, it was submersed in 1x TBE in the electrophoresis chamber and the comb and dams were removed. As reactions already had loading dye present, 5µL of each PCR product was introduced into a single well. 5µL of KAPA Universal DNA ladder (KAPA Biosystems) was also loaded into the first well to allow fragment size determination. The gel electrophoresis was then carried out for 45 mins at constant 150V (safety maximum of 400mA set). Upon

completion, the ethidium bromide stained DNA fragments were visualised using a UV-trans illuminator (Bio Rad Labouratories; Bio-Docit).

2.5.1.2 Sequencing the PCR amplicon products

PCR products were purified using Qiagen PCR purification kits as per the manufacturer's instructions (below). The purified DNA fragments from the PCR amplicons shown in the previous step were submitted either to MWG Eurofins (Germany) by post (15µL of purified PCR DNA plus 3 µL of single primer at 10pM), or submitted to the local Central Biotechnology Sequencing service at the School of Medicine facility. After receiving the results of sequencing we used BLAST software program to align the PCR sequences with a reference sequences was performed (NCBI http://www.ncbi.nlm.nih.gov/).

2.5.1.3 Purification of DNA from PCR products for Sequencing

After testing the PCR products by agarose gel to confirm the amplification of DNA fragment, amplicons were purified. Five volumes relative to the PCR reaction were added to the "PB" binding buffer. This was then transferred into QIAquick spin column attached to a 2ml collecting tube. These were centrifuged at ≥10,000 rpm for one min and the flow through discarded. 750 µl of buffer PE was added to wash bound DNA and the tube re-centrifuged as above and the flow-through discarded. An additional spin for 1 min was performed to air dry the column. The column was transferred to a clean pre labelled 1.5ml microcentrifuge tube and 30 µl molecular-grade water added and incubated for 1 min at room temperature prior to centrifugation for 1 min at 13,000 rpm.

2.5.1.4 Extracting DNA using QIA quick Gel Extraction Kit

When more than one band was amplified, or for sub cloning inserts after restriction enzyme digest, reactions were separated on 1% agar gels containing 0.1% crystal violet instead of ethidium bromide. DNA bands were excised with a sterile sharp scalpel, the piece of gel containing the band then weighed and dropped in a 1.5ml sterile Eppendorf tube, 3 volume of buffer QG was then added (100mg of gel= 100 µl) using the QIA quick gel extraction kit. Gel was dissolved completely at 50◦C and then 1 volume of isopropanol added and the tube inverted. The solution then transferred to QIA quick column and centrifuged at 13,000rpm for 1min. The sample then washed by 0.75 ml of PE buffer, spun for 1min at 13,000rpm. Then the column placed on a sterile pre-labelled Eppendorf tube and overlaid with 50 µl of Sigma biological water to the centre of the column, incubated at RT for 1 min, then spun at 13000 rpm for 1 min. The eluted material contained the band for further analysis or ligation.

2.5.1.5 Extraction and purification of genomic DNA from *Ureaplasma* species

Two hundred ml from each strain of *Ureaplasma* were grown in 250 ml sterile tubes for 48 hours in USM, centrifuged for 3 h at 13,000 *xg* in a cold centrifuge, supernatant was discarded and the pellet was resuspended in 1ml PBS and transferred to a 1.5ml Eppendorf tu-be, spun for an additional 30min at 13,000 rpm, then supernatant were discarded and the pellet was used for DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen, UK). The pre-treatment protocol for gram negative bacteria was followed, including incubation with proteinase K from QIAGEN tissue lyser Kit, at 56°C for 2 hours. Because RNA-free genomic DNA was desired, the optional step of adding 4 µL RNase A (100 µg/mL) was performed. DNA was then extracted and purified in accordance with the manufacturer's spin-column protocol.

2.5.1.6 Precipitation of DNA

The volume of the DNA sample from the previous step was measured. Then 1/10 volume of sodium acetate, pH 5.2, added (final concentration of 0.3 M), mixed well, then added 2.5 volumes of cold 100 % ethanol and mixed well. Tubes were placed on ice or at -20°C for >20 minutes. Tubes were centrifuged at maximum speed in a centrifuge for 15 minutes. Carefully decant supernatant. Add 1 ml 70% ethanol. Mix. Spin briefly (~5 minutes). Carefully decant supernatant. Air dry pellet, re-suspend in the appropriate volume of TE buffer. DNA concentration was then measured in a spectrophotometer**.** Final DNA yield was determined using a spectrophotometer at a wavelength of A260 nm. The spectrophotometer was blanked against TE prior to measuring absorbance of DNA. Purified aliquots of DNA were stored at -80 °C until required.

2.5.1.7 Creation of the pMT85 expression vector for MBA expression

PCR was performed as above using the preMBA and SV3end primers, which have Xba1 sites incorporated into the primers. The appropriate band was isolated by gel extraction and the purified band incubated with Xba1 restriction enzyme (Promega) for 4 h using manufacturer's instructions. The expression vector pMT85 (Aboklaish *et al*., 2014) was cut in parallel and the insert containing the *MBA* gene and natural promoter were ligated into the expression vector, following removal of the restriction enzyme by QiaQuick PCR purification kit (Qiagen), using T4 ligase (Promega) as per manufacturer's instructions. Following overnight ligation at 15°C the pMT85 was transformed into One Shot competent E.coli (Life Technologies) and selected on 100 mg/L gentamicin agar plates. Single colonies were grown up and sequenced to ensure no errors were introduced and then scaled up and transformed into HPA5 by Dr. Ali Aboklaish.

2.5.2 Designing a synthetic gene

The synthetic gene "histag-SV3-*MBA*" was designed using the web site

http://www.lifetechnologies.com/uk/en/home/life-science/cloning/gene-synthesis/geneart-gene-synthesis.html

https://www.lifetechnologies.com/order/geneartgenes/launchConfigurator.action?document_Id=140948

The sequence of serovar 3 MBA (amino acid sequence) was utilised to generate a codon-optimised DNA for expression in *E.coli*. In particular tryptophan is encoded by UGA in *Ureaplasma*, but this is a stop codon in other prokaryotes. Additionally, *Ureaplasma* genomes have a 25% GC content. The synthesised gene was also constructed to have the EF-tu promoter added prior to the ATG start codon:

TGAAGAAGTAATGGTAGCACGTGCAAAAAAATAATTATTTTAACAATTAACAA-
TAAAGTATTTAATTAATAATCTAAAAAGTTAAAATGTTATAATTATAAGGTATAATAC-
CTTTTATTATGTATTCAAAAATTATTTAGAAAATAAATTAATTTTTAAGGAGATTTAAA

Codons for 6 histidine residues were also inserted prior to the TAA stop codon. The sequence was sent to MWG Eurofins for gene synthesis and it was delivered in the plasmid pEX-A2. This was transformed into *E.coli* and selected in ampicillin resistant plasmid (100 mg/L) and this transformed bacteria used in my experiments. Only 2 repeats could be engineered in the synthetic version of serovar 3 MBA as gene synthesis is confounded by recurring repeats (according to the provider). Following transfection of the plasmid into One Shot competent *E.coli* (as per manufacturer's instructions) the MBA protein was separated into membrane and cytoplasmic fraction by Triton-X114 fractionation methods (detailed below) and tested by

Western blot analysis to localise the site of expression of the MBA protein by using monoclonal antibodies that recognise incorporated histidine repeats (Biolegend) or by using anti-MBA monoclonal antibodies 6522, 6523 and 6525 (Virostat Inc.).

2.6 Culturing *E.coli*

2.6.1 Preparing LB broth *E.coli*

Twenty five grams of LB broth powder purchased from(Q Bio gene) was added to 1 L water in an autoclave bottle and mixed with a magnetic stir bar and plate. The bottle was autoclaved for 15 min at 121ºC and allowed to cool to 50 º C then 100 mg of ampicillin (Sigma) was added and allowed to cool and stored at 4ºC until needed.

2.6.2 Preparing agar plates for *E.coli*

The equivalent procedure to broth was followed except that the LB agar powder (Q Bio gene) also contains agar. Following addition of ampicillin at 50ºC as above, the agar was poured into 10 cm diameter sterile plates and allowed to set prior to store at 4ºC until needed.

2.6.3 Triton X 114 extraction of *E.coli* membrane proteins

Triton X-114 is used to separate membrane fractions from solubilised whole cells, as this detergent comes out of solution when heated to 37ºC (cloud point). Membrane-associated proteins can thus be separated from cytoplasmic and insoluble proteins and identified as such in membrane or cytoplasmic fractions following separation by warm centrifugation.

Colonies of MBA-expressing E.Coli (and controls transformed with empty plasmid) were grown overnight in 25ml LB broth at 37°C and 225 rpm in a bacterial shaking incubator. Bacteria were pelleted at 3600 xg for 10 min and the pellets re suspended in 5 ml Triton x114-TBS (10mM Tris, pH 7.4, 150mM NaCl, 1% Triton X114) on ice for 30 min and vortexed every 5 minutes. Insoluble material was removed by centrifugation for 10 min (3600 *xg* ,4ºC). One ml of the supernatant was layered on top of 500 µl sucrose cushion (TBS, 1% Triton x114, 0.6% sucrose) in 1.5 ml Eppendorf tubes, which were then warmed to 37 ºC for 10 min and then spun for 5 min in bench top centrifuge at 25°C at 17,000 *xg*. The top layer was separated (cytoplasmic fraction), while the oily pellet was washed in warm TBS twice and then dispersed by suspension in an equal volume of 10 % SDS (in TBS). Cytoplasmic and membrane fractions were added to an equal volume of LDS loading buffer (Life Technologies) and heated for 5 min at 95°C prior to separation by SDS-PAGE. If not used immediately, the samples were frozen at -80ºC for future use.

2.7 Immunohistochemistry

E.coli colonies from LB agar plates (both SV3-MBA expressing and control strains) were removed and embedded in paraffin wax by Central Biotechnology Services. These paraffin blocks were then sectioned, deparaffinised, then paraffin section rehydrated with xylene 2 x 5 minutes, isopropyl alcohol (IPA) 2 x 5 minutes, and washed in tap water 5 minutes. Antigen retrieval was performed for 1 hour in 10mM citrate acid buffer (pH 6) containing 0.005% Tween 80 at 95◦C prior to being probed for with monoclonal anti MBA antibodies (6522, 6523 and 6525) diluted in 10mM PBS + 0.6% BSA (PBS/BSA) to confirm the localisation of the synthetic MBA protein on the surface of individual *E.coli* bacilli in comparison to staining of *E.coli* that are transformed with an empty plasmid. Unbound monoclonal antibodies were

removed by three washes for 1 min each in PBS/BSA. Bound antibody was detected with 1/100 dilution of peroxidase conjugated donkey anti-mouse IgG (Jackson Immunoresearch) in PBS/BSA then wash and develop 3 minutes in 0.025% DAB + 15-30% H_2O_2. In some cases the bacilli were additionally stained for 10 sec in 0.02% picromethyl blue prior to being dehydrated, cleared in IPA, xylene and mounted with a coverslip.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism (La Jolla, CA). For screening of lysis targets or flow cytometry binding, significant lysis or binding was determined by comparing data for all concentrations of serum relative to a serum-negative control using ANOVA with Bonferroni correction for multiple comparisons in post-hoc analysis. Comparisons of lysis or binding curves with different variables (adult versus fetal sera, sera from pregnant versus non-pregnant ewes, lysis of sensitised versus non-sensitised targets, aged versus fresh erythrocyte targets, no-complement inhibitor versus differing concentrations of CDX-1135, etc.) were analysed by repeated measures ANOVA with Bonferroni correction for multiple tests in post-hoc analysis of individual concentrations of sera being compared. All serum killing assay data were expressed as mean and standard error of the mean (SEM). Comparisons were only considered significant for P values < 0.05.

CHAPTER 3

Comparison of Complement Activity in Adult and Preterm Sheep Serum

(Published paper)

3 Comparison of Complement Activity in Adult and Preterm Sheep Serum

3.1 Introduction

The complement system is a key part of innate immunity. It does not require previous exposure to be effective, but it does make the humoral immune system more effective in clearing pathogens, and complement activation recruits innate and adaptive immune cells to the site of inflammation, where it aids in modulating and refining the adaptive immune response [reviewed in(McGreal, 2012)]. The complement system is composed of a series of serum proteins that circulate in proactive forms that are sequentially cleaved and activated in an amplifying cascade, forming a series of bioactive intermediates following activation. Complement is activated by specific triggers that are designed to detect 'foreign' surfaces. Specific IgG and IgM anti- bodies adopt a complement-activating conformation following binding to recognized antigen epitopes (the classical activation pathway), which leads to the activation of complement component C1. Similarly, many of the innate immune lectins (e.g. mannan- binding lectin (MBL), ficolins, and collectin 11) activate complement via complexes with associated serine proteases following lectin binding to carbohydrates that are composed of sugar moieties or carbohydrate combinations/linkages that are not found in the host (the lectin activation pathway)(Hansen *et al.,* 2010). Both of these triggers for complement activation require calcium to form the active enzymatic convertase complexes [reviewed in (Degn and Thiel, 2013)]. The final method of complement activation does not utilize active recognition molecules, but is a failure to regulate the low-level fluid-phase conversion of the central component C3 to a fluid-phase activating convertase (the alternative activation pathway). This last activation pathway can be assessed independently as it functions in the absence of calcium and has been best characterized through studies of complement activation by yeast cells (Meri *et al.,* 2002).

Complement activation leads to ‘tagging’ of the activating surface by covalent attachment of opsonins (component C3b and processed fragments), which enhance complement receptor-mediated phagocytic engulfment. Further processing of the C3b covalently bound to foreign antigens leads to enhanced antibody production and refinement against those antigens by B-lymphocytes (Caroll,2004) while release of small soluble fragments called chemotaxins and anaphylatoxins (C3a and C5a) act to recruit and prime innate and adaptive immune cells to the site of complement activation.

Complement activation can also result in direct lysis of the activating cells by formation of the membrane attack complex, a non-covalent complex of complement components C5b, C6, C7, C8, and C9 which insert pores into the membrane of activating surfaces. It is this last consequence of complement activation that is measured by the standard CH50 (50% complement haemolysis assay) functional test, determining the dilution of serum required to lyse 50% of a one-per cent suspension of rabbit antibody- coated sheep erythrocytes (Whaley, 1985; Nilsson and Nilsson, 1984). However, erythrocytes are generally protected from complement-mediated lysis in a species-restricted manner (i.e. serum has poor ability to lyse erythrocytes from the same species) due to the expression of complement regulators on the erythrocyte surface (Ish *et al.*, 1993; Kim *et al*., 1995; Rushmere *et al.*, 1997; White *et al*., 1994; Yu *et al.,* 1997). These regulators include CD59, CD55 (also known as decay-accelerating factor), and CD35 (also known as complement receptor (1) as well as cell surface recruitment of serum complement regulators factors H and I [reviewed in(Lindahl *et al.,* 2000)]. Some of these regulators have been characterized for sheep (Kišová-Vargová, 2012; Vanden Berg, 1993; Stohl *et al.*, 2012; Beth *et al*., 2013); however, it is likely that homologues for all of these regulators exist. For this reason, the standard CH50 test using sheep erythrocyte targets is suboptimal for measuring sheep complement activity. Data from previous studies suggest that the close homology between sheep and goat immune

proteins also renders sheep erythrocytes a poor target for goat complement, requiring creation of a modified CH50 assay for goat serum. Herein, I describe an optimized, modified CH50 test that is more sensitive for sheep complement.

Although complement does not require previous exposure to be fully effective, it is not fully functional at birth in humans, and the lysis measured by CH50 requires 2–3 times more neonatal serum than adult serum due to reduced concentrations of complement components[reviewed in (McGreal *et al.*, 2012]. The insufficiency of neo- natal complement is further increased in neonates born prematurely, with a correlation of lower complement component concentration, and greater amounts of serum for the CH50 values, with increasing degree of pre-maturity (McGreal *et al.,* 2012). However, complement activity during gestational development animal models of human pregnancy is under-investigated. The sheep model has gained popularity in examining gestational inflammation and preterm birth pathology including gestational reperfusion injury, (Castillo-Melendez *et al.,* 2013; De Matteo *et al.,* 2010; Wolfson *et al.* ,2012), in utero *Ureaplasma parvum* infection (Kemp et al., 2011) and Escherichia coli lipopolysaccharide-mediated inflammation (Dean *et al*., 2011; Zhang *et al*., 2012). Moreover, complement has been shown to be activated or play a role in the underlying pathogenesis in non-sheep models and humans for these conditions (Beeton *et al.,* 2012; Sun *et al*., 2011; Li *et al.,* 1999). Soluble human CR1 (sCR1) has successfully been used to experimentally identify the contribution of complement activation to pathogenesis by inhibition of complement in rats, guinea pigs, pigs, and mice (Lillegard *et al*., 2013; Weiser *et al*., 1997; Souza *et al.*, 2005; Wagner *et al.*, 1999) using soluble recombinant forms from various commercial sources (TP10, later renamed CDX-1135, and APT070), but to the best of our knowledge, not in sheep.

3.2 Aims

1-To develop an assay to measure the complement activity in sheep sera as traditional methods use sheep erythrocytes as a model, which are protected by sheep complement regulators.

2-To compare the complement function in preterm lambs sera at three different gestational ages: 85, 95 and 125 days gestation relative to adult sheep sera.

3- To investigate the ability of full-length soluble CR1 (CDX-1135) to suppress sheep complement activation in an *ex vivo* model.

3.3 Results

3.3.1 Screening Target Erythrocytes for Lysis by Sheep Serum

Lysis of guinea pig, rabbit, and human erythrocytes by diluted maternal adult sheep sera is shown in Fig.3.1. No significant lysis was observed for human erythrocytes without pre-incubation with both rabbit anti-CD55 and anti-CD59 antibodies, and no lysis of chicken erythrocytes, including following pre-sensitization with rabbit anti-chicken erythrocyte antibody, was observed (data not shown). Erythrocyte lysis relative to concentration of sheep serum changed with longer incubation at 37°C, and higher serum concentrations achieved complete lysis early on, but lower concentrations required longer incubation times to achieve maximum lysis (Fig.3.1). Little increase in lysis was observed after 30 min. Guinea pig erythrocyte lysis was only marginally greater if the cells were pre-sensitized with rabbit anti guinea pig erythrocyte antibody (Fig.3.1b and d), whereas human erythrocytes pre-incubated with both rabbit anti-CD55 and anti-CD59 antiserum required longer incubation to achieve similar cell lysis to guinea pig erythrocyte targets (Fig.3.1a). Rabbit erythrocytes were

also lysed by sheep serum, even in the absence of pre-incubation with an anti- rabbit erythrocyte antibody, but required higher concentrations of sheep serum to achieve significant lysis (Fig.3.1c). Ageing the human erythrocytes for 2–4 weeks at 4°C prior to use in the assay greatly increased their susceptibility to lysis (aged human erythrocyte lysis is shown in Fig.3.1a, but a comparison between fresh and aged human erythrocytes is available in the Appendix Figure Supp.1). Lysis of target erythrocytes was the same when comparing serum obtained from non-pregnant ewes to serum from pregnant ewes.

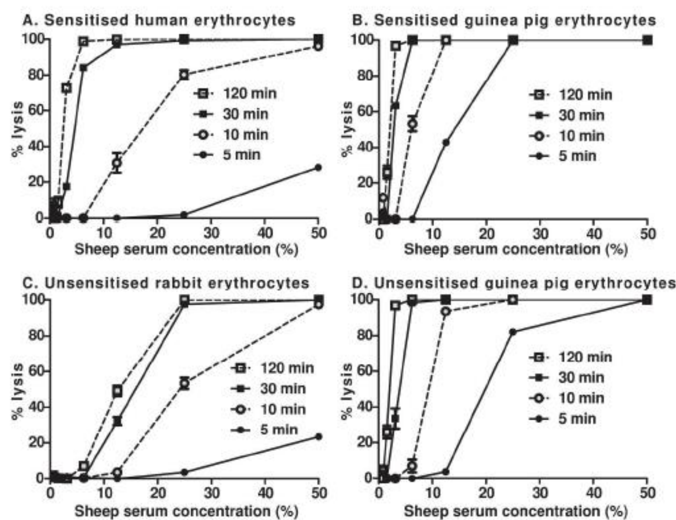

Figure 3.1 Lysis of rabbit antibody pre-sensitised human (A) or guinea pig (B) erythrocytes compared to lysis of rabbit (C) or guinea pig (D) erythrocytes that were not pre-coated with anti-erythrocyte antibody. Target erythrocytes were incubated with increasing concentrations of maternal sheep sera and lysis measured following incubation at 37°C for 5, 10, 30 or 120 min.

3.3.2 Complement-mediated Lysis by Neonatal Sheep

The ability of cord serum obtained from 125-day gestational age caesarean-delivered lambs to lyse our target erythrocytes was also examined (Fig.3.2). The lysis of rabbit anti-erythrocyte pre-sensitized human and guinea pig cells was similar to the maternal sheep sera (Fig.3.2a and 3.2b); however, target cell lysis was much lower at earlier time points, and maximum lysis was about twofold lower relative to maternal sera (Fig.3.1a and b). Foetal serum was unable to lyse more than 20% of target rabbit erythrocytes (Fig.3.2c), even after incubation for 120 min. Furthermore, a reasonable response curve required to evaluate serum concentrations yielding 50% haemolysis for unsensitized guinea pig erythrocytes (Fig.3.2d).It took 90 min longer than if the guinea pig cells were pre-coated with antibody (Fig.3.2b). The ability of sera taken from foetal sheep at earlier 95- and 85-day gestational ages was also examined. Lysis of pre-sensitized guinea pig erythrocytes was lower for sera at 25 and 12.5% concentration for sera from 85-day and 95-day gestational age sera compared with 125-day gestational age sera following short incubation times (Fig.3.3a), whereas these differences between lysis were shifted to lower concentrations of 6.25 and 3.15% when allowed to incubate for an hour (Fig 3.3b).

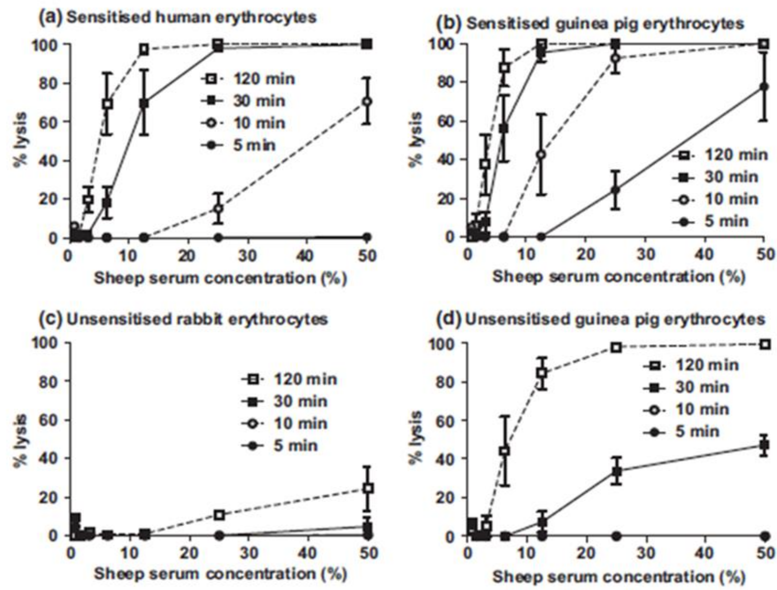

Figure 3.2 Lysis of rabbit antibody pre-sensitised human (a) or guinea pig (b) erythrocytes compared to lysis of rabbit (c) or guinea pig (d) erythrocytes that were not pre-coated withanti-erythrocyte antibody. Target erythrocytes were incubated with increasing concentrations of 125-day gestational age foetal sheep sera and lysis measured following incubation at 37°C for 5, 10, 30 or 120 min.

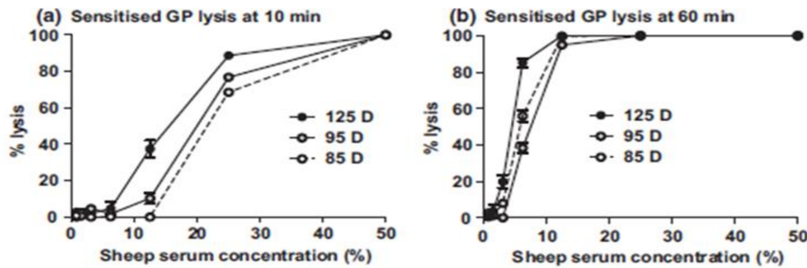

Figure 3.3 Lysis of rabbit antibody pre-sensitised guinea pig erythrocytes by sera from fetal sheep at 125, 95 or 85 days of gestational development. Target erythrocytes were incubated with increasing concentrations of sera and lysis measured following incubation at 37°C for 10 min (a) or 60 min (b).

3.3.3 Lysis by the Alternative Pathway in Sheep Sera

As lysis of the rabbit and guinea pig erythrocytes occurred in the absence of pre-sensitizing antibody, the contribution of the alternative pathway was examined by investigating erythrocyte lysis in the absence of calcium. Unlike the classical and lectin pathways, the alternative pathway can function in the presence of calcium chelator EGTA (Fig.3.4). Twice as much serum was required to achieve the same degree of rabbit erythrocyte lysis in the absence of calcium (alternative pathway), compared with lysis in the presence of calcium. Moreover, the maximum alternative pathway lysis (achieved after 60 min incubation) was equivalent to the cell lysis observed after only 10 min in the presence of calcium (Fig.3.4a). Similar results were observed for un sensitized guinea pig erythrocytes in the absence of calcium, with four-fold more serum required to achieve the same degree of guinea pig erythrocyte lysis by the alternative pathway alone (Fig.3.4b). Foetal sheep sera (125 day gestation) failed to achieve

50% lysis of guinea pig erythrocytes via the alternative complement pathway, even after 120 min incubation (data not shown).

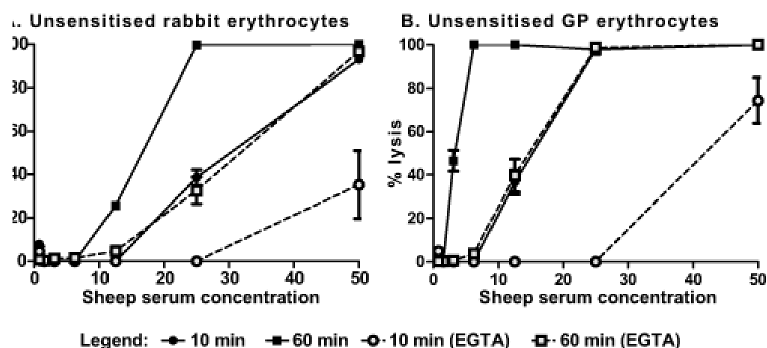

Figure 3.4 Lysis of rabbit (a) or guinea pig (b) erythrocytes that were not pre-coated with anti-erythrocyte antibody. Target erythrocytes were incubated with increasing concentrations of maternal sheep sera and lysis measured following incubation at 37°C for 10 or 60 min. Lysis was performed in the presence of EGTA (alternative complement pathway only) and compared with serum diluted in normal complement fixation buffer (all complement pathways active).

3.3.4 Endogenous Antibodies in Maternal Sheep Sera Binding to Erythrocyte Targets

Flow cytometry was utilized to measure sheep serum antibody binding to unsensitized rabbit and guinea pig erythrocytes from five maternal and foetal pairs (Fig.3.5). Incubation of erythrocytes with maternal sheep sera at a concentration >10% resulted in the aggregation of both guinea pig or rabbit erythrocytes and, consequently, an inability to analyse them by flow cytometry. In contrast, incubation of rabbit or guinea pig erythrocytes with 50% foetal sera (125 day gestation) did not result in a significant binding of sheep antibody to the surface of the target erythrocytes (Fig.3.5). This identifies the mechanism underlying the differential ability

between foetal and maternal sera to lyse un sensitized target erythrocytes. No significant difference was observed for erythrocyte antibody binding between pregnant and non-pregnant sheep, and no significant antibody binding was observed for any of the foetal serum from gestational ages 85, 95 or 125 days.

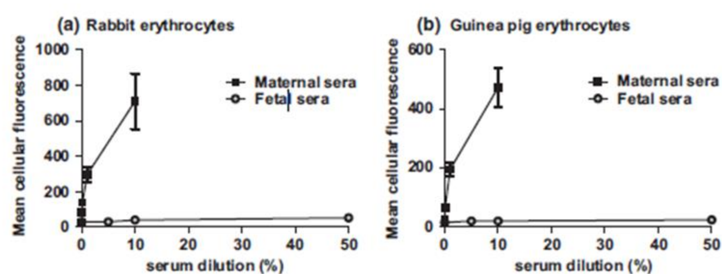

Figure 3.5 Binding of sheep antibodies from 125 day gestational age and maternal sheep sera to un sensitised rabbit (A) or guinea pig (B) erythrocytes as determined by flow cytometry Mean and standard error for five matched maternal foetal pairs are shown.

3.3.5 Failure to Inhibit Sheep Sera-mediated Lysis by Human Complement Inhibitor

By comparing the lysis of antibody pre-sensitized guinea pig erythrocytes by sheep sera in the presence or absence 1 or 4 μM (final concentration) of complement inhibitor CDX-1135, I observed no inhibition of maternal sheep sera above a serum concentration of 6.25% (Fig3.6b); however, under the same conditions, human serum showed strong inhibition even at concentrations as high as 50% (Fig.3.6b). Inhibition of human serum by CDX-1135 remained unchanged when measured again after 120-min incubation (data not shown). The ability to inhibit human serum was reduced if fourfold less CDX-1135 was used (Fig.3.6a). The ability of CDX-1135 to inhibit rat sera lysis of sensitized guinea pig erythrocytes was comparable with that observed for human sera (data not shown), indicating that this form of soluble human CR1 is fully capable of regulating complement from other species. However, these findings demonstrate that an alternative method of complement inhibition will be required to examine the contribution of complement to pathology in sheep models.

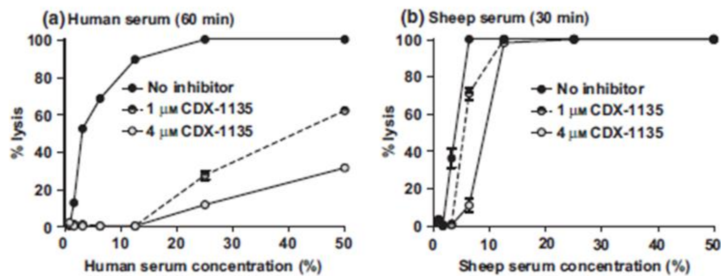

Figure 3.6 Lysis of rabbit antibody pre-sensitised guinea pig erythrocytes by human serum (A) or adult sheep serum (B)Inhibition of target cell lysis by 4.0 or 1.0 µM (final concentration) of complement inhibitor CDX-1135 as compared to serum lysis in the absence of inhibitor. As sheep serum lyses erythrocytes faster, an earlier incubation is shown; however, the trend is the same for all incubation time points.

3.4 Discussion

The complement system is composed of a series of over 30 proteins in the serum component of the blood, produced by the liver and some blood cells. This system has evolved to detect invading pathogens and eliminate them directly or indirectly through recruitment and refinement of the cellular innate and adaptive immune systems. As a result, host cells; in particular erythrocytes need to protect them against bystander lysis following complement activation. The dominant surface complement regulator is CD59, and it has been shown to protect cells in a largely species-restricted manner (Ish *et al*., 1993; Kim *et al*., 1995; Rushmere *et al*., 1997; White *et al.*, 1994; Yu *et al*., 1997). Due to the close genetic homology between goats and sheep, the inability of goat serum to lyse sheep erythrocytes have recently been high-

lighted (Moreno-Indias *et al*., 2012). Sheep CD59 has been characterized, and blocking monoclonal and polyclonal antibody has been raised (van den Berg *et al.*, 1993) however, I was unable to achieve significant lysis of sheep erythrocytes even when pre-sensitized with rabbit antisheep erythrocyte antibody, and blocking anti-CD59 antibodies were used (data not shown).Similar to the studies by Moreno-Indias *et al*.,(Moreno-Indias *et al.*, 2012). I found that human erythrocytes were a good substitute and their sensitivity was increased by ageing the cells for 2–4 weeks at 4°C prior to use; however, guinea pig erythrocytes were found to be the best haemolysis assay target, and both the cells and the rabbit anti-erythrocyte antibody are available commercially. The contribution of complement to pathogenesis extends beyond infection. Damage arising from reperfusion injury, either caused by hypoxia, vascular occlusion or transplantation, is largely due to the activation of complement immediately following reperfusion (Gorsuch *et al*., 2012).

Complement has also been found to be a significant component underlying autoimmune disease as well as renal disease and age-related macular degeneration (McCaughan *et al*., 2013; AMBAti *et al*., 2013). As a result, a wide variety of complement regulators are currently under development for use in renal disease patients, age-related macular degeneration, transplantation, stroke, and myocardial infarction patients (Barilla-Labarca and Furie, 013).These range from blocking monoclonal antibodies against complement component C5 (such as Eculizumab) to recombinant forms of naturally occurring complement regulators such as factor H or complement receptor 1 (CR1;CD35). Given the differences in homology of complement components, the latter group has been more extensively examined in animal models. Soluble forms of CR1 have been favoured in animal models as CR1 is known to regulate all the pathways, and it has multiple C3b and C4b binding sites in the full-length molecule that mediates both accelerated decay of multiple convertases and factor I-cofactor activity, leading to the inactivation of C4b and C3b (Crehan *et al*., 2012). I found a recombinant form of sCR1 that

is composed of the 30 N-terminal domains was a poor inhibitor of sheep complement even at concentrations effective at regulating human serum (Fig.3.6b) and despite previous studies showing effective reduction of complement-mediated pathology by CDX- 1135 in other animal models, including rats (Lillegard *et al*., 2013, Weisman *et al*., 1990).The use of sheep as a non-primate model of human disease is increasing, especially for studies of disease in pregnancy and pregnancy complications. For example, neonatal asphyxia induced by transient occlusion of the umbilical cord before birth has been shown to lead to reperfusion injury and to mimic the intrauterine hypoxia that complicates a proportion of human births (Castillo-Melendez *et al*., 2013). Further, hypoxic injury following preterm birth and associated new born respiratory disease is also studied in sheep models (Wolfson *et al*., 2012). More recently, sheep models have been developed to investigate intrauterine inflammation induced by injection or infusion of lipopolysaccharides or bacterial infection (De Matteo *et al*., 2010; Dean *et al*., 2011; Kemp *et al.*, 2011; Zhang *et al*., 2012). None of these studies has investigated the contribution of the complement system to the disease pathogenesis, largely due to the current lack of knowledge on the sheep complement system. I have optimized an assay that allows the comparison of sheep sera from different conditions. I found no difference in haemolytic capacity between pregnant and non-pregnant adult sheep sera, but found that complement activity in preterm foetal sheep was decreased relative to matched maternal sera, but still had considerable activity even in sera from preterm lambs at 85 days gestation (150 days is normal gestation), which makes them considerably more immune competent than the equivalent gestational age humans [reviewed in(McGreal 2012)]. The inability of foetal sheep sera to lyse rabbit and unsensitized guinea pig erythrocytes was the biggest difference observed. However, this was related to the complete absence of cross-reacting antibodies in foetal serum that bound to rabbit and guinea pig erythrocytes (Fig.3.5). This identifies an additional difference

between humans and sheep: in humans, maternal IgG actively transfers to the foetal circulation from about 13 weeks gestation and reaches 50% maternal concentration by 28– 32 weeks [reviewed in(Palmeira *et al.*, 2012)], equivalent to gestational age 95–125 days in sheep, whereas, in sheep, it has been suggested that IgG may cross the placental barrier to the allantoic sac(Morris, 1980; Omwandho, 1997) , but not to the amniotic cavity or foetal circulation(Morris , 1980); this difference between the species probably results from the haemochorial structure of the human placenta in contrast to the syndesmochorial placentation of the sheep. In fact, other than primates, it appears that only rabbits and guinea pigs have the capacity to transfer maternal IgG to foetuses across the placenta (Pentsuk and van der Laan, 2009) this means that any immune response observed in foetal sheep and other ruminants has to reflect the foetal response. This may also suggest that the immune system of ruminants may be more competent at birth. Herein, I describe a new assay to evaluate the functional complement activity in sheep. I found that serum from normal Caesarean-delivered preterm lambs had lower, but still substantially active, complement systems. I also found that maternal sheep serum contains significant cross-reacting antibodies that bind guinea pig and rabbit erythrocytes, which are absent from matched preterm sheep serum, resulting in a requirement to pre-sensitize guinea pig erythrocytes to measure foetal serum haemolysis activity. As the potent complement inhibitor, CDX-1135 was not effective in inhibiting sheep complement; further studies are required to identify an inhibitor that has application to pathogenesis studies in sheep models of *in utero* infection and hypoxia.

In conclusion, I found that serum from normal Caesarean-delivered preterm lambs had lower, but still substantially active complement systems. I also found that maternal sheep serum contains significant cross-reacting antibodies that bind guinea pig and rabbit erythrocytes, that are absent from matched preterm sheep serum, resulting in a requirement to pre-sensitise

guinea pig erythrocytes to measure fetal serum haemolysis activity. As the potent complement inhibitor, CDX-1135 was not effective in inhibiting sheep complement;

further studies are required to identify an inhibitor that has application to pathogenesis studies in sheep models of *in utero* infection and hypoxia.

CHAPTER 4

Increased *Ureaplasma* bacteraemia in lambs of lower gestational age is proportional to increased insufficiency of the complement system with greater prematurity

4 Increased *Ureaplasma* bacteraemia in lambs of lower gestational age is proportional to increased insufficiency of the complement system with greater prematurity.

4.1 Introduction

Like most parts of the innate immune system, the complement system does not require previous exposure to be effective. The complement system encompasses a series of >30 serum proteins that interact through an amplification cascade following activation by antibodies, immune complexes, pattern recognition molecules or foreign microbial surfaces. Complement also acts to integrate the innate and humoral immune responses through recruiting innate and adaptive immune cells to the site of activation (via chemotaxin and anaphylotoxin release), improving the humoral response through a natural adjuvant activity, and increasing engulfment of microbes through decorating the surface with opsonins (Henri *et al.*,1979; John *et al*., 1984; Dunkelberger and Song, 2010). Complement also has direct anti-microbial activity mediated by the formation of lytic pores on activating surfaces, which makes it a pivotal barrier to initial invasion by pathogens. However, as we have previously reviewed, the complement system does not have full potency at birth in humans due to lower circulating levels of some of the components and this insufficiency is greater with increasing prematurity (McGreal, 2012).The reduced capacity of the complement system at term birth, which is exacerbated with increasing prematurity, has been proposed to be responsible for the increased susceptibility of neonates to bacterial infection and sepsis (Levy, 2007; Strunk *et al*. 2011). I have recently found decreasing complement function in sheep with increasing prematurity (Ahmed *et al.*, 2014) which is often used as an experimental model for *in utero* infection. Sheep are commonly used to investigate the effects of *Ureaplasma parvum* infection, which is the most common infectious organism associated with preterm birth (Epstein, *et al.* 2000; Moss, *et al.,* 2005; Knox, *et al.* 2010). *Ureaplasma parvum* is one of the smallest self-replicating micro-

organisms identified to date and is unusual in that its minimal genome confers a failure to make a cell wall like gram-positive and gram-negative bacteria (Waites *et al.,* 2012). The cell wall has been shown to confer a protective effect against complement mediated killing particularly in gram-positive bacteria, and *U.Parvum* has been shown to be relatively susceptible to lysis by human complement (Beeton *et al.,* 2012).

4.2 Aims of this chapter are

- To check the capacity of *Ureaplasma* following experimental intrauterine infection to establish an *in vivo* infection and replicate inside the amniotic fluid and cause bacteraemia in vulnerable foeti.
- To follow the evolution of the Ureaplasma-cidal activity between 95 and 125 days in preterm foetuses compared to adult sheep.
- To define the kinetics of *in vitro Ureaplasma* killing by non-immune adult and preterm sheep sera.
- To investigate the susceptibility of the plasma and amniotic fluid –recovered strains to adult and preterm sheep sera.
- To investigate the decreasing ability of preterm sheep sera to lyse inert complement targets (sensitised guinea pig erythrocytes) correlated with bactericidal activity between two gestational ages, as well as amongst sera samples of the same gestational age.
- To correlate the bactericidal efficiency of serum from bacteraemic and non-bacteraemic lambs to complement function (measured by 50% haemolytic assay).

4.3 Results

4.3.1 Determining kinetics for infection in experimentally infected sheep

The kinetics of acute *Ureaplasma* infection were determined by time course sampling via surgically implanted catheters (Figure 4.1) following injection of 10^7CCU of *Ureaplasma* into the amniotic fluid via a separate isolated catheter to avoid contamination. At 2 h post-inoculation a mean of 176.5 +/- 47.0 CCU/ml was measured. The titre of *Ureaplasma* in the amniotic fluid then doubled at 6 h and increased by 7.7-fold at 12 h, but didn't show a significant increase until 24 h when it increased to 133-fold relative to the 2 h levels (23,455 +/- 8,710 CCU/ml; $p<0.05$). *Ureaplasma* titres continued to increase to a peak at 48 hr and then appeared to slightly decrease at 5 days, but no statistical significance was found comparing the *Ureaplasma* titre between 24, 48 and 72 hours (Figure 4.1).

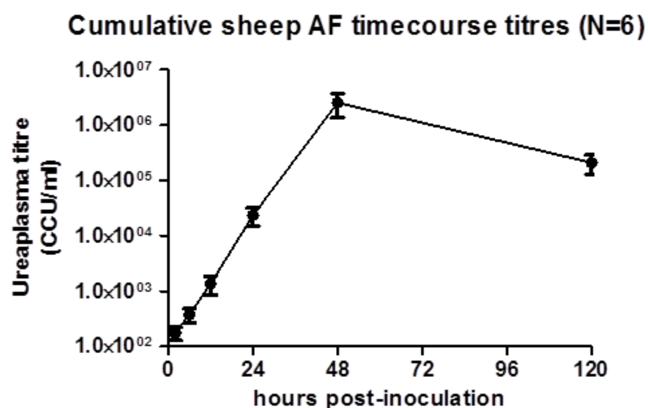

Figure 4.1 Growth kinetics for *U. parvum* parent strain (HPA5) *in vivo.* Mean and standard deviation of *Ureaplasma* titres in samples taken via surgically implanted intrauterine catheter at 2, 6, 12, 24, and 120 h after experimental intrauterine infection. Each point represents 6 separate pregnant ewes and average titre for each time point/ewe was determined in triplicate.

Comparing the AF HPA5 titres from three groups of sheep following different length of infection showed relatively stable level of *Ureaplasma* in the AF from 2 days up to 6 weeks post –infection (Figure 4.2).

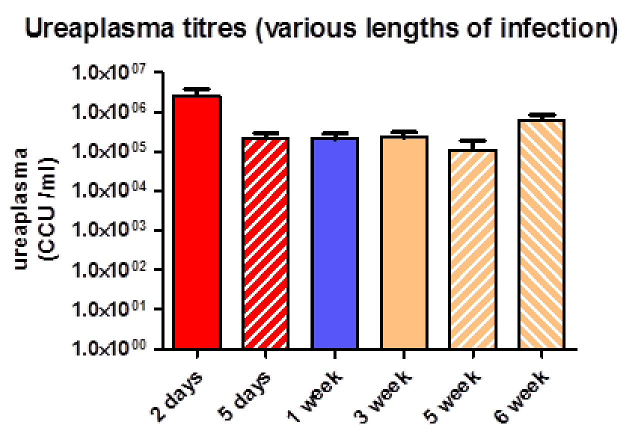

Figure 4.2 Amniotic fluid (AF) titres for HPA5 following various lengths of infection. Red bars indicate samples taken from the catheterised animals in Figure 4.1. The blue bar indicates delivery samples following a 1 week infection in 125D GA animals administered by ultrasound-guided needle injection into the amniotic fluid. The beige bars indicate ultrasound guided needle samples at 3 and 5 weeks following infection at 80D GA as well as samples taken at delivery (6 weeks). Each bar is the average and standard error of 6 animals per group.

4.3.2 Plasma titres of *Ureaplasma* in the circulation of lambs of different GA

To examine the rate of fetal bacteraemia following experimental intrauterine *Ureaplasma* infection, three different groups of pregnant sheep were inoculated with *Ureaplasma*. Amniotic fluid sampling catheters do not remain clear for longer periods of study; therefore, inoculation of these groups was performed by ultrasound-guided needle, and samples were then collected at Caesarean-section delivery. Lambs were surgically delivered at either 94 d or 125 d GA. For all groups the *Ureaplasma* titre in the amniotic fluid was similar (range = 1-6x10^5 CCU/ml; data not shown), but variation was seen in the number of animals with culturable *Ureaplasma* in their plasma (Table 4.1). Group 1 was experimentally infected at 116d GA and the lambs were delivered at 125d GA (10 d post-inoculation) while group 2 were infected at 83d GA and delivered at 125d GA (42 d post-inoculation).In the third group *Ureaplasma* was injected at early age (70D GA) and delivered at day 94 (24 d post-infection). Only one lamb at 125 d GA was found to have *Ureaplasma* recoverable from the plasma and this was from the shorter infection group. However, 50% of the lambs in group 3, infected for 24 days, had *Ureaplasma* that could be cultured from their plasma samples. No bacteremia was detected in 125 gestational day animals after 42 day chronic infection. The single positive 125 d GA lamb had 100 CCU/ml viable *Ureaplasma* recovered from their plasma and the range of viable *Ureaplasma* in cord plasma from 94 d GA lambs ranged from 10-10,000 CCU/ml (Figure 4.3).

Table 4.1 Rate of *Ureaplasma* recovery from amniotic fluid and plasma of preterm lambs infected *in utero* with HPA5 for variable durations, *Ureaplasma* infection at early age (70D GA) caused bacteraemia in 50% of infected lambs. While at 125 days only 1 out of 10 animals infected for 10 days had detectable *Ureaplasma*. No bacteraemia was detected in 125 gestational day animals after 42 day chronic infection.

GA infected	GA at delivery	Length of infection	Amniotic fluid	Cord Blood
116 days	125 days	10 days	10/10	1/10
83 days	125 days	42 days	7/8	0/8
70 days	94 days	24 days	8/8	4/8

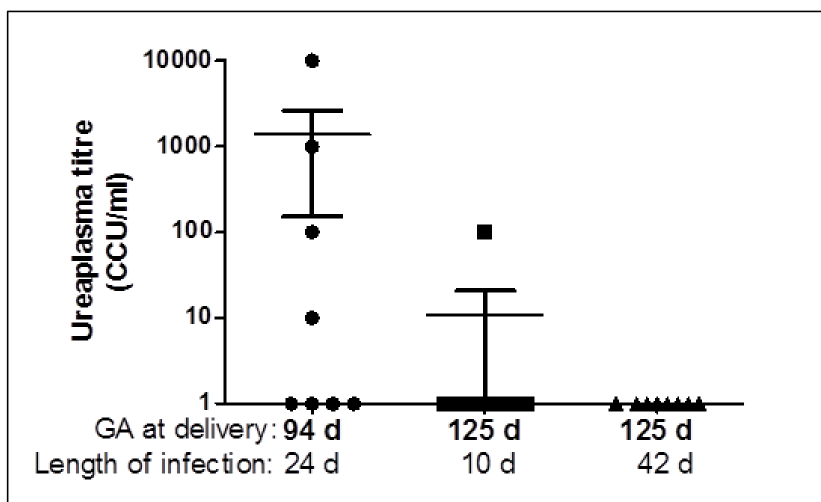

Figure 4.3 *Ureaplasma* titres in cord plasma as determined by culture in lambs delivered at 94 d or 125 d GA. Each point determined in triplicate.

4.3.3 Immunoblot analysis of antibody response in preterm lambs

Anti-*Ureaplasma* antibody response in the sera of all lambs were determined by reactivity of sera by immunoblot to *Ureaplasma* proteins (both the inoculating strain HPA5 and unrelated *Ureaplasma* serovar 6 strain HPA61) separated by non-reducing polyacrylamide electrophoresis. No evidence of anti-*Ureaplasma* antibody response was found for sera following 10 or 24 d infections (groups 1 and 3).While 3/8 of the 125 d GA lambs were found to have anti-*Ureaplasma* antibodies that specifically reacted with the inoculating strain (Figure 4.4). The 50 kDa bands were identified as the major surface antigen (multiple banded antigen) through co-identification with specific monoclonal antibodies (data not shown).

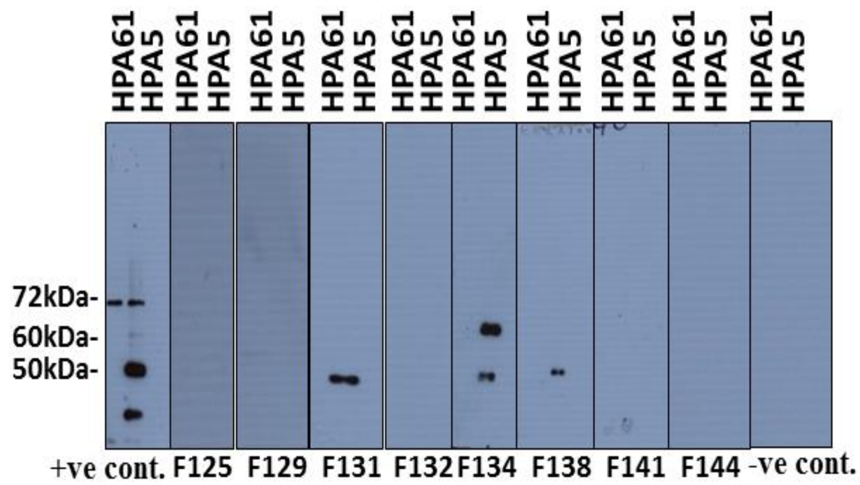

Figure 4.4 Screening sera from preterm lambs (F=foetus) infected with *Ureaplasma* for 42 days and delivered at day 125 GA against the inoculating (HPA5) and antigenically distinct serovar 6 (HPA61).Representative results of 3 repeat experiments shown.

4.3.4 *Ureaplasma*-cidal activity of serum from adult sheep and preterm lambs

The capacity of sera from non-immune adult sheep and preterm sheep of gestational ages 125D and 95D were examined for their ability to kill the *Ureaplasma* strain used to inoculate the pregnant sheep for experimental *in utero* infection. Adult sera, despite the fact that the animals had not been exposed to *Ureaplasma* previously and have no anti-*Ureaplasma* reacting antibodies when tested by Western blot against purified strains, showed strong bactericidal activity against HPA5. Adult sera, at dilutions as low as 6.25%, was capable of completely killing the total input of *Ureaplasma* (Figure 4.5). Whereas, dilutions below 50% of 125D sera showed some survival of challenged *Ureaplasma* and sera from 95D animals only decreased the input *Ureaplasma* by a maximum of 450 +/- 190 fold relative to *Ureaplasma* titres incubated with heat-inactivated sera. Therefore the most immature sera were significantly deficient in bactericidal activity, consistent with bacteraemia being observed in lambs delivered at 94D GA following *in utero* experimental *Ureaplasma* infection above.

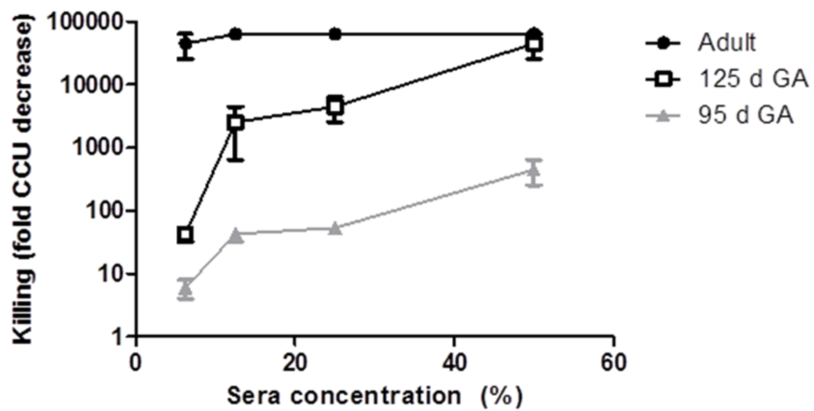

Figure 4.5 *Ureaplasma* killing by diluted non-immune sera from adult sheep (black circle), 125 d GA preterm lambs (open square) and 95 d GA preterm lambs (grey triangles) following 1 h incubation at 37°C. Killing determined by relative decrease in *Ureaplasma* titre compared to incubation with matched heat-inactivated sera. Each point represents average and standard deviation of sera from 4 separate animals.

4.3.5 Kinetics and calcium dependence for sera killing of *Ureaplasma*

The rate of bactericidal activity of 50% non-immune sera from adult sheep, and 95 d GA preterm lambs were examined by stopping complement activation with the addition of the calcium and magnesium chelator EDTA following varying lengths of incubation with sera. The residual survival of *Ureaplasma* was then assessed by removing the sera and resuspending in culture medium. The kinetics of *Ureaplasma* killing by both adult and preterm sera were comparable: the majority of bactericidal activity occurred in the first 10 minutes following serum addition (Figure 4.6), with a negligible increase in *Ureaplasma* killing for preterm sera even if incubated for an additional 4 hours (data not shown). Isolated function of the alternative complement pathway can be assessed if serum killing is performed in the presence of calcium-chelator EGTA. Under these conditions, no alternative pathway killing was observed for *Ureaplasma* indicating killing is mediated by the classical or lectin pathways.

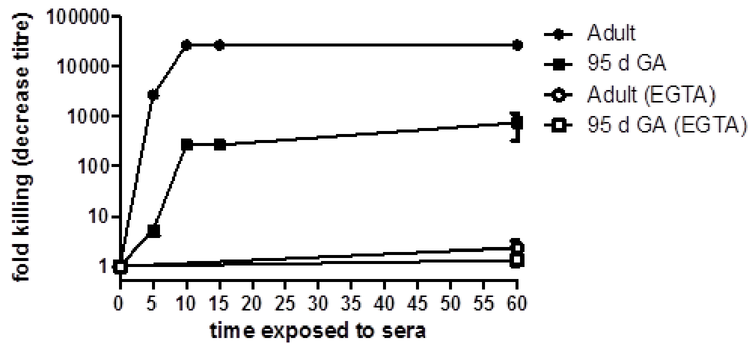

Figure 4.6 *Ureaplasma* killing kinetics of non-immune adult sheep and 95 d GA sera. Fifty percent dilutions of sera in the presence of calcium and magnesium (all complement pathways active, black circle = adult, black square = 95 d GA lamb) were incubated with *ureaplasma* for various times before blocking further complement activation with addition of EDTA prior to titration of surviving *Ureaplasma.* No killing of *Ureaplasma* was observed if sera was (adult = grey circle, 95 d GA = grey square) diluted in the presence of EGTA and magnesium (only alternative complement pathway active). Each point represents average and standard error of sera from 4 separate uninfected animals, killing calculated by decreased titre relative to *Ureaplasma* titre following incubation with matched heat-inactivated sera.

4.3.6 Investigation of serum susceptibility of recovered plasma and amniotic fluid *Ureaplasma* strains

Ureaplasma strains recovered from plasma were examined to determine if they were less susceptible to serum killing than strains recovered from amniotic fluid from bacteraemic and non-bacteraemic lambs. Recovered isolates were separated into 3 group: (1) Plasma-recovered isolates; (2) amniotic fluid recovered isolates from the same animals that had *Ureaplasma* recovered from their plasma; and (3) amniotic fluid-recovered isolates from animals that did not have bacteraemia. Susceptibility to 50% adult sheep sera and 50% 95 d GA sera from control uninfected animals are shown in Figure 4.7. Isolates cultured directly from plasma were as susceptible to sera as amniotic fluid-recovered isolates from matched bacteraemic or non-bacteraemic animals. All recovered isolates were equally resistant to non-immune sera from uninfected control 95 d GA, except (paradoxically) for one of the plasma-derived isolates from lamb (115).

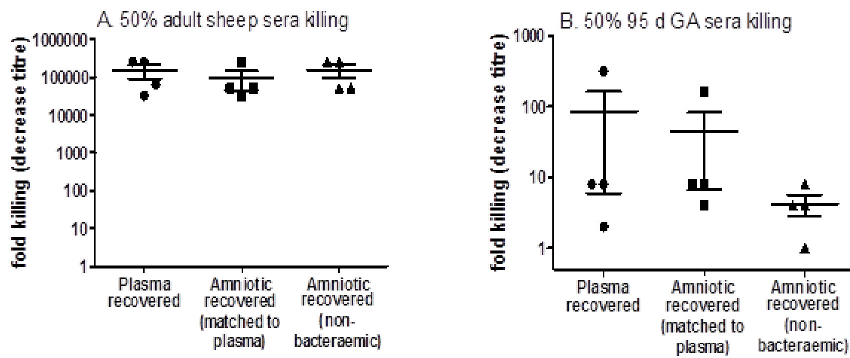

Figure 4.7 Serum susceptibility of plasma-recovered and amniotic fluid-recovered strains (infected for 24 days) to non-immune sera from 50% adult sheep or 50% 95 d GA preterm lamb sera. Pooled sera from uninfected animals 95 days and adult were used and each point was determined in duplicate. Representative data shown from 3 repeated experiments. Killing calculated as decreased titre relative to *Ureaplasma* titre following incubation with matched heat-inactivated sera.

4.3.7 *Ureaplasma*-cidal activity of sera from bacteraemic versus non-bacteraemic lambs.

As no advantage of serum resistance in plasma isolates was observed, the capacity of the sera from these two groups of 94 d GA preterm lambs was compared for their capacity to kill HPA5 parental strain inoculated into the pregnant sheep. Endogenous *Ureaplasma* in bacteraemic sera was reduced by filtration through a 0.22 micron filter and the remaining endogenous *Ureaplasma* was measured by titration and subtracted from the matched results at the conclusion of the assay. The serum killing from the 94 d GA *Ureaplasma*-infected animals was higher for non-bacteraemic than bacteraemic with one exception (lamb 116) (Figure4.8). However, the overall range for both non-bacteraemic and bacteraemic lambs reflected the range found for serum taken from uninfected 95 d GA control animals. However, it was noted for the serum with the lowest killing was from the animal with the highest plasma titre of *Ureaplasma* (lamb 115) and that the serum from the animal with the highest serum killing was from the animal with the lowest plasma titre (lamb 116). As no anti-*Ureaplasma* antibodies were present in the immunoblot analysis (data not shown), this suggested that killing capacity of the sera was directly linked to *Ureaplasma* titres in the serum.

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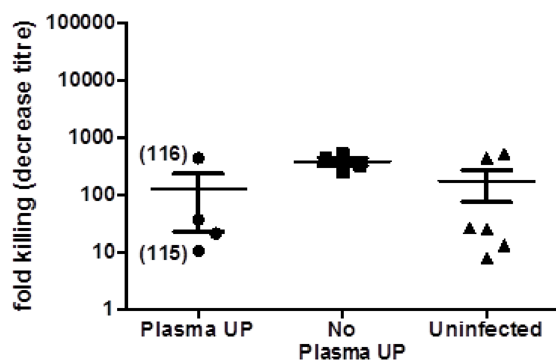

Figure 4.8 Serum killing of *Ureaplasma* original strain by 50% sera from bacteraemic (plasma *Ureaplasma*), non-bacteraemic (no plasma *Ureaplasma*) and uninfected 95 d GA preterm lambs. Each point represents the average of 3 separate killing assays. Killing calculated as decreased titre relative to *Ureaplasma* titre following incubation with matched heat-inactivated sera. Individual points for lambs 115 and 116 are identified

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4.3.8 Complement activity of sera from bacteraemic versus non-bacteraemic lambs.

The complement activity (measured by lysis of guinea pig erythrocytes) was examined for the sera from infected and uninfected 95 d GA lambs. The complement activities in the serum from bacteraemic lambs were much lower than those found to have no *Ureaplasma* in their plasma (non-bacteraemic). Furthermore the lowest complement function was observed in the lamb with the highest *Ureaplasma* titre (lamb 115; 10^4 culture units/ml in plasma) while highest complement activity for the bacteraemic group was found in the lamb with the lowest *Ureaplasma* plasma titre (lamb 116; 10 culture units/ml in plasma; (Figure 4.9A). The complement activity for the non-bacteraemic *Ureaplasma* infected lambs was all relatively uniform and higher than the average for the bacteraemic lambs (Figure 4.9B). Complement function for both groups were representative of the range observed from age matched (95 d GA) uninfected lambs (Figure 4.9C), indicating *Ureaplasma* infection (or bacteraemia) did not influence the overall development of the complement system. A correlation between *Ureaplasma*-cidal activity and complement activity in 95 d GA sera, irrespective of experimental *Ureaplasma* infection, was found (R2=0.30, $p<0.05$; Figure 4.10). Furthermore, complement function of 125D GA serum, which was shown to have significantly greater *Ureaplasma*-cidal activity was found to be higher than all 95 d GA serum tested, but not as high as adult sera (Figure 4.9D).

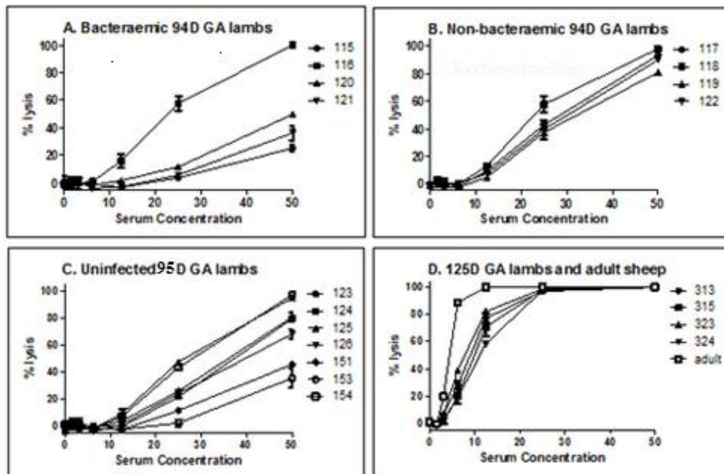

Figure 4.9 Complement activity in bacteraemic (A), non-bacteraemic (B) and uninfected (C) 95 d GA preterm lambs serum. Complement activity for 125 d GA preterm lambs and adult sheep (D) are also shown. Complement activity measured as haemolysis of rabbit antibody-sensitised guinea pig erythrocytes by diluted sera after 30 min incubation at 37oC. Each point determined in triplicate.

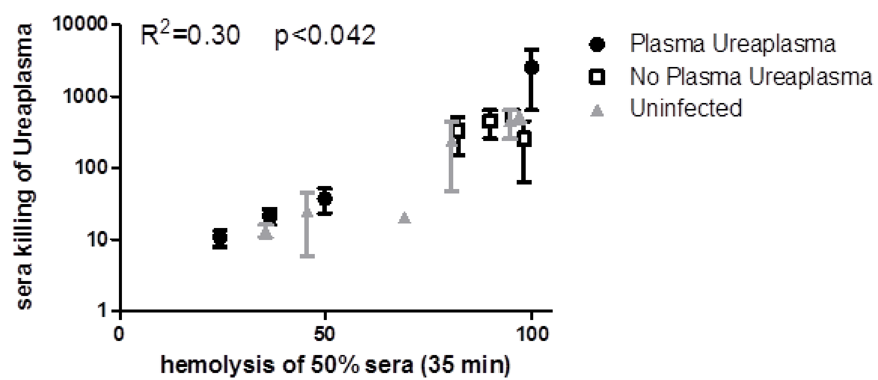

Figure 4.10 Correlation between complement activity (haemolysis) and 50% serum killing of 95 d GA sera (50% dilution, 30 min incubation at 37°C for both conditions) from bacteraemic (plasma Ureaplasma), non-bacteraemic (no plasma Ureaplasma) and uninfected preterm lambs. Each point determined in triplicate. Spearman's coefficient (R2=0.30, p<0.05) shown for untransformed data.

4.4 Discussion

Neonates, particularly those born prematurely, are known to be more susceptible to morbidity and mortality due to infections. The innate immune system represents the primary defence against infection in neonates and the complement system is a significant component of this defence. Term human neonates are known to have an under-developed complement system relative to adults, and complement insufficiency increases with increasing prematurity (McGreal, 2012). Previously I developed a haemolysis assay to show that sheep could be used to model the developmental complement insufficiency of prematurity (Ahmed *et al*., 2015) and here I extended this model to examine the role of complement insufficiency in an experimental intrauterine bacterial infection. Consistent with the *in vitro* complement function assays, the *in vitro* bactericidal capacity of sera diminished with increasing prematurity. This was also reflected by the increased incidence of bacteraemia detected in experimentally infected lambs at 94 d GA (50%) relative to 125 d GA (10%) lambs. Within the 94 d GA cohort, the bactericidal serum activity was found directly correlated to the complement function in the serum (R2=0.30, $p<0.05$) with one exception, lambs with bacteraemia were found to have lower complement function compared to non-bacteraemic lambs. Further, this one exception was also found to have the lowest plasma titre of *Ureaplasma*. The correlation between *Ureaplasma* bactericidal activity and complement function was not influenced by experimental infection, suggesting that the natural variation in complement activity at 94 d GA determined whether *Ureaplasma* infection in the amniotic fluid became systemic or not.

The only group found to develop specific anti-*Ureaplasma* antibodies was infected at 83 d GA and delivered at 125 d GA, and only 3 of 8 animals were found to respond. Inability to sample fetal blood except at delivery makes it impossible to determine if the seropositive

group represent those that were bacteraemic at 94 d GA, but it is a tempting speculation as it is just short of 50% of the infected animals. The anti-*Ureaplasma* antibodies are of fetal immune origin, as we have previously shown no maternal transfer of IgG occurs in uninfected sheep (Ahmed *et al.*, 2015) and experimental *Ureaplasma* infection did not alter this as we found no characteristic maternal anti-guinea pig erythrocyte reactivity in the sera from any of the preterm lambs (data not shown).

The HPA5 strain has previously been shown to be susceptible to human serum killing in the absence of specific anti-*Ureaplasma* antibodies (Beeton *et al.,* 2012). As with human serum studies with this strain, sheep serum bactericidal activity appears to be mediated by the classical pathway; however, no reagents to examine the lectin pathway (also calcium-dependent) in sheep are available and the contribution of the ovine lectin pathway to *Ureaplasma* killing cannot be ruled out. It was unexpected that the kinetics for both adult and preterm sheep serum killing was identical, rather than a prolonged killing curve for the preterm serum. However, no significant increase in *Ureaplasma* killing was observed if incubation with sera was increased from 10 min and 4 h for either adult or preterm sheep sera.

The use of sheep as a non-primate model of human disease is increasing, especially for studies of disease in pregnancy and pregnancy complications. For example, neonatal asphyxia induced by transient occlusion of the umbilical cord before birth has been shown to lead to complement-mediated reperfusion injury and to mimic the intrauterine hypoxia that complicates a proportion of human births (Castillo-Melendez *et al.,* 2013). Further, hypoxic injury following preterm birth and associated newborn respiratory disease is also studied in sheep models (Wolfson *et al.,* 2012). More recently, sheep models have been developed to investigate intrauterine inflammation induced by injection or infusion of lipopolysaccharides or microbial infection (Dean *et al.,* 2011; Hillman *et al.,* 2014; Kemp , 2011; Maneenil *et al.,* 2015;

Zhang *et al.,* , 2012) . None of these studies has investigated the contribution of the complement system to the disease pathogenesis or to the role of complement in controlling systemic spread, largely due to the current lack of knowledge on the sheep complement system.

In conclusion, I provided the first detailed kinetic analysis of establishment of *Ureaplasma* infection in sheep amniotic cavity following experimental infection. Amniotic fluid titres of *Ureaplasma* rapidly increased to maximal levels by 48 hours post inoculation that was stable for weeks following the infection. *Ureaplasma* could be cultured from 50% of cord blood (indicative of systemic spread of amniotic fluid infection) for Caesarean-delivered lambs that were infected at 70 d GA and delivered at 94 d GA, but only from 10% of lambs infected at 116 d GA and delivered at 125 d GA. No anti-*Ureaplasma* immune response was observed in either of these groups and serum complement function was directly related to the *Ureaplasma* titres in the plasma *in vivo* and the capacity of preterm lamb sera to kill *Ureaplasma in vitro*. Lambs that were experimentally infected at 83d GA and delivered at 125 d GA did not have any *Ureaplasma* detected in their plasma and only 3 of 8 of those infected for 42 days had evidence for an antibody response to *Ureaplasma*; however, presence of anti-*Ureaplasma* in sera had no effect on the amniotic fluid titres measured at delivery.

CHAPTER 5

Characterisation of monoclonal antibodies used for typing different strains of *Ureaplasma* species

5 Characterisation of monoclonal antibodies used for typing different strains of *Ureaplasma* species.

5.1 Introduction

Ureaplasma species are the smallest, free living mucosal parasites that inhabitant the human urogenital tract. It can be isolated from 40-70% of sexually active women and has been reported to increase to as high as 90% during pregnancy but drop to 25% after menopause according to reports, while in men it is found to a lesser extent (19.2 %) and in can be isolated in 5 % of children where girls are about eight times more infected than boys (Foy *et al*., 1975; Iwasaka *et al.,* 1986*;* Viscardi, 2010; Volgmann *et al.,* 2005,). *Ureaplasma* play a role in diseases of the genital tract and adverse pregnancy outcomes and fetal and neonatal pathological sequel (Cassell *et al.*, 1993; Kramer, 2011). Following Shepard's initial differentiation of *Ureaplasma* from *Mycoplasma* based on agar plate colony morphology (T for "tiny" colony, rather than *Mycoplasma* "fried egg" morphology) (Shepard, 1954), a maximum separation into 14 "serovars" was subsequently published by Robertson and Stemke in (1982) using a panel of polyclonal rabbit anti-sera and a combination of modified metabolic inhibition test and a colony indirect epifluorescence methods. In the years that followed, it became clear that these 14 serovars could be grouped into two separate sub-types that were initially called "biovar" 1 and 2(Robertson and Stemke, 1982). However, in 2002 Robertson *et al*., utilised conserved differences in DNA-DNA hybridisation, distinctive restriction fragment length polymorphism(RFLP) patterns, and other genomic differentiators to divide the 14 serovars into two distinct species: *U. parvum* (serovars 1, 3, 6 and 14) and *U. urealyticum* (serovars 2, 4, 5, and 7-13)(Robertson *et al.,* 2002). A conserved 45 bp size difference in a PCR amplicon using primers recognising the promoter and coding region of the major surface protein (multiple banded antigen; MBA) was found capable of separating clinical *U. parvum* from *U. urealyticum* strains (Teng, 1994). These authors also found that different sized amplicons for related

primer sets in this region could also separate *U. urealyticum* serovars 2, 5, 7, 8, 9 and 11 from *U. urealyticum* strains 4, 10, 12 and 13, as well as uniquely identifying *U. parvum* serovar 6 from all other isolates(Teng, 1994) .The MBA is a lipid-anchored protein that is expressed on the surface of *Ureaplasma* and is composed of a signal peptide, a lipid addition signal sequence and a relatively well conserved non-repeating region of approximately 100 residues at the N-terminus. However, the C-terminus region is composed of repeats that vary in sequence between serovars and in repeat number amongst strains of the same serovar (Zheng *et al*., 1996). Kong *et al.,* (2000) found that the predicted amino acid sequence for the repeat region of each *U. parvum* serovar was slightly different and that *U. urealyticum* serovars could be separated into a unique serovar 10 repeat (genotype B) and two groups (with identical MBA N-terminal repeats) encompassing serovars 2, 5 and 8 (genotype A) and serovars 4, 12 and 13 (genotype C), but that serovars 7, 11 (genotype E) and 9 (genotype D) could not be differentiated by MBA repeat sequence in their study. Development of monoclonal antibodies against the MBA have also been reported (Cheng et al., 1993; Cheng *et al.*, 1994; Naessens *et al*., 1998; Watson *et al.*, 1990; Zheng *et al*., 1996),some of these recognise single serovars, while others recognise groups of *U. parvum* or *U. urealyticum* sub-groups. However, all of these antibodies belong to independent research groups and are not readily available. In this chapter I provide the first characterisation of commercially available monoclonal antibodies and I show results for the first single monoclonal antibody capable of specifically detecting the MBA of all *U. parvum* and *U. urealyticum* strains simultaneously.

5.2 Aims

1-To find the regions and similarities between the *MBA* gene within the best characterised *Ureaplasma* species serovar 3 and serovar 10 relative to the other prototype strains to localise and define the C-terminal repeats characteristic for the remaining serovars.

2-Characterisation of a panel of commercially available monoclonal antibodies raised against the MBA for binding and differentiation of prototype strains of *Ureaplasma parvum* and *Ureaplasma urealyticum*.

3-To develop a recombinant serovar 3 MBA expressed in *E.coli* to confirm specificity and to determine the capacity of monoclonal antibodies to be used for immunohistochemistry.

5.3 Results

5.3.1 Defining the regions and similarities among the *MBA* gene within the best characterised *Ureaplasma* type: serovar 3

First it is important to define the regions and similarities among the *MBA* gene within the best characterised *Ureaplasma* type: serovar 3. There are 3 fully annotated genomes available for serovar 3 in GenBank (strains 700970, 27815 and SV3F4)(Figure5.1) . The first 2 strains have a single *MBA* gene copy in their genomes, while the clinical strain SV3F4 has 2 identified copies (Figure5.2). However, only one of these *MBA* genes in the latter sequence aligns perfectly to the first two strains (Wu *et al*., 2014). Kong *et al.* (2000) had identified serovar specific repeats for *U. parvum* that allows the identification of the *MBA* genes in the whole shotgun sequence database (Table 5.1) as well as many of the *MBA* genes for *U. urealyticum*

(Kong *et al*., 2000). Table 5.1 shows lists of the prototype serovar strains with available sequences in GenBank and the identification of patterns consistent with the genotype classification of Kong *et al.* (2000), including some of my proposed modifications. The analysis of the *MBA* genes is difficult as most of the available genes are from shotgun sequences that have not been completed to seal the genome; therefore some sequences may reflect sequencing fragments. It is well known that repeats regions, like the C-terminus of the *MBA* gene, cause a break in the contig assembly. Many of the putative *MBA* genes also do not start with a consensus sequence which would include the lipid anchor sequence. Comparing the consensus *U. parvum* serovar 3 N-terminus to the only completely sequenced and annotated *U. urealyticum* genome (serovar 10) identifies a single MBA protein with the C-terminal repeat TQPGSGST. The alignment of the N-terminal region of this protein compared to the consensus N-terminus from serovar 3 is shown in Figure 5.3. Cross-examining the *U. urealyticum* MBA protein database with this consensus N-terminus sequence from serovar 10 MBA identified the best candidates for MBA from the remaining *U. urealyticum* serovars listed in (Table 5.2).

CLUSTAL O(1.2.1) multiple sequence alignment

```
27815       MKLLKNKKFWAMTLGVTLVGAGIVAIAASCSNSTVKSKLSNQFAKSTDGKSFYAVYEIEN
700970      MKLLKNKKFWAMTLGVTLVGAGIVAIAASCSNSTVKSKLSNQFAKSTDGKSFYAVYEIEN
SV3F4_1     MKLLKNKKFWAMTLGVTLVGAGIVAIAASCSNSTVKSKLSNQFAKSTDGKSFYAVYEIEN
            ************************************************************

27815       FKDLSNDDKKSLSNIEFNAALTSAENKTESTLEKGHLVGEKIYVKLPREPKPNEQLTIIS
700970      FKDLSNDDKKSLSNIEFNAALTSAENKTESTLEKGHLVGEKIYVKLPREPKPNEQLTIIS
SV3F4_1     FKDLSNDDKKSLSNIEFNAALTSAENKTESTLEKGHLVGEKIYVKLPREPKPNEQLTIIS
            ************************************************************
                                        ↓Start of repeats
27815       KSGLIKTSGLLISDNLNYQTEKVNFETTQPGKEQPAGKEQPAGKEQPAGKEQPAGKEQPA
700970      KSGLIKTSGLLISDNLNYQTEKVNFETTQPGKEQPAGKEQPAGKEQPAGKEQPAGKEQPA
SV3F4_1     KSGLIKTSGLLISDNLNYQTEKVNFETTQPGKEQPAGKEQPAGKEQPAGKEQPAGKEQPA
            ************************************************************

27815       GKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPA
700970      GKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPA
SV3F4_1     GKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPA
            ************************************************************

27815       GKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQ------PA
700970      GKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPA
SV3F4_1     GKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKEQPAGKETTGK-----------

            *********************************************  .
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Figure 5.1 Alignment of amino acid sequences for serovar 3 strains 700970, 27815 and SV3F4. The non-repeating region is present in the N-terminal 129 residues that are then followed by multiple repeats of PAGKEQ repeats in the C-terminus.

NCBI Blast 2 sequences alignment

```
MBA#1  1    MKLLKNKKFWAMTLGVTLVGAGIVAIAASCSNSTVKSKLSNQFAKSTDGKSFYAVYEIEN
60
            MKLLKNKK W +T+   L+G  ++   ASCS+S ++SKLS++  +  D  ++Y  Y+I N
MBA#2  1    MKLLKNKKIWVLTISSVLIGVSVITSIASCSSSNIESKLSDKLVEDEDN-NYYVAYQISN
59

MBA#1  61   FKDLSNDDKKSLSNIEFNAALTSAENKTEST------LEKGHLVGEK---IYVKLPREPK
111
            ++ L + +KK L  + F+ ++T  + KT+++      + +G L  ++    Y+KL R+P+
MBA#2  60   YEKLKDFEKKQLEKVTFSTSITPTD-KTKNSQVFNAKVNEGILFNQQAKTFYIKLVRKPE
118

MBA#1  112  PNEQLTIISKSGLIKTSGLLISD-NLNY  138
              E + I+  + ++KT G ++++ NLN+
MBA#2  119  VGESIIILPNNSILKTHGFVVTNENLNF  146
```

Figure 5.2 Alignment of the two MBA proteins in strain SV3F4 Only 33% identity was found with 8% gaps for the N-terminus region only. The second MBA protein also lacked a C-terminal repeat region.

CLUSTAL O(1.2.1) multiple sequence alignment

```
SV10      MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
SV3       MKLLKNKKFWAMTLGVTLVGAGIVAIAASCSNSTVKSKLSNQFAKSTDGKSFYAVYEIEN
          ***********:**********:**:*****.*.******.*:.**.* *******:***

SV10      FDDLNENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
SV3       FKDLSNDDKKSLSNIEFNAALTSAENKTESTLEKGHLVGEKIYVKLPREPKPNEQLTIIS
          *.**.::***:*.: ***.*:********.:  ****: :*********** :******.

SV10      KGGLLKTASLVLPDNLNYQTENIGFETQPGSGSTTQP
SV3       KSGLIKTSGLLISDNLNYQTEKVNFETTQPGKEQ---
          *.**:**:.*:: ********:: ***   . .
```

Figure 5.3 Alignment of N-terminus of confirmed serovar 10 MBA protein against the conserved serovar 3 MBA protein N-terminus However only 109/151(72%) of the residues were identical, consistent with species divergence.

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Table 5.1 Analysis of multiple banded antigen sequences available in GenBank.

Species	Serovar	GenBank number	Accession number	Repeat amino acid sequence
U. parvum	1	27813 (1 gene)	AAD09744.2	PGKEQQ
U. parvum	3	700970 (1 gene), 28715 (1 gene) SV3F4 (2 genes-only 1 with repeat)	AAF30784.1; ACA32903.1; BA073668.1	PAGKEQ
U. parvum	6	27818 (1 gene)	AAD09745.2	PGKE
U. parvum	14	33967 (3 genes)	AAD09743.2; EDT87551.1; EDT87494.1;	PAGKEQQ
U. urealyticum	10	33699 (6 genes-only 2 with repeat)	AAD02692.2; ACI60338.1	TQPGSGST
U. urealyticum	2	27814 (10 genes-only 1 with repeat)	AAD02696.2	TKPGSGET
U. urealyticum	5	27817 (6 genes-only 2 with repeat)	AAD02698.2; EDU06213.1	TKPGSGET
U. urealyticum	8	27618 (10 genes – only 1 with repeat[2])	AAD02700.2	TKPGSGET
U. urealyticum	9	33175 (3 genes-only 2 with repeat)	AAD02701.2; EDX53694.1	KKPETG-STEGGS[1]TEG
U. urealyticum	4	27816 (2 genes)	AAD02697.2; EDY74356.1	TSPEKPGNGT
U. urealyticum	12	33696 (4 genes – only 2 with repeat[3])	AAD02694.2; EDX53195.1[3]	TSPEKPGNGT
U. urealyticum	13	33698 (1 gene)	AAD02695.2	TSPEKPGNGT
U. urealyticum	7	27819 (5 genes-only 1 with repeat[4])	EDU56636.1[4]	GAQSGNGSGSASGS?
U. urealyticum	11	33695 (3 genes-only 1 with repeat)	EDU67198.1	GAQSGNGSGSASGS[4]

1. My analysis has found that the repeat is not completely conserved and that this residue is either an S or a T to detect all copies of the repeat in the C-terminus for gene UUR9_0631.

2. There are 3 other serovar 8 MBA genes that appear to have the conserved N-terminus, but without identifiable repeat.

3. One of the genes with the expected C-terminus repeat unit does not start with the homologous N-terminus sequence but instead begins with MNYQTEKVEFETQPGNGT

4. Only one of the serovar 11 MBA genes has the conserved N-terminus and it has a C-terminus repeat of GAQSGNGSGSASGS, and the serovar 7 MBA gene with this conserved C-terminus repeat does not start with the homologous N-terminus sequence but instead begins with MNYQTEKVDFKSTHTPEPTPTPTPAPKEDKAVVSN-VEFSDVDAKAKTAKVKLTFALVVQLKDESQKLLKL

Table 5.2 MBA protein sequences similar to the conserved N-terminus of serovar 10 MBA

Serovar	Accession number	Identity	Repeat
SV5 (27817)	EDU06213.1	148/151	TKPGSGET
SV5 (27817)	AAD02698.2	148/151	TKPGSGET
SV2 (27618)	AAD02696.2	148/151	TKPGSGET
SV8 (27618)	AAD02700.2	148/151	TKPGSGET
SV4 (27816)	EDY74356.1	147/150	TSPEKPGNGT
SV4 (27816)	AAD02697.2	147/150	TSPEKPGNGT
SV13 (33698)	AAD02695.2	147/150	TSPEKPGNGT
SV12 (33698)	AAD0694.2	147/150	TSPEKPGNGT
SV12 (33698)	EDX53543.1	144/149	AEVDKLTKLIKEKEAKVAEVQKAIEE-GEKVVKTKQATKTTAENELTAAQKVVDDK-KAEKTTLDKELADAKAQLDKANKKDPKDQ
SV2 (27814)	EEH02498.1	139/143	None
SV9 (33175)	AAD02701.2	141/146	KKPETGSTEGGSTEG
SV9 (33175)	EDX53694.1	141/146	KKPETGSTEGGSTEG
SV8 (27618)	EEH01641.1	141/146	KEEKVKAVKAKLDAAEKELTTAKS-DVETKQKAVDAAKTKLDQATKESTDANQS-LETAKENQKTKQQAVDSAKEELKVAEGSTEN
SV8 (27618)	EEH01837.1	141/146	KEEKVKAVKAKLDAAEKELTTAKS-DVETKQKAVDAAKTKLDQATKESTDANQS-LETAKENQKTKQQAVDSAKEELKVAEGSTEN
SV2 (27814)	EEH01994.1	140/145	NKDEKIQKAEEKLAKAKEELNKVNELVTT-KDTAVKEATKALETAKADAKTKEAAVTTA-TRELEEAKKSNEA
SV13 (33698)	EDT49352.1	143/149	None
SV11 (33695)	AAD02693.2	137/147	None
SV7 (27819)	AAD02699.2	137/147	None
SV7 (27819)	EDU56613.1	137/145	None
SV11 (33695)	EDU67198.1	137/147	GAQSGNGSGSASGS

As shown from (Table 5.2) serovars 2, 5 and 8 had the closest homology to the serovar 10 N-terminus with only 3 polymorphisms. Moreover, all these homologues had the expected TKPGSGET repeat, and it is interesting that serovar 5 had two copies in the genome. The next closest group of homologues included serovars 4, 12 and 13 with the expected TSPEK-PGNGT repeat. However, one variant of SV12 with a long 84 residue repeat was also found in the database. Two unusual MBA homologues with a 84 residue repeat different from SV12 above were found for serovar 8 and a variant of serovar 2 with a 71 residue repeat. MBA homologues with no identifiable repeat in the C-terminus were found for two serovar 7 MBA genes, as well as putative genes for serovar 2, 11 and 13. The potential repeat of GAQSGNGSGSASGS was identified for serovar 11 in an MBA homologue, but the potential MBA protein with this repeat for serovar 7 does not retain high homology for the N-terminal sequence. Furthermore, two different MBA putative proteins were identified for serovar 10 (ACI60338.1 and AAD02692.2) were found in the database with 100% identity, both with the expected TQPGSGST repeat. However, variants (other than the validated annotated serovar 10 genome) of the *U. urealyticum* may be due to errors in the shotgun genome sequencing data.

5.3.2 Visualisation of MBA proteins from *U. parvum* by Western blot

Using the above prototype strains, I next utilised Western blot to visualise MBA proteins from *U. parvum* (Figure 5.4) using some of the monoclonal antibodies (mAbs) previously published by Watson *et al.,* (1990) (Table 5.3) to validate the panel of monoclonal antibodies to be tested from Virostat plc. For those monoclonal antibodies that detected all *U. parvum* species, the reactivity against all *Ureaplasma spp*. was then examined (Figure 5.5) The previously published (Watson *et al.,* 1990) mAbs, behaved mostly as expected: 5B1.1 detected only serovars

3 and 14, 10C6.6 detected only serovar 3, and 8A1.2 did not react with any of the *U. parvum* MBAs (Figure 5.4). 8B5.2 did not react as well as expected and did not seem to detect serovar 3. However, equivalent patterns to the previously published monoclonal antibodies were found in the commercially available antibodies: 6525 behaved exactly as 5B1.1 (although in one experiment it did seem to weakly cross-react with serovars 1 and 6; Figure 5.5), while 2 other mAbs (4H2 and 2G9) reacted in a mirror fashion by detecting the MBAs of serovar 1 and 6, but not serovars 3 and 14. However, three of the commercial mAbs detected all the *U. parvum* MBA species equivalently (6522, 6523 and 2B11), performing better than the previously published mAb 8B5.2 (Figure 5.4). These antibodies differed when extended to detecting the MBAs from *U. urealyticum* prototype strains (Figure 5.5). The reactivity of 2B11 appears to be restricted only to *U. parvum* MBA, while 6523 reacted strongly with *U. parvum* MBA and weakly with *U. urealyticum* serovars 7 and 11. However, 6522 appeared to detect the MBA from both *U. parvum* and *U. urealyticum* equivalently, although the intensity of the MBA from serovar 4 was negligible (Figure 5.5) or weak (Figure 5.6). Moreover, 6522 detected a range of MBA from clinical isolates (Figure 5.6). Identical MBA species were detected by both serovar 10 specific antibody 8A1.2 (Figure 5.7) and 6522 (Figure 5.6) confirming that the reactivity of the commercial antibody is restricted to the MBA. The summary for the reactivity of the commercial antibodies is shown in (Table5.4).

Table 5.3 Reactivity of monoclonal antibodies provided by Prof. Gail Cassell.

Clone	Recognised species	Published reactivity
8B5.2	*U. parvum* *U. urealyticum*	SV1, 3, 6, and 14 strongly Weakly all except SV2 and 5
10C6.6	*U. parvum*	SV3 only
5B1.1	*U. parvum*	SV3 and 14
8A1.2	*U. urealyticum*	SV10 only

Table 5.4 Reactivity of commercial Virostat monoclonal antibodies.

Clone	Recognised species	Published reactivity
6522	*U. parvum* *U. urealyticum*	SV1, 3, 6, and 14 strongly All strongly, except SV4 weakly
6523	*U. parvum* *U. urealyticum*	SV1, 3, 6, and 14 strongly SV7 and 11 weakly
6525	*U. parvum*	SV3 and 14
2B11	*U.parvum* *U. urealyticum*	SV1, 3, 6, and 14 strongly No reactivity
2G9	*U. parvum*	SV1 and 6
4H2	*U. parvum*	SV1 and 6

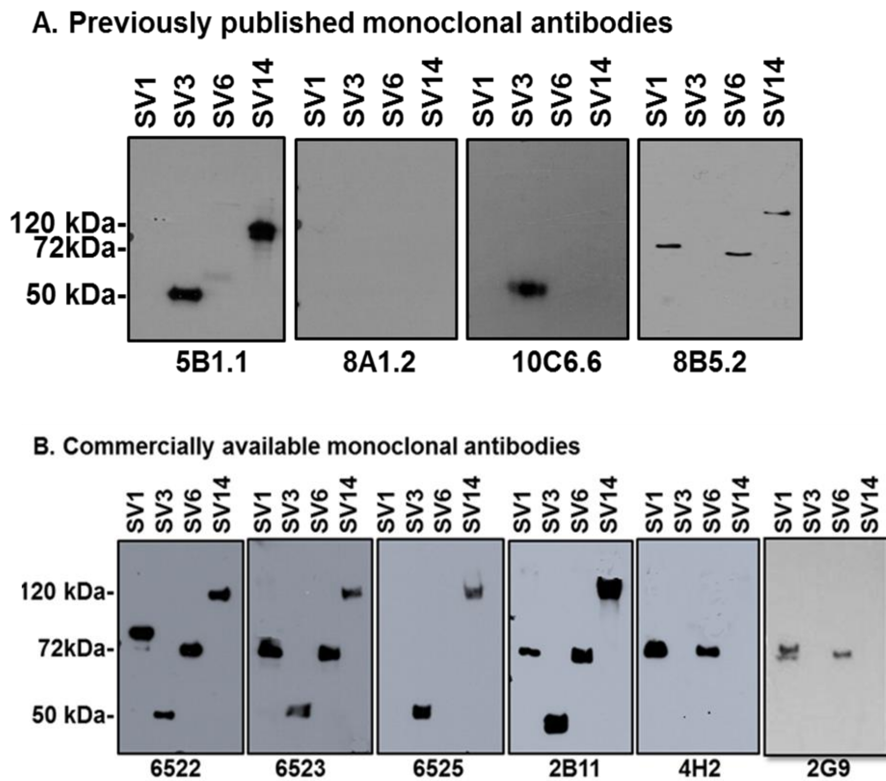

Figure 5.4 Western blot analysis of whole bacterial proteins from prototype *U. parvum* serovars A. MBA proteins detected by previously characterised antibodies from Watson *et al.* (2000) separated on a 7.5% polyacrylamide gel. B. MBA proteins detected by Virostat plc. monoclonal antibodies on a 7.5% polyacrylamide gel. Representative blots shown for multiple repeats. Molecular mass for each serovar was maintained for each detected MBA, validating the reactivity of the monoclonal antibodies.

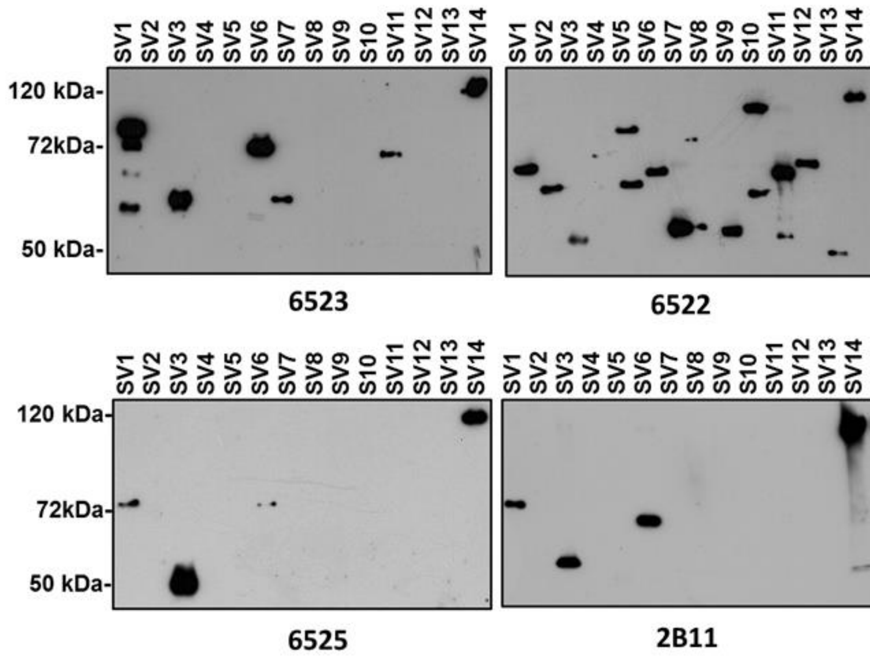

Figure 5.5 Western blot analysis of whole bacterial proteins from prototype *U. parvum* and *U. urealyticum* strain separated on a 7.5% polyacrylamide gel and transferred to nitrocellulose prior to probing with commercial antibodies 6522, 6523, 6525 and 2B11. Relative molecular mass of MBA bands for specific serovar 1, 3, 6, 7, 11 and 14 strains where identical when detected by more than one antibody. Representative blot from multiple repeats of the experiment are shown.

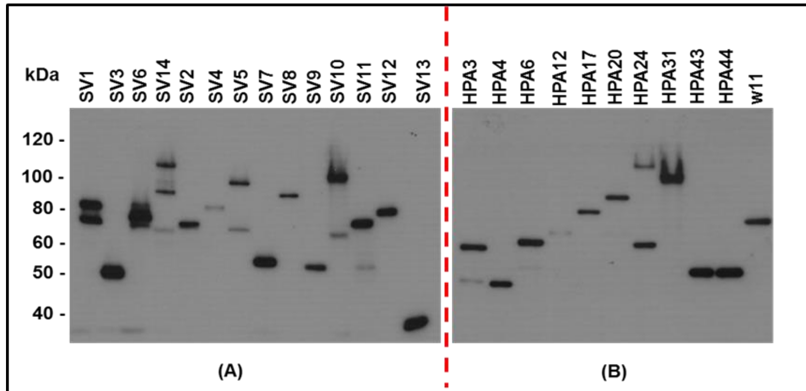

Figure 5.6 Western blot analysis of whole bacterial proteins from prototype *U. parvum* and *U. urealyticum* strains (A) and 11 clinical isolates (B) by 6522 following separation on a 7.5% polyacrylamide gel and transfer to nitrocellulose. Figure reprinted from thesis of Ali Aboklaish with permission.

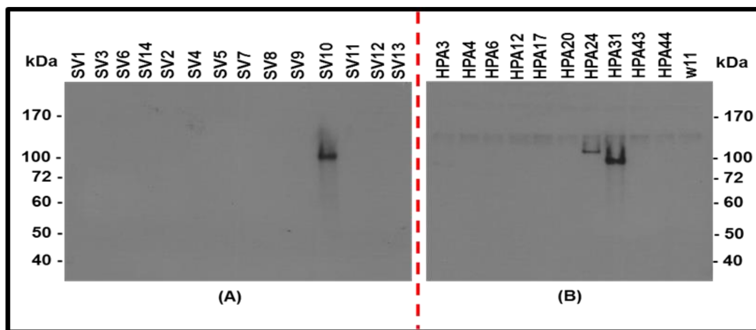

Figure 5.7 Identical Western blot analysis of whole bacterial proteins from prototype *U. parvum* and *U. urealyticum* strains as in Figure 5.6except that it was probed with the serovar 10 specific mAb 8A1.2 Comparison of the detected MBA species shows exactly the same pattern as 6522 for those strains. Sequencing of C-terminus of clinical isolates HPA24 and 31 confirmed they were the only strains with the unique TQPGSGST repeat.Figure reprinted from thesis of Ali Aboklaish with permission.

5.3.3 Use of monoclonal antibodies to detect serovar 3 by immunohistochemistry

The capacity of these antibodies to detect serovar 3 by immunohistochemistry was also investigated. However, this is technically very difficult as the size of individual *U. parvum* bacteria is between 200-400 nm and below the resolution of a light microscope. Therefore, a scaled up culture of characterised serovar 3 strain HPA5 was pelleted at 17,000 xg and the pellet was embedded in paraffin wax, sectioned and processed for peroxidase-conjugated secondary staining using the monoclonal antibodies. As the pellet was colourless, blue agar beads were added to the pellet prior to embedding to locate the pellet. Antigen recovery steps utilising boiling citrate solution were required to facilitate mAb signal. All of 6522, 6523 and 6525 gave strong signals relative to the isotype matched negative control (Figure 5.8). However, the *U. parvum* pellet appears amorphous and is not a very appealing example of immunohistochemistry success. Therefore, a recombinant form that could be expressed on *E. coli* was created so that more convincing immunohistochemistry could be performed. Figure 5.9 shows the sequence of serovar 3 MBA that was optimised for expression in *E.coli* (this is required as the endogenous tryptophan codon in the serovar 3 sequence is UGA – a stop codon in *E. coli*) and Figure 5.10 shows the process by which the synthetic MBA was created. Only 2 repeats could be engineered in the synthetic version of serovar 3 MBA as gene synthesis is confounded by recurring repeats (according to the provider). Following transfection of the plasmid into *E.coli* the MBA protein was expressed and was found to segregate into the membrane, rather than the cytoplasmic fraction, by Triton-X114 fractionation methods and Western blot analysis (Figure 5.11). The protein was detected both by monoclonal antibodies that recognise histidine repeats as well as by 6525. Immunohistochemistry of *E.coli* colonies removed from ampicillin containing Luria agar plates and embedded in paraffin wax and sectioned show that 6522, 6523 and 6525 all detect the synthetic MBA protein and the distribution clearly show the outline of individual *E.coli* bacilli (Figure 5.12).

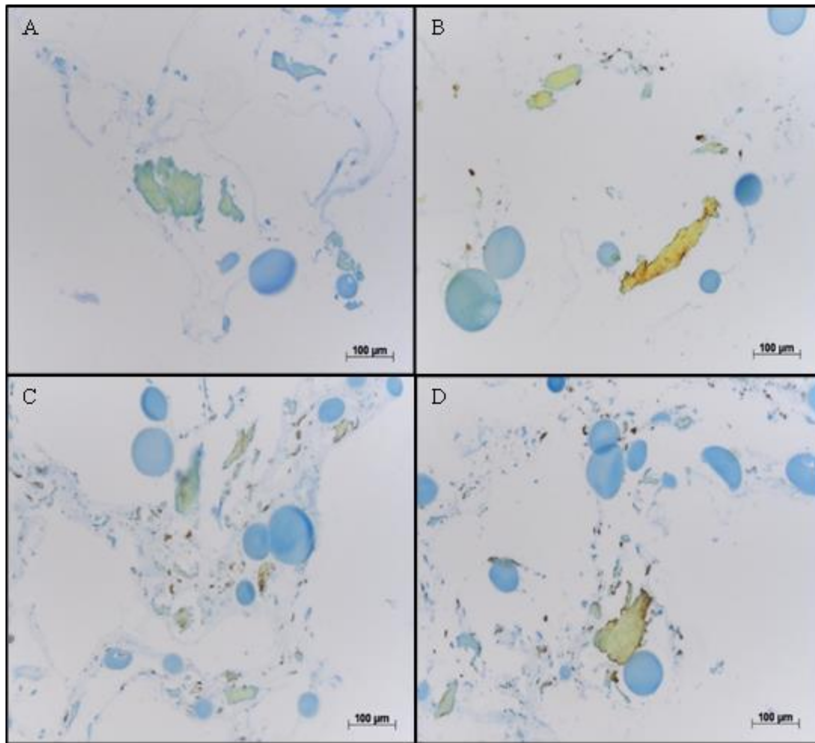

Figure 5.8 Immunohistochemistry of a serovar 3 (strain HPA5) pellet of U. parvum embedded in paraffin wax. No signal was seen when an isotype matched negative control monoclonal antibody was utilised, while brown peroxidase staining was distinct when the pellet was incubated with antibodies 6522, 6523 and 6525. The blue beads were added for section orientation and the scale bar is shown.

```
                  Expasy translate tool prediction:
   M  K  L  L  K  N  K  K  F  W  A  M  T  L  G  V  T  L  V  G
E.coli: atgaaactgctgaaaaacaaaaaattctgggcaatgaccctgggtgttaccctggttggt
SV3:    atgaaattattaaaaaataaaaaattctgagctatgacattaggtgttaccttagttgga

   A  G  I  V  A  I  A  A  S  C  S  N  S  T  V  K  S  K  L  S
E.coli: gcaggtattgttgcaattgccgcaagctgtagcaatagcaccgttaaaagcaaactgagc
SV3:    gctggaatagttgctatagcagcttcatgttctaattcaactgttaaatctaagttaagt

   N  Q  F  A  K  S  T  D  G  K  S  F  Y  A  V  Y  E  I  E  N
E.coli: aatcagtttgcaaaaagcaccgatggtaaatccttttatgccgtgtatgaaatcgaaaac
SV3:    aaccaatttgctaaatcaacagacggtaaaagtttttatgcggtttacgaaattgaaaac

   F  K  D  L  S  N  D  D  K  K  S  L  S  N  I  E  F  N  A  A
E.coli: ttcaaagatctgagcaacgatgacaaaaaaagcctgagcaacatcgaatttaatgcagca
SV3:    tttaaagatctaagtaatgatgataaaaaatcattaagtaacattgaatttaatgctgct

   L  T  S  A  E  N  K  T  E  S  T  L  E  K  G  H  L  V  G  E
E.coli: ctgaccagcgcagaaaacaaaaccgaaagcaccctggaaaaaggtcatctggtgggtgaa
SV3:    cttacatcagctgaaaacaaaacagaaagtacacttgaaaaaggtcatttagttggtgaa

   K  I  Y  V  K  L  P  R  E  P  K  P  N  E  Q  L  T  I  I  S
E.coli: aaaatctatgttaaactgcctcgtgaaccgaaaccgaatgaacagctgaccattattagc
SV3:    aaaatttacgttaaattacctcgtgaaccaaaacctaatgaacaattaactattattagt

   K  S  G  L  I  K  T  S  G  L  L  I  S  D  N  L  N  Y  Q  T
E.coli: aaaagcggtctgattaaaaccagcggtctgctgattagcgataatctgaattatcagacc
SV3:    aaaagtggattaatcaagacttcaggtttgttaatatctgataatttgaattatcaaaca

   E  K  V  N  F  E  T  T  Q  P  G  K  L  Q  P  A  G  K  E  Q
E.coli: gaaaaagtgaactttgaaaccacccagcctggtaagcttcagcctgctggcaaagaacaa
SV3:    gaaaaagtgaactttgaaactacacaaccaggtaaagaacaaccagcaggtaaagaacaa

                 P  A  G  K  E  Q  H  H  H  H  H  H
      E.coli: ccggcaggtaaagagcagcaccatcaccatcaccattaaaagctttaa
```

Figure 5.9 MBA amino acid sequence utilised to generate codon-optimised sequence for expression in *E.coli*. Original *U. parvum* sequence is provided for comparison. The use of TGA as a tryptophan in *Mollicute* sequences is highlighted in green. Two added BamH1 restriction sites (highlighted in grey) engineered into the sequence are also shown that will remove the repeat sequence; however, this resulted in one sequence alteration of E to L (highlighted in red) in the recombinant version.

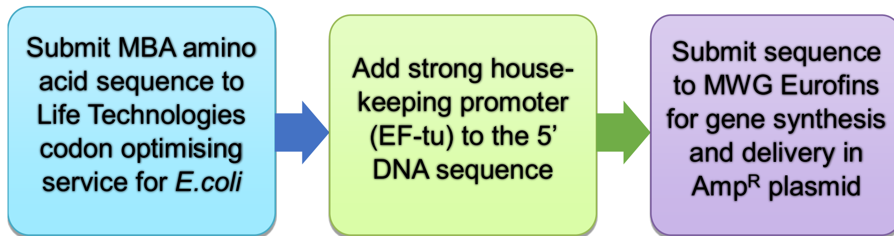

Figure 5.10 Process by which the sequence in above figure was generated and configured prior to submisssion for gene synthesis. The promoter utilised consistent of -170 bp to ATG start codon for the *tuf* Gene for serovar 3 (UU522 in ATCC strain 700970 full genome annotation).

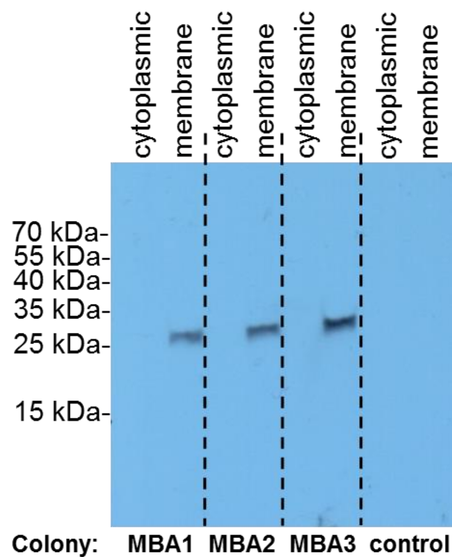

Figure 5.11 Western blot analysis following TritonX-114 partitioning to separate membrane bound and cytoplasmic fractions .Monoclonal anti-MBA 6525 was used to detect recombinant MBA expression from 3 separate colonies of E.coli transfected with a plasmid expressing the gene outlined in figure 5.9 (MBA1-3) compared to a colony that was transfected with an irrelevant gene. The predicted mass of the protein (by http://www.bioinformatics.org/sms/prot_mw.html) was 18.97 kDa however this does not take into account the removal of the signal peptide and addition of the lipid anchor. Figure shown is representative of repeated experiments.

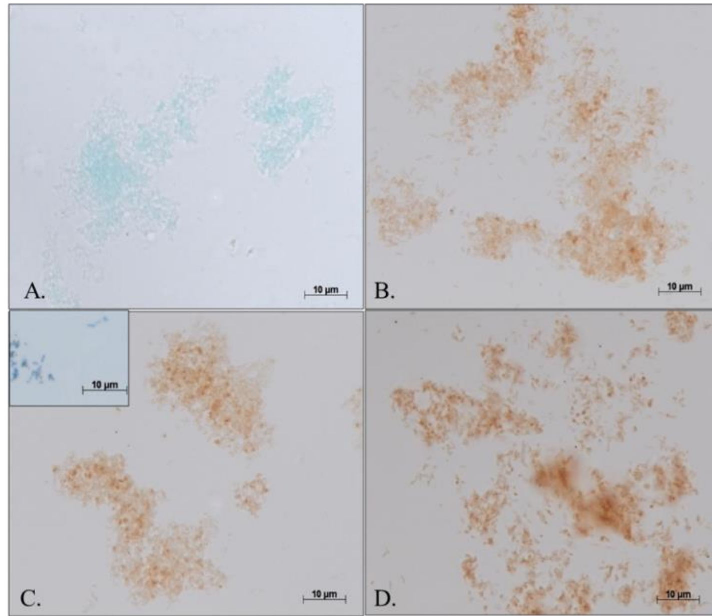

Figure 5.12 Immunohistochemistry visualisation of synthetic serovar 3 MBA expressed by transformed *E.coli* by monoclonal anti-MBA 6522 (B) 6523 (C) and 6525 (D) as compared to 6522 staining of *E.coli* that are transformed with an empty plasmid (A). Counterstaining *E.coli* bacilli with Gram-stain obscures the peroxidase staining (inset C), therefore, no counter stain was used in the larger images, although outline of individual bacilli at the edge of colonies are distinguishable in B-D. Images are taken with oil-emersion 100x objective lens, scale bar included to indicate magnification.

5.4 Discussion

Ureaplasma is one of the smallest self-replicating organisms with a minimal genome, ranging in size from 0.75 to 0.95 Mbp (Paralanov *et al.,* 2012) . These genomes are predicted to encode on average 604 (*U. parvum*) or 664 (*U. urealyticum*) protein encoding genes, with 515 genes universally conserved across all serovars. Separating the serovars by phylogenetic analysis has been performed in the past showing a clear separation of *U. parvum* from *U. urealyticum* (Harasawa *et al.,* 1996; Maniloff, 2002), but it is the conserved domain of the MBA gene that has been used to separate the phylogeny of the different serovars(Knox *et al.*, 1998; Kong et al., 1999). The MBA protein is an excellent target for separation of the *Ureaplasmas* into distinct sub-groups as it is highly expressed, often being the obvious variation between strains when separated by SDS-PAGE and stained with Coomassie blue. It is therefore likely to represent a significant immunological target. However, all serovars cannot be completely resolved from one another based on MBA sequence (e.g. serovars 2, 5 and 8). Molecular methods of separating the serovars based on targets external to the *MBA* gene have been successful for the prototype strains(Xiao *et al.,* 2010); unfortunately application of these typing schemes to clinical isolates did not maintain clear serovar differentiation(Sung *et al.,* 2011) , likely due to a propensity for *Ureaplasma* species to undergo extensive horizontal gene transfer(Paralanov *et al.,* 2012) .

There have been several reports for the development of mAbs that recognise serovars in the past. The first report was by Watson *et al*. (1990) where a panel of monoclonal antibodies were characterised and antibodies that recognised individual serovars 3, 8 and 10 were characterised (Watson *et al.,* 1990). Further characterisation of these antibodies by peptide mapping identified the key aspects of the amino acid sequence of the repeat region motifs that differentiated serovar 3 specific mAbs 10C6.6 (KEQPA) and 3B1.5 (EQP) from an antibody

(5B1.1) that recognised both serovars 3 and 14 (GK) equally (Zheng *et al.,* 1996) . However, this last motif exists in the repeat of all *U. parvum* strains, so should not be specific only to serovars 3 and 14. Other mAbs that have been characterised bind specifically to serovar 4 (Cheng *et al.*, 1993), serovar 9(Naessens et al., 1998), and serovar 1, 3, or 6 (Cheng *et al.,* 1994). However, the one commonality to these reagents is that they are sequestered in individual research labouratories. Here I have characterised a panel of commercially available monoclonal antibodies and found the first monoclonal antibody (6522) that appears to recognise all strains of *U. parvum* and *U. urealyticum* (with the possible exception of serovar 4). I also found an antibody that only recognises epitopes common to *U. parvum* (2B11) as well as one that recognises all *U. parvum* and weakly binds to serovars 7 and 11 (which belong to the same genotype)(Kong *et al.,* 2000) . Comparison of the entire MBA sequence between all *Ureaplasma* strains shows 4 potential regions of complete homology with hydrophilic and charged residues and some regions that could be the targets of differential recognition (Figure 5.13). Equally, the antibody 2B11 could recognise the areas that are conserved between *U.parvum* serovars, but altered for *U. urealyticum* serovars. The weak recognition of serovars 7 and 11 in addition to *U. parvum* by 6523 is difficult to explain as there is no obvious region of the conserved N-terminal region that separates these two serovars from the remaining *U. urealyticum* strains. With regards to previously published monoclonal antibodies, visual inspection of the amino acid sequence of the repeat regions shows differences that justify how antibodies could specifically recognise serovars 1, 3, 6, 9, and 10, but the shared repeat regions for serovar 4, 10, 12 and 13 and 2, 5 and 8 make it difficult to understand antibodies that only detect serovar 4(Cheng *et al.,* 1993) and serovar 8(Watson *et al.*,1990). I have validated the bands recognised by the commercial antibodies against previously characterised antibodies, to confirm it is the MBA protein that the commercial antibodies are binding.

Using the MBA as a method to classify strains is not without potential disadvantages. The MBA is phase variable. When grown in the presence of rabbit polyclonal anti-MBA antibodies, expression has been found to be shut off(Zimmerman *et al.,* 2009) , as have selection of non-adherent sub-populations(Monecke *et al.,* 2003) .The mechanism of phase variation is speculated to involve tyrosine recombinases (particularly XerC) and inversion of promoter regions driving expression of the open-reading frames(Zimmerman, 2013: Zimmerman *et al.,* 2015). The tendency for recombination can also result in multiple copies of the *MBA* gene being present in the genome and comparative genomic analysis has suggested that repeats characteristic of different serovars can be found in a single genome of the same strain (Paralanov *et al.,* 2012); however, no evidence of co-expression of separate serovar repeat expression was provided in that report.

It is interesting that phase variation of *MBA* could be induced by co-culturing with polyclonal anti-MBA antibodies (Zimmerman *et al.,* 2009), and may well relate to the original serotyping methods where polyclonal anti-serovar sera were found capable of inhibiting the metabolism of matching strains through an unknown mechanism. Watson et al. (1990) confirmed that 4 of 6 monoclonal antibodies also had differing capacities to inhibit metabolism of matching serovars. Whether different epitopes on the MBA relate to metabolic inhibition, or whether only those monoclonal antibodies that recognise epitopes available in the native protein inhibited metabolism remains unknown(Watson *et al.,* 1990). While it is difficult to speculate a mechanism for how antibody binding to a lipid anchored bacterial protein would result in bactericidal or bacteriostatic activity, the future of anti-MBA monoclonal antibodies as therapeutics in exploiting this phenomenon remains open.

Conclusion, here I provide a detailed analysis that differentiates between the reactivity of a panel of monoclonal antibodies raised against the MBA. The results will enable other researchers to speciate clinical isolates, or assign specific serovars in some cases, based on differential detection by Western blot or immunohistochemistry. More importantly, these reagents will enable researchers to begin to examine correlations between MBA size or numbers of MBA isoforms present in each sample, rather than just nucleic acid determinations. This may be of particular importance for future investigations as experimental investigations in pregnant sheep have found increased pathology when less than 5 MBA isoforms were observed compared to pregnant sheep infected with 9 or more MBA isoforms for the same strain(Knox *et al.,* 2010) .

CLUSTAL O(1.2.1) multiple sequence alignment

```
Signal sequence-expected to be removed ↓
sv6        MKLLKNKKFWAMTLGVTLVGAGIVAIAASCSNSTVKSKLSSQFVKSTDDKSFYAVYEIEN
sv1        MKLLKNKKFWAMTLGVTLVGAGIVAIAASCSNSTVKSKLSNQFAKSTDDKSFYAVYEIEN
sv14       MKLLKNKKFLAMTLGVTLVGAGIVAIAASCSNSTVKSKLSNQFAKSTDGKSFYAVYEIEN
sv3        MKLLKNKKFWAMTLGVTLVGAGIVAIAASCSNSTVKSKLSNQFAKSTDGKSFYAVYEIEN
sv4        MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
sv13       MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
sv12       MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
sv8        MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
sv5        MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
sv2        MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
Sv10       MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
sv9        MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
sv11       MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
           ********* *:**********:**:*****.*.******.*:.**.* *******:***

sv6        FKDLSDNDKKSLNDIEFNAALTSVENKTENLVTKGHLVGEKIYVKLPREPKPNEQLTIIN
sv1        FKDLSDNDKKSLNDIEFNAALTSVENKTENLVTKGHLVGEKIYVKLPREPKPNEQLTIIN
sv14       FKDLSNDDKKSLSNIEFNAALTSAENKTESTLEKGHLVGEKIYVKLPREPKPNEQLTIIS
sv3        FKDLSNDDKKSLSNIEFNAALTSAENKTESTLEKGHLVGEKIYVKLPREPKPNEQLTIIS
sv4        FDDLNENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
sv13       FDDLNENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
sv12       FDDLNENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
sv8        FDDLTENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
sv5        FDDLTENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
sv2        FDDLTENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
Sv10       FDDLNENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
sv9        FDDLTENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
sv11       FDDLTENDKKALNETEFNVAITSVENKTENATIKGHLLNKKIYVKLPREPKAKEQLTIIN
           *.**.::***:*.: ***.*:**.*****.   ****: :*********** :******.

sv6        KSGLIKTSGLLIPNNLNYQTEKVNFETAPKTQEPGKEPGKEPGK-E--PGKEPG---KEP
sv1        KSGLIKTSGLLIPDNLNYQTEKVNFETASKTEEPKENVGEQP-GKEQQPGKEQQ---P--
sv14       KSGLIKTSGLLISDNLNYQTEKVNFETTQPGKEQQPAGKEQ-----QPAGKEQQ---P-A
sv3        KSGLIKTSGLLISDNLNYQTEKVNFETTQPGKEQPA-GKEQPAGKEQPAGKE-Q---P-A
sv4        KGGLLKTASLVLPDNLNYQTEKVEFETQPGNGTTSPE---KPGNGTTSPEKPG-------
sv13       KGGLLKTASLVLPDNLNYQTEKVEFETQPGNGTTSPE---KPGNGTTSPEKPG-------
sv12       KGGLLKTASLVLPDNLNYQTEKVEFETQPGNGTTSPE---KPGNGTTSPEKPG-------
sv8        KGGLLKTASLVLPDNFNYQTENIGFETKPGSGETTKP---GSGETTKPGSGETT------
sv5        KGGLLKTASLVLPDNFNYQTENIGFETKPGSGETTKP---GSGETTKPGSGETT------
sv2        KGGLLKTASLVLPDNFNYQTENIGFETKPGSGETTKP---GSGETTKPGSGETT------
Sv10       KGGLLKTASLVLPDNLNYQTENIGFETQPGSGSTTQP---GSGSTTQPGSGSTT------
sv9        KGGLLKTASLVLPDNFNYQTEKVDFENAPTPTPEPTP---TPTPKEDKAIVSNVEFSEVN
sv11       KGGLLKTASLALPDNLNYQTEKVDFKSTHTPEPTPTP---TPAPKEDKAVVSNVEFSDVD
           *.**:**:.* : :*:*****:: *:.
```

Figure 5.13 Alignment of MBA from all *Ureaplasma* species for potential epitopes recognised by 6522. Green highlighted regions indicate regions of conserved homology with hydrophilic and charged residues that could serve as immunogenic epitopes. Amino acids prior to the first cysteine are expected to be removed as a signal sequence.

CHAPTER 6

Evolution of *Ureaplasma parvum* During *in utero* infection

6 Evolution of *Ureaplasma parvum* during *in utero* infection

6.1 Introduction

The most commonly isolated bacteria from uterus of women in spontaneous preterm labour with intact membranes are mixture of low virulence bacteria like *Ureaplasma* species, *Mycoplasma hominis*, peptostriptococci, *Gardenella vaginalis*, and bacteroids (Holst *et al.,* 1994; Rodney *et al.,* 2006). One of the most common but underestimated pathogens of female reproduction, as mentioned in 2005 by Volgmann and colleagues (Volgmann *et al.* 2005) is *Ureaplasma*, a member of the class *Mollicutes*, found to be the most common organism isolated from the amniotic fluid and tissues of those born prematurely between (23-32wk) and those with low birth weight in an inversely proportional rate with their gestational age (Kallapur *et al.* 2013; Novy et al. 2009; Watts et al. 1992). Additionally, many reported studies showed that *Ureaplasma* long thought to be a harmless commensal is potentially pathogenic for humans particularly during pregnancy where it can establish chronic intrauterine infection in spite of its extreme susceptibility to fetal serum and may end in devastating reproductive sequel (Alfa *et al.,* 1995; Cassell *et al.,* 1986; Viscardi *et al.,* 2010; Waites *et al*., 2006;). Experimental *in utero* infection in sheep with *U. parvum* has been used to investigate the pathogenicity of these bacteria which is associated with induction of preterm birth (Jennifer *et al*., 2010; Knox *et al.,* 2010; Moss *et al.,* 2005).

Ureaplasma are fastidious and require a highly supplemented culture environment to grow and reproduce. It hydrolyses urea as the sole source of energy and requires cholesterol for synthesis of its lipid cell membrane and it is a facultative anaerobe (Masover *et al.,* 1977). This explains the preference of *Ureaplasma* to adhere and colonise the urogenital track mucosa and live as normal commensals in a large proportion (62%) of sexually active women. Colonisation is increased during pregnancy (80-90%) and drops to less than (25%) during

puerperium and menopause which may reflect a hormonal effect on mycoplasma infection (Iwasaka *et al.,* 1986). On the other hand, *Ureaplasma* can cross the chorioamniotic membranes and get access to the amniotic cavity during pregnancy either by ascending from the lower genital tract across the cervix (which is the most plausible route) or by haematogenous distribution through the placenta, or by descending into the uterus from the peritoneal cavity. Iatrogenic infection can also occur during invasive procedures like amniocentesis or chorionic villous sampling, as these methods mechanically bypass anti-infective mechanisms which defend normal pregnancy. Most *Ureaplasma* infections are sub-clinical and difficult to detect except by amniotic fluid aspiration and analysis by molecular techniques or by cultivation (Waites *et al.,* 2009; Gerber *et al.,* 2003).

Once inside the host, it will be confronted by the immediate innate immune response and later by the adaptive immune response. In spite of that, *Ureaplasma* can survive, colonise and replicate inside the host tissues. The ability of *Ureaplasma* to escape the host immune response during its colonisation in the uterus has been examined by the scientists for many years(Citti *et al.*, 2010; Dando, *et al.* 2012). *Ureaplasma* has many virulence factors to overcome the host immune response including an IgA protease, phospholipase A and C, urease and the ability to generate hydrogen peroxide, which help the organism to overcome host defence and enable it to hydrolyse and invade host tissue (Viscardi, 2012; Kallapur *et al.* 2013). Yet, the most important immunodominant antigen in *Ureaplasma* is a surface-exposed lipoprotein referred to as the multiple banded antigen (MBA), which has two domains; the variable part (consisting of a number of uniform repeating units) and a conserved part (consisting of a signal peptide and a lipid–anchor signal and a conserved non-repeating stalk). The variable repeating units define its serovar specificity but for *U. parvum* these have cross reactive epitopes (Teng *et al.,* 1994; Viscardi, 2012). The *MBA* gene can undergo continual antigenic variation either by changing the number repeats (by expansion of the multiple C-terminal short amino acid

(QPAGKE) repeats; i.e. size variation) or by synchronised inversion of the promoter region which switches off MBA expression in favour of expressing an adjacent gene in the opposite genomic orientation (called phase variation). This is thought to respond to immunological pressure to allow *Ureaplasma* to persist for longer duration and promote host adaptation (Monecke *et al.*, 2003; Zimmerman *et al.* 2009; Viscardi, 2012; Zimmerman, 2014). Loss of the major surface antigen (MBA), which could be targeted by the adaptive immune system, is thought to help *Ureaplasma* to evade the host immune response (Paralanov *et al.,* 2012).

Zimmerman *et al.,* (2009) demonstrated that MBA can undergo size variation through changing the number of the C-terminal repeats and he proved that some MBA negative *Ureaplasma* strains can be created by exposing the protein to repeated immunological pressure with antibodies against the *MBA* gene. This resulted in switching on of the hypothetical gene (UU376) instead of the original *MBA* gene (UU375) through a mechanism of DNA inversion event in a gene locus mediated by the XerC recombinase (Zimmerman *et al*., 2009; Zimmerman *et al.,* 2011; Zimmerman, 2014).

6.2 Aims

- To further characterise the strains recovered from chronic intrauterine infection from Chapter 4, using the characterised reagents of Chapter 5.
- To compare the serum killing susceptibility of strains recovered from infected sheep following various lengths of infection.
- To purify recovered isolates by repeated colony isolation and compare MBA size to serum killing phenotype.
- To examine *MBA* gene size and sequence, as well as total genome sequence, for phenotypically altered, purified recovered strains to the original annotated genome sequence of the infecting HPA5 strain.

6.3 Results

6.3.1 Analysis of MBA from strains recovered from amniotic fluid.

In Chapter 5, I have fully characterised monoclonal antibodies recognising the MBA so that I may return to strains recovered from amniotic fluid following experimental intrauterine infection in Chapter 4 for more in depth analysis. Figure 6.1 reviews the *Ureaplasma parvum* titration in amniotic fluid samples recovered from catheterised sheep and two separate groups of sheep with amniotic fluid recovered at delivery and by ultrasound guided needle sampling.

Western blot analysis of amniotic fluid recovered strains from the catheterised sheep (120 hr) and samples recovered from amniotic fluid at surgical delivery after 1 week infection are shown in(Figure 6.2). MBA isoforms detected by monoclonal antibody 6525 show doublets for amniotic fluid isolates from sheep 12.068 and 12.073; but these are similar in mass to the single isoform observed in original inoculating HPA5 strain. Two of the strains recovered

from the non-catheterised group (amniotic fluid samples obtained at surgical delivery) also show a doublet MBA isoform (Figure 6.2). However, the new emerging MBA isoforms are approximately 20 kDa increased in size. Western blot analysis of isolates grown directly from ultrasound-guided amniocentesis samples taken at 3 and 5 weeks post-infection, continue to show increased numbers of MBA isoforms (Figure 6.3), and by 5 weeks no original 50 kDA MBA isoforms are detectable. To determine if multiple MBA isoforms were expressed by single isolates, I plated each of the strains recovered out by amniocentesis on Ureaplasma selective agar plates (Figure 6.4) and picked single colonies to be inoculated into broth. I repeated the colony purification twice in duplicate, designating the first round by a or b (i.e. 13.136a and 13.136b), and a number to the second round (i.e. 13.136a1, 13.136a2, 13.136b1, and 13.136b2). Figure 6.5 shows the isolates arranged by increasing MBA isoform size. In all cases sub-strains containing only a single MBA isoform could be isolated (140b2 and 132b1 could subsequently be cloned to single MBA species).

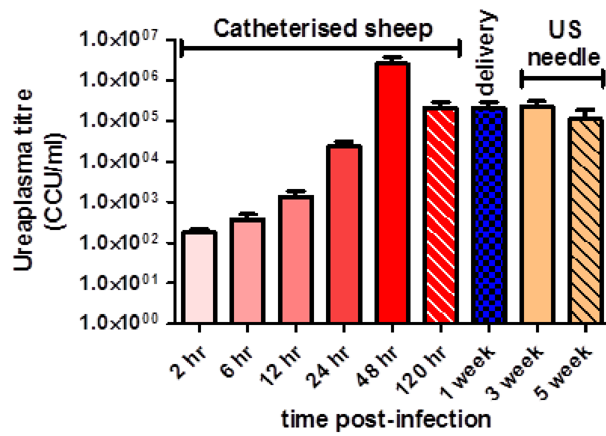

Figure 6.1 *Ureaplasma* titration in amniotic fluid samples retrieved from 3 separate groups of pregnant sheep experimentally infected with strain HPA5 Group 1: repeat amniotic fluid catheter samples (red; N=6), Group 2: amniotic fluid collected at Caesarean delivery (blue; N=6), and Group 3: amniotic fluid samples collected by ultrasound guided needle at 3 and 5 weeks post-infection (beige; N=6). Mean and standard error of the mean shown for individual animal titres (which represents the average of each animal determined in triplicate).

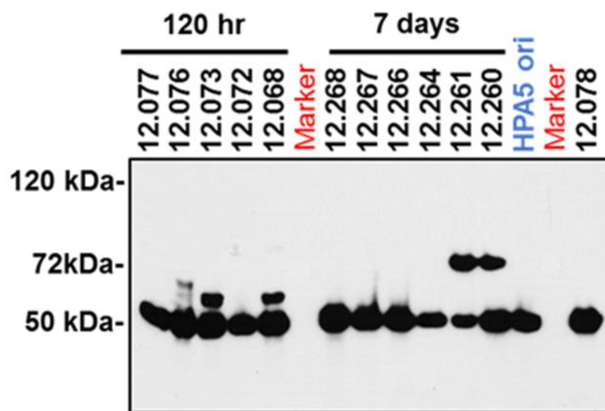

Figure 6.2 Western blot analysis of *Ureaplasma parvum* isolates grown directly from amniotic fluid samples collected from the final catheter time point (12.068-12.078) or from samples collected at surgical delivery following a week of intrauterine infection (12.260-12.267) by strain HPA5 (inoculating strain included as control= ori). MBA isoforms detected by the monoclonal antibody 6525).Western blot represents identical results from three separate experiments.

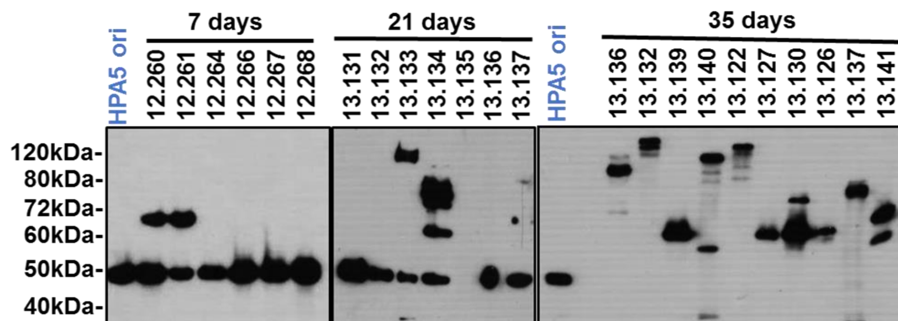

Figure 6.3 Western blot analysis of *Ureaplasma parvum* isolates grown directly from amniotic fluid samples. For comparison the 1 week post-infection samples from figure 6.2 are included. Isolates grown from ultrasound-guided amniocentesis samples from seven HPA5-infected sheep were collected at 3 weeks (13.131-13.137; no viable *Ureaplasma* was isolated from 13.135) as well as from 10 sheep following 5 weeks of HPA5 infection (sheep 13.132. 13.136 and 13.137 are common to both groups, but the remaining 7 were sampled for the first time at 5 weeks). MBA isoforms detected by the monoclonal antibody 6525. Western blot represents identical results from three separate experiments, inoculating original HPA5 strain included for reference (HPA5 ori).

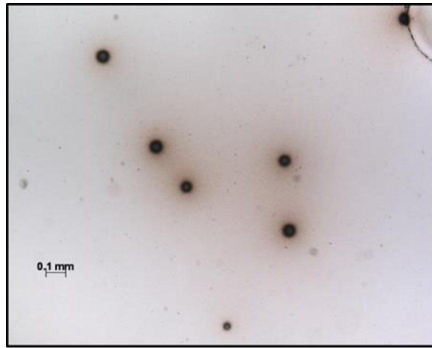

Figure 6.4 Colonies of HPA5 recovered by amniocentesis after 5 weeks of intrauterine infection. Strains grown in *Ureaplasma* selective broth were inoculated on to *Ureaplasma* selective agar and incubated for 48 hr at 37oC. Individual colonies were picked with a 10 µl pipette tip and inoculated into broth. Process repeated twice to isolate purified sub-strains. Agar plate visualised under a 4x objective and scale bar added to the image by OpenLab software utilised to capture image via CCD camera.

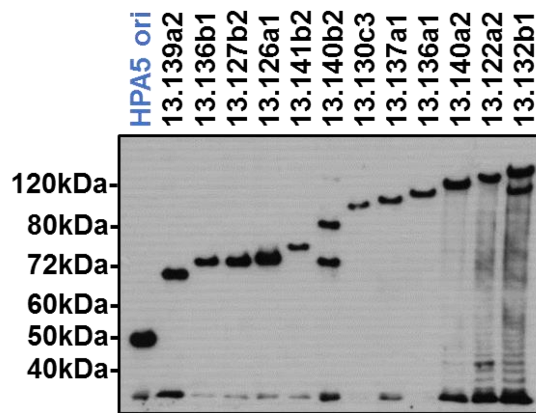

Figure 6.5 Western blot analysis of amniotic fluid recovered HPA5 strain of *Ureaplasma parvum* at 5 weeks post-infection. Isolates were colony purified twice prior to culture scale up and separation by SDS-PAGE Isoforms are arranged by increasing MBA isoform mass (increased by 20->70kDA compared to inoculating strain (HPA5 ori). MBA isoforms detected by the monoclonal antibody 6525. Western blot represents identical results from three separate experiments.

6.3.2 Characterisation of complement susceptibility of recovered isolates

Now that it had become clear that the HPA5 strain appear to be altering their MBA isoforms with prolonged intrauterine infection, I examined the susceptibility of three of these strains to sera from 95 dGA, 125 dGA and adult sheep (N=3 uninfected controls each). In chapter 4, I show that the complement activity of fetal sera decreases with increasing prematurity and I had examined the susceptibility of isolates from amniotic fluid and plasma in strains isolated from 94 dGA lambs following a 24 day infection. However, I only examined a 50% dilution of adult and 95 dGA sera (Figure4.7). Therefore, for these isolates I examined a titration of sera between 50% and 6.25%. Figure 6.6 shows that the original HPA5 strain and strains recovered from amniotic fluid after 1 week post-infection are equivalent in their susceptibility to sera. However, strains recovered after 3 weeks post-infection were less susceptible and after 5 weeks post-infection were almost completely resistant to 125 dGA sera and resistant to seronegative (M18) human serum and start becoming more resistant to uninfected pregnant sheep sera (Figure 6.6).

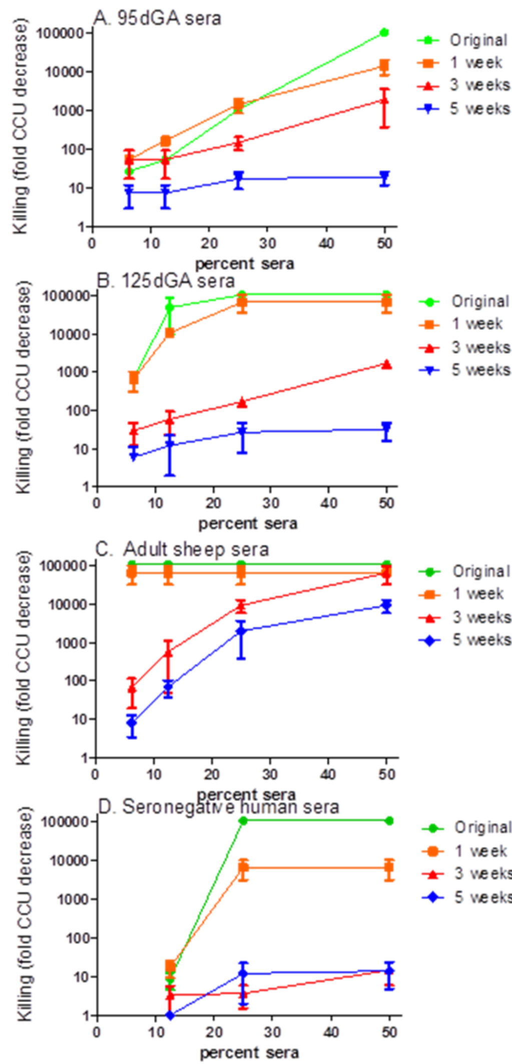

Figure 6.6 Serum killing assays for *Ureaplasma*, comparing original inoculating HPA5 strain to strains recovered after 1, 3, and 5 weeks post-infection in sheep Sera from 3 control uninfected 95 day gestational age (dGA), three 125 dGA and 3 adults (matched to the 125 dGA group), as well as M18 (seronegative human serum) were diluted in CFB and incubated with bacterial strains for 30 min at 37ºC. Data averaged from 3 strains chosen at random for 1, 3, & 5 weeks are shown. Mean and standard error for cumulative data shown.

I also examined the complement susceptibility of the colony purified isolates from Figure 6.5 to see if there were individual differences between strains purified from different sheep 5 week post-infection by incubation with 50% 125 dGA sera (Figure 6.7). These strains were isolated from 7 separate sheep and have known MBA isoform mass (Figure 6.5). They are arranged from smallest MBA (13.139a2) to largest MBA (13.132b1). The susceptibility to killing by sera from uninfected 125 dGA lambs was also compared to the highly sensitive parental HPA5 strain as well as to ATCC prototype *Ureaplasma parvum* serovar 3 (700970) and *Ureaplasma urealyticum* serovar 8 (27618)(Figure 6.7). Serovar 8 was found to be as sensitive to sheep sera as HPA5, while ATCC serovar 3 was found to be slightly less sensitive (Figure 6.7; $p<0.05$). Three of the isolates (13.139a2, 13.126a1 and 13.122a1) appear to be less resistant than the others, however no significance was found between these groups by ANOVA. In chapter 4, I also found one of the ten 125 dGA lambs infected for 10 days had detectable HPA5 in their plasma (14.231), I have included this bacteremic strain for comparison and found that this strain was resistant to 125 dGA sera (Figure 6.7). Furthermore, in chapter four I also found that the strain isolated from the plasma of 95 dGA lamb 14.115 was more sensitive to 95 dGA sera than the other isolates and it was included for comparison in these experiments as well (Figure 6.7). Consistent with the initial observations, plasma strain 14.115, was also more sensitive to125 dGA sera and was not significantly more resistant than the parental strain (although it approached significance at $P=0.0546$). For completeness, in Figure 6.8, I also include the Western blot analysis comparing the strains isolated from the amniotic fluid and plasma from 94 dGA infected animals from chapter 4 (14.115, 14.116, 14.120 14.121), as well as matched plasma and amniotic fluid isolates from another experiment (14.138 and 14.139) and the single plasma-isolated strain from 125 dGA infected animals (14.231; no amniotic fluid samples were collected for comparison in these animals). It is notable that the unique sensitive strain from the plasma of 94 dGA animal 14.115 is the only one (for both plasma and amniotic fluid isolates) that retains the original MBA mass compared to HPA5 (Figure 6.8) and that animal 14.231 is the

only strain isolated from one of ten 125 dGA animals infected for 10 days, exists as a single larger MBA isoform mass (Figure 6.8).

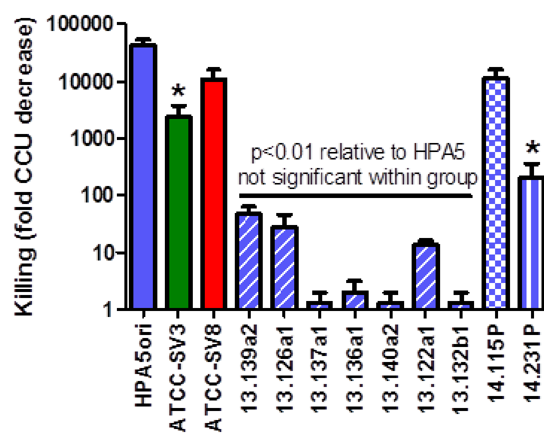

Figure 6.7 Serum killing assays for *Ureaplasma*, comparing susceptibility of original inoculating HPA5 strain to ATCC prototype serovar 3 and serovar 8 and to strains recovered from plasma of infected sheep (14.115P and 14.231P from figure 4.7) and the colony purified strains obtained from amniotic fluid following 5 weeks intrauterine infection (from figure 6.5). Strains were incubated for 30 min with 50% sera from 125 dGA uninfected lambs (pooled). Data shown represent combined data from 3 replications of the same experiment (mean and standard error of the mean). *=$p<0.05$ significant difference, ANOVA comparison between colony purified samples as a subset failed to identify a significant difference.

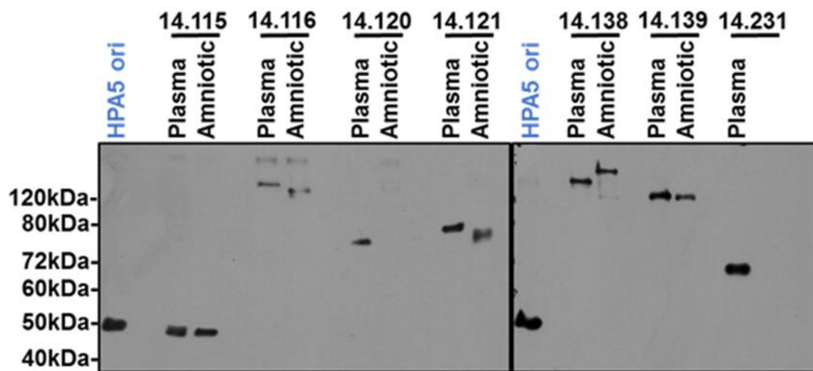

Figure 6.8 Western blot analysis of isolates recovered from the amniotic fluid or plasma at surgical delivery of 94 dGA lambs infected for 24 days with the exception of 14.231 which was the only plasma isolate out of ten 125 dGA lambs infected for 10 days) with HPA5 strain of *Ureaplasma parvum*. Strains were scaled up directly from samples. Samples 14.115, 14.116, 14.120, and 14.121 were characterised in chapter 4; however, samples 14.138 and 14.139 were treated with a potentially confounding variable and have been included only for comparison of MBA isoforms between plasma and amniotic fluid from the same animal. Only serum sensitive strains from 14.115 retain the original MBA mass compared to the inoculating (HPA5 ori) strain. MBA isoforms detected by the monoclonal antibody 6525. Western blot represents identical results from three separate experiments.

6.3.3 Investigation of variation in *MBA* gene size

The mass of the MBA protein increases with longer intrauterine experimental infection and this appears to be coincident with the decreased susceptibility of the recovered strains to be killed by sheep complement. Therefore, primers were designed based on the prototype serovar 3 strain (ATCC 700970) to amplify the *MBA* gene from the surrounding genomic DNA (Figure 6.9). The primers include the previously published primers (Teng *et al.,*1994)) that amplify the region from -125 bp in the promoter (UMS-125) to 226bp in the coding region (UMA226) that is used to detect *Ureaplasma parvum* strains in clinical samples. Combinations of these primers were used to amplify peri-*MBA* gene DNA from the original HPA5 strain and the colony purified strains from Figure 6.5 (Figure 6.10). Two of the amplified *MBA* genes were selected for cloning into the TopoTA cloning vector pcr2.1 (Figure 6.11) and the results of this sequencing are shown in Figure 6.12. Analysis of these sequences determined that the predicted amino acid sequence for the *MBA* gene in the original HPA5 strain had 17 repeat units (EQPAGK), compared to the 43 repeats found in the prototype ATCC strain. This accounts for the much smaller MBA mass for original HPA5. By contrast the *MBA* gene from 13.136b1 was predicted to contain 36 repeat units, which is consistent with the 342 bp increase in the PCR amplicons relative to HPA5 ori (Figure 6.11). However, this would only account for a predicted 11.6 kDa to be added to the mass of MBA by Western blot, which seems a bit small for the 20 kDa difference observed by Western blot (Figure 6.5).

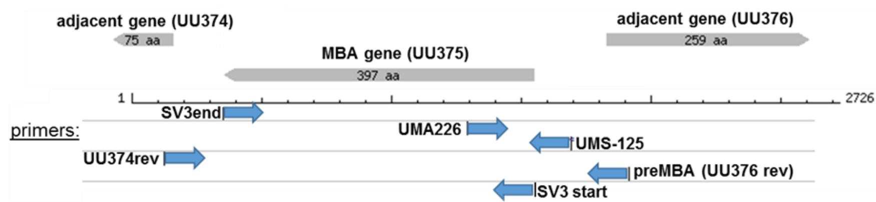

Figure 6.9 Diagram showing primer direction and placement relative to the sequence of the MBA gene and the adjacent genes in the serovar 3 *Ureaplasma parvum* genome.

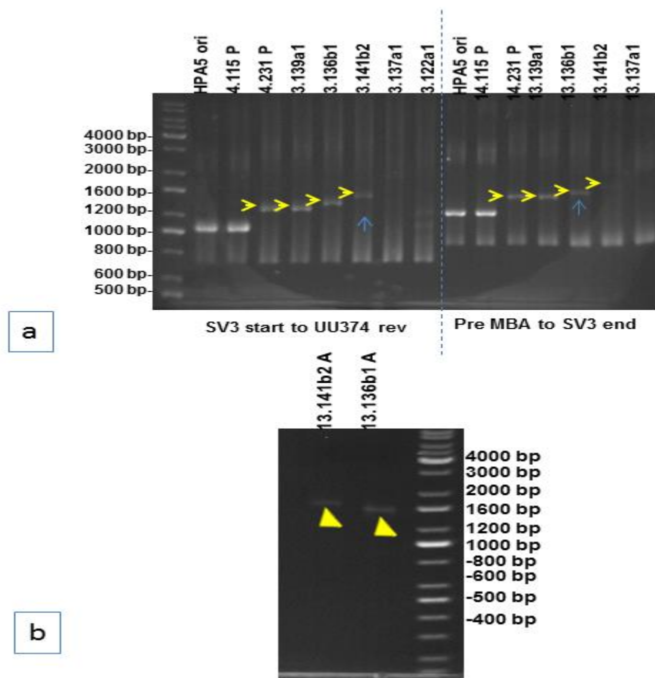

Figure 6.10 (a) showing primer direction and placement relative to the sequence of the *MBA* gene and the adjacent genes in serovar 3. *Ureaplasma parvum* genome amplicons separated on a 1% agarose gel, visualised by ethidium bromide and UV light. All PCR reactions were run for 45 cycles with 54ºC annealing temperature and 2.5 minute polymerase extension time, and the primer pairs used are listed below the bands (separated by a dotted line). Yellow arrow heads indicate the specific PCR products, which show increasing size relative to the original (HPA5 ori) inoculating strain; blue arrows indicate bands purified for cloning into pMT85 for transposon expression. (b) Showing the gel purified bands, 13.136b1From UU374 start (entire promoter from MBA) to SV3end and 13.141b2 from UMA-125(partial promoter) to UU374start (intergenic region from SV3 MBA stop to next gene).

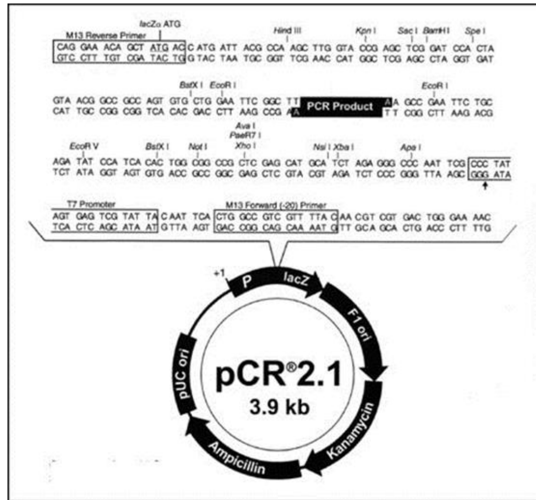

Figure 6.11 Diagram for Topo-TA cloning vector pCR2.1

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actagtaacggccgccagtgtgctggaattcgcccttgtgtctagagagatggttgttgg
pCR2.1 vector sequence                   Xba1 in primer sequence
cttggagctgaagctgcttcaaagttcacttttctgtttgataattcaaataataaaac
Start of UU376 gene (reverse)^
ttattaaaaataatatagatatataattaaattttaatcataatttataataaaaaata
          <-complete UU376/UU375 intergenic region->
tctaataatgttatcgataatgcagaaaaataaaaaaataaaaaaaatagtaaaaattat

ataattaaaagtgcaagtgctaaataaaaagtatttgcaatctttatatgttttcgttaa

aattaaaaattaattactgtagaaattatgtaagatcaccaaatcttagtgttcatattt

tttacatatattaaataaaaacaataaaatgacatattttttatattaggagaatcataa
start of MBA (UU375) gene>
atgaaattattaaaaaataaaaaattctgagctatgacattaggtgttaccttagttgga
 M  K  L  L  K  N  K  K  F  W  A  M  T  L  G  V  T  L  V  G
gctggaatagttgctatagcagcttcatgttctaattcaactgttaaatctaagttaagt
 A  G  I  V  A  I  A  A  S  C  S  N  S  T  V  K  S  K  L  S
aaccaatttgctaaatcaacagacggtaaaagtttttatgcggtttacgaaattgaaaac
 N  Q  F  A  K  S  T  D  G  K  S  F  Y  A  V  Y  E  I  E  N
tttaaagatctaagtaatgatgataaaaaatcattaagtaacattgaatttaatgctgca
 F  K  D  L  S  N  D  D  K  K  S  L  S  N  I  E  F  N  A  A
cttacatcagctgaaaacaaaacagaaagtacacttgaaaaaggtcatttagttggtgaa
 L  T  S  A  E  N  K  T  E  S  T  L  E  K  G  H  L  V  G  E
aaaatttacgttaaattacctcgtgaaccaaaacctaatgaacaattaactatcattagt
 K  I  Y  V  K  L  P  R  E  P  K  P  N  E  Q  L  T  I  I  S
aaaagtggattaatcaagacttcaggtttgttaatatctgataatttgaattatcaaaca
 K  S  G  L  I  K  T  S  G  L  L  I  S  D  N  L  N  Y  Q  T
gaaaaagtgaactttgaaactacacaagcaggtaaagaacaaccagcaggtaaagaacaa
 E  K  V  N  F  E  T  T  Q  A  G  K  E  Q  P  A  G  K  E  Q
ccagcaggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaagaacaaccagca
 P  A  G  K  E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q  P  A
ggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaa
 G  K  E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q  P  A  G  K
gaacaaccagcaggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaagaacaa
 E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q
ccagcaggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaagaacaaccagca
 P  A  G  K  E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q  P  A
ggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaa
 G  K  E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q  P  A  G  K
gaacaaccagcaggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaagaacaa
 E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q
ccagcaggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaagaacaaccagca
 P  A  G  K  E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q  P  A
ggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaa
 G  K  E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q  P  A  G  K
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 E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q
ccagcaggtaaagaacaaccagcaggtaaagaacaaccagcaggtaaagaacaaccagca
 P  A  G  K  E  Q  P  A  G  K  E  Q  P  A  G  K  E  Q  P  A
ggtaaagaacaaccagcaggtaaagaaactactggaaaataatctagaaagggcgaattc
 G  K  E  Q  P  A  G  K  E  T  T  G  K  - Xba1 in primer sequence
tgcagatatccatcacactggcggccgctcgagcatgcatctagagcccaatcgctaaag
pCR2.1 vector sequence
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Figure 6.12 Sequence from pCR2.1 vector containing *MBA* gene and surrounding DNA (including predicted open-reading frame translation) Xba1 sites used to clone into expression vector pMT85 are also underlined. Two mutations in the gene, compared to HPA5 original, are highlighted in grey: the first is silent; the second alters proline to alanine.

6.3.4 Transposon delivery of a larger *MBA* gene to HPA5 to increase serum resistance

The sequenced 13.136b1 amplicon included the promoter region and the entire coding region. Therefore, an experiment was designed to express a larger MBA in HPA5 to see if it could transfer resistance to 125 d GA serum killing. The 13.136b1 amplicon was transferred to the transposon plasmid pMT85 using the flanking Xba1 site (Figure 6.12) and Dr. Ali Aboklaish transformed the original HPA5 strain with the second copy of *MBA* gene in pMT85. Three separate colonies that contained the gentamicin resistance gene from pMT85 were examined by Western blot and compared to HPA5 transformed with pMT85 containing irrelevant genes. As can be seen from Figure 6.13, these strains co-expressed two isoforms of MBA: one that corresponds to the original mass of HPA5 and the other that corresponds to the mass of 13.136b1. Unfortunately, this hybrid strain remained sensitive to killing by 125 dGA sera when challenged for 30 min at 37°C (data not shown). This suggests that serum resistance was mediated by some other alteration to the HPA5 genome.

6.3.5 Comparison of whole genome sequence between original HPA5 and recovered strains

HPA5 original strain and the recovered strains 13.122a2, 13.126a1, 13.127b1, 13.127b2, 13.136a1, 13.136b1, 13.137a1 and 13.139a2 were scaled up to 700 ml cultures, pelleted at 13,000 xg for 3 h and the genomic DNA extracted by Genelute columns. The entire amount of DNA was submitted to Public Health England (via Dr. Victoria Chalker) and the assembled contigs were returned to me (Table 6.1). These contigs ranged in size from 425,777 bp to

1,418 bp and each strain yielded about 8 to 12 contigs. Using the Geneious 8.0.5 analysis software these assembled pieces of DNA were compared to the fully annotated genome for the original strain HPA5 (completed by Dr. Mike Beeton and Dr. Brad Spiller). As can be seen from Figure 6.14, the contigs from each strain range from 8 to 14 stretches of DNA that covers the entire genome. Direct copies of the 5S, 23S and 16S rRNA operon cause problems for the alignment (any repeat >200bp is read as the same sequence). In the Geneious program analysis rRNA sequences are red arrows, tRNA are pink arrows, genes are yellow arrows (which almost always correspond to green predicted protein arrows). Comparing sequence differences between all recovered sequences that are conserved compared to the original strain annotated sequence, very few genetic alterations are observed (other than increasing repeat regions for the *MBA* gene; UU375) as listed in Table 6.2. Although 3 single nucleotide polymorphisms (SNP) were detected in all strains for hypothetical open-reading frame UU046, all were synonymous and would not change the amino acid sequence of a resultant protein. Similarly two additional SNPs were found in intergenic regions for all recovered strains between genes UU584 and UU583 and between two tRNA sequences that both code for species that recognise arginine codons in mRNA. The only potential significant conserved change relative to the original strain was the 17 SNPs distributed between the two copies of the ammonium transporter gene. However, how this could reduce complement sensitivity is unclear. There are other points in the genome that seem to be mutational hot-spots, for example 5 out of 9 recovered strains showed a mutation in the hypothetical gene UU111(Figure 6.14) that would lead to a lost protein product (frame-shift and loss of start codon). Most of the remaining alterations in one or more recovered strain occurred in polyA, polyT or polyTA tracks in intergenic sequences that are unlikely to have had a significant effect on gene expression (Table 6.2). On overall analysis, the alteration to the number of C-terminal repeats in the *MBA* gene

was the most significant conserved change in recovered strains relative to the original serum-sensitive strain, despite the failure of dual MBA strains to have transferred serum resistance.

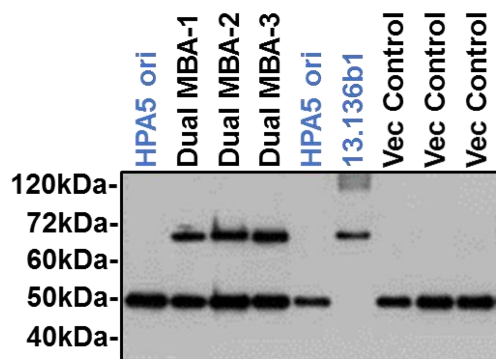

Figure 6.13 Western blot analysis of HPA5 original strain (HPA5 ori), the colony purified 13.136b1 recovered isolate and HPA5 strains transformed with mini-transposon vector pMT85 without an insert (vec control) or containing the 13.136b1 *MBA* gene and endogenous promoter (entire intergenic region between genes UU375 and UU376; dual MBA). Three separate colonies of the transformed HPA5 were grown up separately and processed, showing that transposon delivery does not alter MBA isoform and the delivered second copy of *MBA* is universally expressed in all transposon mutants resistant to selection in 128 g/L of gentamicin. MBA isoforms detected by the monoclonal antibody 6525. Western blot represents identical results from three separate experiments.

Table 6.1 Details of genomic DNA contigs.

Strain	basepairs	Strain	basepairs	Strain	basepairs	Strain	basepairs
127b2	425,777	**122a1**	89,490	**126a1**	35,502	**136b1**	2,171
139a2	389,297	**127b1**	81,007	**126a1**	34,514	**127b1**	1,698
122a1	328,621	**136b1**	80,997	**139a2**	34,473	**139a2**	1,606
127b1	328,602	**127b2**	80,995	**137b1**	34,473	**137b1**	1,606
137b1	328,509	**136a1**	80,984	**122a1**	34,473	**136b1**	1,606
136b1	326,261	**136a1**	79,826	**126a1**	21,498	**136a1**	1,606
136a1	260,494	**139a2**	49,820	**126a1**	5,632	**127b2**	1,606
136a1	165,386	**122a1**	79,785	**136b1**	5,615	**126a1**	1,606
126a1	142,235	**137b1**	79,778	**127b1**	4,954	**122a1**	1,606
126a1	100,295	**127b2**	79,777	**122a1**	4,954	**127b1**	1,605
137b1	97,204	**127b1**	79,742	**122a1**	2,832	**127b2**	1,578
126a1	97,187	**136b1**	74,219	**127b1**	2,828	**127b1**	1,572
136b1	93,503	**126a1**	74,118	**137b1**	2,765	**136b1**	1,526
139a2	90,409	**126a1**	64,300	**136a1**	2,765	**139a2**	1,430
136a1	90,388	**126a1**	54,824	**126a1**	2,765	**137b1**	1,430
137b1	90,373	**139a2**	46,542	**139a2**	2,701	**136b1**	1,430
136b1	90,373	**139a2**	46,542	**136b1**	2,701	**127b2**	1,430
127b2	90,373	**137b1**	46,542	**127b1**	2,701	**127b1**	1,430
127b1	90,373	**122a1**	46,542	**122a1**	2,701	**126a1**	1,430
122a1	90,373	**126a1**	46,538	**136b1**	2,436	**122a1**	1,430
127b1	89,491	**139a2**	36,559	**127b1**	2,386	**136a1**	1,418

Contigs are listed by decreasing size, listed next to the strain of origin. Each strain is composed of 8 to 14 contigs and the largest contig (for 127b2) represents a 61.8% coverage of the complete HPA5 genome.

Table 6.2 Summary of genetic sequence alterations in recovered strains

Strain	Gene	Genome Position	Alteration	Comments
All	UU046 (hypo)	89,530-81	3 SNP	Silent mutations
All	UU584-UU583	164,189	A to G	150 bp upstream from UU583
All	$tRNA_{arg3}$-$tRNA_{arg2}$	271,261	T to G	33 bp upstream from $tRNA_{arg2}$
All	UU375 (MBA)	425,557	T to A	Silent mutation
All	UU219 (amt2)	591,695	A to G	
All	UU219 (amt2)	591,833-93	12 SNP	
All	UU218 (amt1)	593,377	A to G	
All	UU218 (amt1)	593,653	A to C	
All	UU218 (amt1)	593,673	A to G	
All	UU218 (amt1)	593,680	A to G	
122a1, 136b1, 139a2	UU111 (hypo)	2,806	Del A	Frame-shift
126a1	UU111 (hypo)	3,033	ATG to ATA	Start codon loss
136a1	UU111 (hypo)	2,692	G to T	Truncation of geneat 114/200 a.a.
136b1	UU065 (pepF-1)	62,059	Del A	Frame-shift
130c3	UU043-UU042	97,195	C to A	95 bp upstream from UU043
127b1 **136b1**	UU043-UU042	97,212	Del AA Del A	Poly A track 112 pb upstream from UU043
137b1	UU565 (hypo)	189,690	C to T	Pro to Ser
136b1, 127b2 **126a1,130c3, 139a2**	c0206-c0207	237,037	Del T Ins T Ins T	Poly T track 156 bp upstream from c0207
130c3	UU518 (hypo)	251,315	G to T	Thr to Lys
122a1, 130c3	$tRNA_{arg3}$-$tRNA_{arg2}$	271,260	Del T	Poly T track
136a1 **136b1**	UU498-UU497		Ins A Del A	Poly A track
137b1 **139a2**	UU497-UU496		Del T Del TT	
130c3, 139a2 **136b1**	UU474-UU473		Ins TA Del TA	
136b1	UU454-UU453	347,173	G to T	16bp upstream from UU454
139a2	UU359-UU358	444,214	Ins A	Poly A track 113 bp upstream from UU359
136a1	UU349-UU348	457,152	C to G	40 bp upstream from UU349
139a2	UU339-UU338	468,864	G to A	74 bp upstream from UU339
139a2, 136b1	UU264-UU263	557,204	Ins TA	Poly TA track 29 bp upstream UU263
122a1 **127b1/2, 137b1**	UU187(rpoB)	626,289 625,728	C to G C to G	Ala to Gly Gly to Ala

Ins = Inserted nucleotide, Del = Deleted nucleotide, SNP = single nucleotide polymorphi

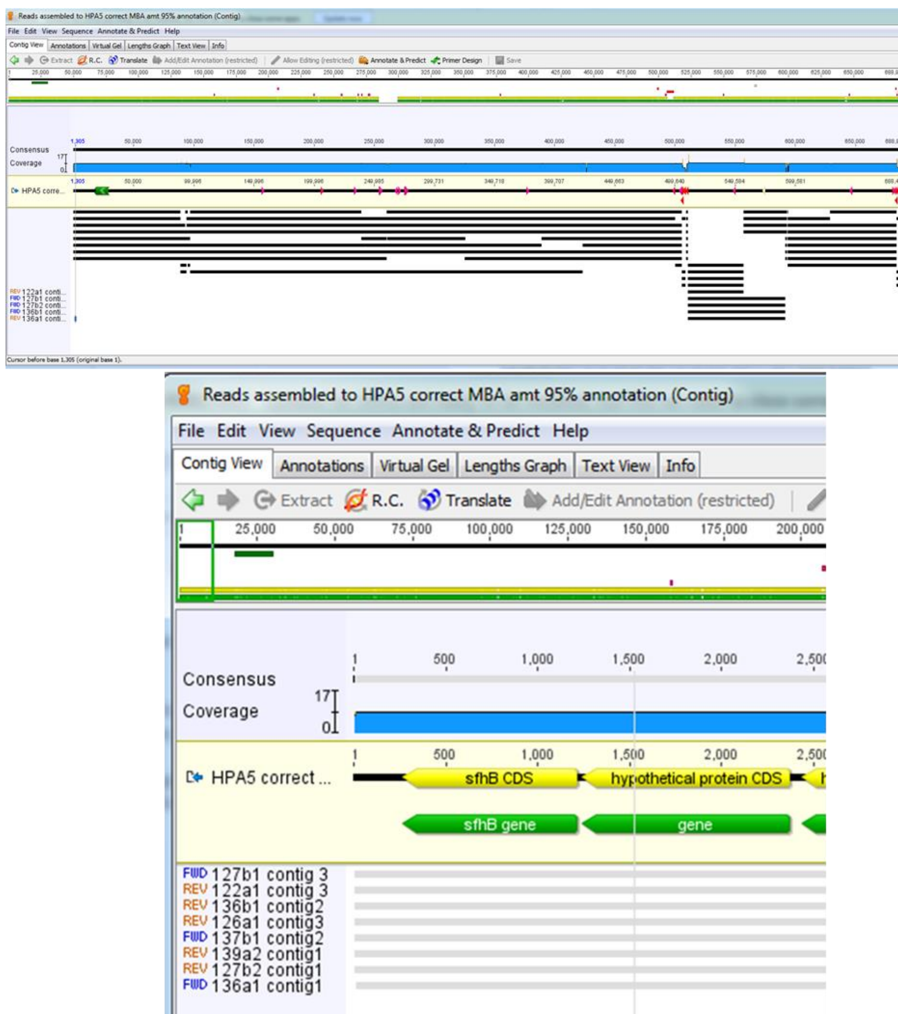

Figure 6.14 Geneious 8.0.5 alignment of recovered strain genomic DNA sequence contigs to the completed and annotated HPA5 strain sequence. Top panel shows alignment of all contigs from table 6.1, while the lower panel shows a zoomed view showing the complete agreement in sequence identity to the parent HPA5 strain.

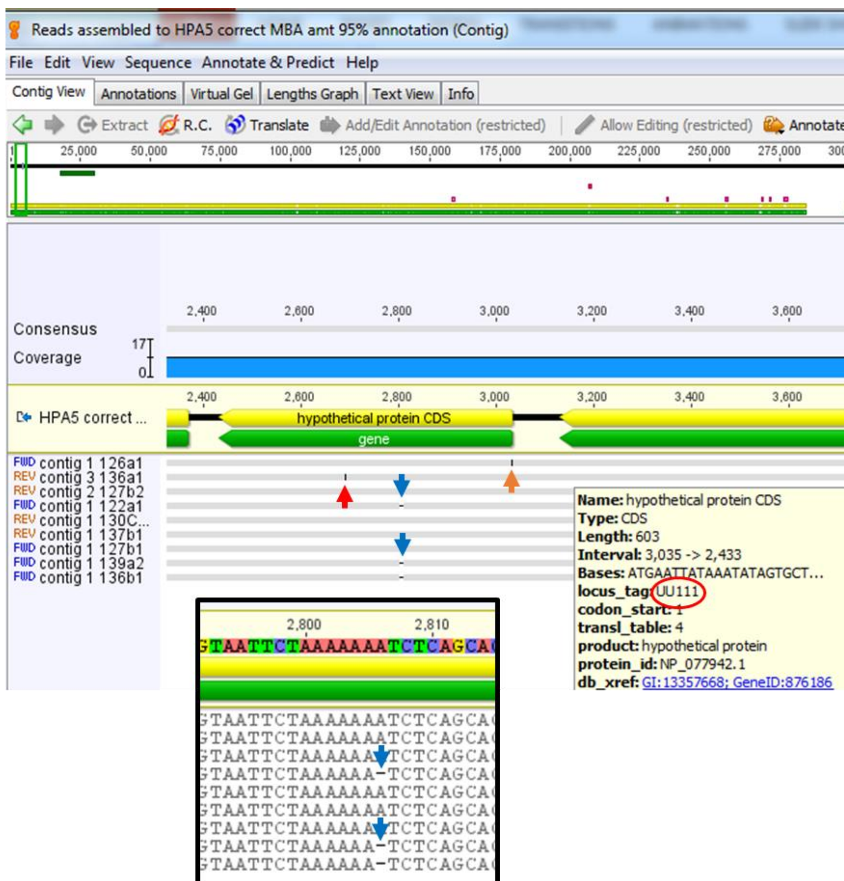

Figure 6.15 Geneious 8.0.5 alignment of recovered strain genomic DNA sequence contigs to the completed and annotated HPA5 strain sequence. This screen shot shows an example of the pan-genome analysis comparing between recovered strains and original infecting HPA5 strain. In this example the gene UU111 (circled) showed variations for the recovered strains: 13.122a1, 13.139a2 and 136b1 which had a deletion of a single A nucleotide (blue arrows, zoomed in within the inset), while strain 13.126a1 has a SNP converting the first codon from ATG to ATA, removing the start codon, and strain 13.136a1 has a SNP creating a stop codon sequence that would truncate the resulting protein at 114 amino acids of the predicted full length 200 amino acids.

6.4 Discussion

There has not been an equivalent study to this one previously carried out. Phenotypic alterations in *Ureaplasma parvum* have been induced *in vitro* by serial challenge to increasing concentrations of macrolide antibiotics (Pereyre *et al.,* 2007). This resulted in mutations in the genes encoding 23S rRNA and ribosomal proteins L4 and L22, which resulted in high level macrolide resistance. Furthermore, studies running in parallel to this thesis (Ali Aboklaish thesis) have found that serial *in vitro* challenge of *Ureaplasma parvum* strains with sublytic concentrations of seropositive human serum resulted in induced serum resistance. However, the HPA5 strain has been previously reported to be very susceptible to serum killing (Beeton *et al.,* 2012), and HPA5 was the only strain examined found to be incapable of undergoing *in vitro* induction of serum resistance (Aboklaish thesis). Furthermore, induced resistance to human sera was found to coincide with induced expression of a 41 kDa protein (Aboklaish *et al.,* 2014). While these strains had induced resistance to human sera, no alteration to sensitivity to sheep sera was observed and no expression of the 41 kDa protein was observed in HPA5 strains recovered from chronically infected pregnant sheep after 5 weeks.

Alterations in the major surface protein associated with complement resistance have been reported for the mouse pathogen Mycoplasma pulmonis (Simmons and Dybvig, 2003). For these studies transposon mutants were characterised and it was found that strains with large variable surface antigens (Vsa) were resistant to mouse complement, while short Vsa strains were susceptible to killing. In a subsequent publication by the same group (Simmons *et al.,* 2004; Warren and Dybvig, 2003), they found that the increased resistance was directly related to the increase in glycocalyx surrounding the cell as the repeat unit for *Vsa* included a signal for N-linked glycosylation. Similar to their studies, susceptibility to sheep serum killing was high for original HPA5 and 14.115P strains (Figure 6.7) which had the smallest MBA isoform ,

compared to 14.231P and 13.139a2 (Figure6.8) which had an MBA approximately 20 kDa larger. However, the difference in the size of these MBA isoforms was the number of PAGKEQ repeats, which do not contain a known glycosylation motif. Lack of a known glycosylation motif does not exclude a novel *Ureaplasma* glycosylation mechanism; however, Schiff acid reagent staining (reacts with sugars) of whole cell proteins by Western blot did not identify the MBA protein as glycosylated (Aboklaish thesis).

Capacity for MBA size variation following experimental intrauterine Ureaplasma infection has been reported for prolonged chronic infection previously (Knox *et al.,* 2010), and the pathology in these animals was directly related to the number of MBA isoforms. They found fewer than 5 isoforms were detected in severe chorioamnionitis, whereas more than 7 isoforms was associated with lower pathology. The later showed that the number of MBA variants increased with length of infection (Robinson *et al.,* 2013), similar to what I have found. Further, investigations found that the presence of maternal anti-*Ureaplasma* antibodies correlated to increased cytokine levels and chorioamnionitis (Dando *et al.,* 2012), and the authors found that incubation of recovered isolates with polyclonal anti-MBA resulted in phase variation shut-off of MBA expression. However, my studies have found that maternal antibodies are incapable of crossing the placenta (Chapter 3) and in my studies no evidence of MBA negative recovered strains or of significant maternal or fetal anti-*Ureaplasma* response (Chapter 4) was found. Irrespective of the previous findings, my data is the first to show a change in phenotype for the recovered isolates that evolves over a span of weeks and more importantly that full genetic sequence analysis of the phenotypically altered evolved strains shows that increased number of MBA repeats is one of the few alterations to have occurred in the genome. It was disappointing that artificial delivery by transposon mutagenesis of a longer *MBA* gene to the serum-susceptible original HPA5 strain did not mediate resistance; however, an alternative

explanation could be that the shorter form is the dominant determinant meditating susceptibility. If time had permitted I would have liked to use transposon mutagenesis to deliver the original HPA5 *MBA* gene to one of the resistant recovered strains to see if that resulted in rescued serum sensitivity to test this hypothesis.

In conclusion this chapter has demonstrated an adaptive strategy whereby *Ureaplasma parvum* changes its genetic code resulting in increased resistance. The mechanism underlying this change is unknown and the process took time to manifest (it was not present after a week and the phenotype continued to adapt after 3 weeks). While there were a few alterations that were conserved amongst all of the strains recovered after 5 weeks chronic infection, only alterations to the length of *MBA* gene and sequence alteration to the duplicate ammonium transporter genes occurred in coding regions of the genome.

CHAPTER 7

GENERAL DISCUSSION

7 General Discussion

7.1 Overview

This thesis has addressed and dealt with different questions about *Ureaplasma* a bacteria from class *Mollicutes* and I focused on the reason why preterm babies are more liable to acquire this infection *in utero* or after birth and how this bacteria can adapt to the host in spite of its extreme sensitivity to human and sheep sera. The first results chapter investigated the functional complement activity of preterm neonatal sheep in comparison to the adult sheep and how its efficiency evolves with increasing gestational age and that maternal sheep antibodies were incapable of crossing the placenta. The second results chapter (4) provided the first detailed kinetic analysis of establishment of *Ureaplasma* infection in the amniotic cavity following experimental infection. It also dealt with level of bacteraemia in *Ureaplasma*-infected lambs: how it increases proportionally to increased insufficiency of the complement system with greater prematurity. The third result chapter (5) characterised a panel of commercial and non-commercial monoclonal antibodies and provided a detailed analysis that differentiates between the reactivity of a panel of monoclonal antibodies raised against a highly expressed major antigenic component of *Ureaplasma* termed the multiple banded antigen (MBA) which is regarded an excellent target for separation of the *Ureaplasmas* into distinct sub-groups. These results will enable other researchers to speciate clinical isolates, or allocate specific serovars in some cases, based on differential detection by Western blot or immunohistochemistry. Also these reagents will enable researchers to begin to examine correlations between MBA size or numbers of MBA isoforms present in clinical isolates and pathology. Finally, the last result chapter (6) demonstrated an adaptive strategy whereby *Ureaplasma parvum* becomes more resistant to serum killing with infections longer than 1 week *in utero*. It also addressed surface protein modulation by which *Ureaplasma* may influence recognition by the host immune system, particularly variation in the multiple banded antigens (MBA). While

increasing *MBA* size appeared to be dominant genomic alteration in the recovered serum-resistant strains, artificial addition of a larger *MBA* to the original strain failed to transfer serum resistance. However, in several experiments, where recovered strains were compared to the original inoculating strain, there was a consistent drive towards MBA isoforms with increased numbers of repeats. The only conclusion that can be drawn is that the increased MBA isoform size is selected for and there is an unknown mechanism in *Ureaplasma parvum* that specifically modifies the *MBA* gene, while retaining high fidelity for the rest of the 619,000 bp genome during replication.

7.2 Complement activity in preterm infants

The innate immune components represent the first line defence against invading pathogens but because of the suboptimal development of the cellular and humoral components of this system during fetal life, the foetus start life more susceptible to infection. In 2009, Soto *et al.* had reported the increase in complement activation markers in the amniotic fluid of pregnant women with spontaneous preterm labour as a result of infection through measuring C3a, C4a, and C5a and correlating higher levels to initiating labour prematurely (Soto *et al.*, 2009). The use of sheep as a non-primate model of human disease is increasing, especially for studies of disease in pregnancy and pregnancy complications. Sheep models have been developed to investigate intrauterine inflammation induced by injection or infusion of lipopolysaccharides or bacterial infection (Kemp *et al.*, 2011; Matteo *et al.* 2010; Zhang *et al.* 2012; Dean *et al.* 2011). None of these studies has investigated the contribution of the complement system to the disease pathogenesis largely due to the current lack of knowledge on the sheep complement system activity and its role in lamb pathophysiology. The complement system is a key part of innate immunity; it does not require previous exposure to be effective, it orchestrates

both immune and inflammatory reactions and supports the immune system in clearing pathogens, and remodelling damaged tissues. Complement activation recruits innate and adaptive immune cells to the site of inflammation where it aids in modulating and refining the adaptive immune response (reviewed in McGreal *et al.* 2012). I optimised an assay that allows the comparison of sheep sera complement activity from different sources (adult non-pregnant, maternal and preterm fetal sera). No difference was found in haemolytic capacity between pregnant and non-pregnant adult sheep sera, but found that complement activity in preterm fetal sheep was decreased relative to matched maternal sera, but still had considerable activity even in sera from preterm lambs at 85 days gestation (term=150days), which makes them considerably more immune competent than the equivalent gestational age humans (reviewed in McGreal *et al* 2012). From these studies, I demonstrated that fetal sheep sera unlike their mothers are unable to lyse rabbit and un-sensitised guinea pig erythrocytes which was the biggest difference observed. This was attributed to the complete absence of cross-reacting antibodies in fetal serum that bound to rabbit and guinea pig erythrocytes, which were abundant in adult sheep sera when examined by flow cytometry. However, adult sheep are unlikely to have been exposed to rabbit and guinea pig erythrocytes either; therefore, the obvious conclusion is that non-sheep glycosylation patterns on these erythrocytes resemble those on microbes which the sheep are readily exposed to as these sheep are sourced from normal farming practice. Anecdotal evidence for this is found in investigation of glycosylation differences of IgG between species. Ragu *et al.,* (2000) found that sheep IgG only contain oligosaccharides with N-glycolylneuraminic acid (NGNA) while IgGs from guinea pig and rabbit have post-translational modification with oligosaccharides containing both N-acetylneuraminic acid (NANA) and NGNA (Ragu *et al.,* 2000). Bacteria including E. coli, have been shown to synthesize this form of glycosylation for use in their cell walls (Severi *et al.*, 2007) supporting this hypothesis. Failure to transfer IgG from mother to foetus is not unique to sheep. In fact,

other than primates, it appears that only rabbits and guinea pigs have the capacity to transfer maternal IgG to foetuses across the placenta (Pentsuk and van der, 2009). This means that any immune response observed in fetal sheep and other ruminants must reflect the fetal immune response alone. In contrary to our findings Silverstein *et al., (*1964) titrated the complement activity in fetal lambs bled at different gestational ages and reported very low complement activity in embryonic lambs before 123 days of gestation and even at term (Silverstein *et al.*, 1964). While in 1968, Colten and colleagues reported sheep complement activity was detectable as early as 39 days of gestation and increased rapidly thereafter to become stable at 60 days gestational age. They further reported an absence of detectable complement activity in the amniotic fluid itself and found that 2 days after birth complement activity was equivalent to the adult levels (Colten *et al.,* 1968). In 1978, Renshaw and Littenecker measured the complement activity using a similar assay to mine (CH_{50}) in new born lambs and found the ewe: lamb ratio of activity ranged between1.48 to 5.3 (Renshaw and Litteneker, 1978). Studies in humans around this time found similar findings for the CH50 values in human neonates. Fireman (1969) found that the ratio of maternal to new born haemolytic activity was 1.87(Fireman *et al.,*1969)., while Adinolfi (1970) reported a ratio of 2.01(Adinolfi *et al.,*1970).A full review for the correlation between gestational age and complement has been published by McGreal *et al.* (2012).

7.2.1 The complement inhibitor CDX-1135

Although complement plays an important role in immune surveillance, it acts as a double edge sword, as it can cause tissue damage and pathology following inflammation and reperfusion injury. Anti-complement therapies have been developed to protect tissues from side effects

of complement activation by the use of recombinant complement regulators (Morgan and Harris, 2003). In my experiment, I found that the most potent complement inhibitor for human sera, CDX-1135, a recombinant form of sCR1 (soluble human complement receptor 1) was capable of inhibiting rat sera and human sera, but had negligible inhibition of sheep sera. CDX-1135 is composed of the 30 N-terminal domains of the potent complement regulator CD35 found mainly on human erythrocytes, but has been proven to be as effective in solution when added as a soluble form. Previous studies showing effective reduction of complement-mediated pathology by CDX-1135 in other rat models include significantly reduction by CDX-1135 of placental mean arterial pressure, C3a production, and vascular endothelial growth factor (VEGF) levels (Lillegard *et al.,* 2013) and reduction of reperfusion-induced complement-mediated myocardial infarction by 44% (Weisman *et al.*, 1990). Therefore, my studies show that an alternative regulator of sheep complement must be found, before the role of complement activation in hypoxia, infection and inflammation damage to the developing neonate using this model, can be addressed experimentally.

7.3 Ureaplasma and preterm labour

The genus *Ureaplasma* which belong to the class *Mollicutes*, have long thought to be commensals of the urogenital tract in humans. However, they are found to be pathogenic, particularly in subpopulations who are immunologically compromised. In pregnant women it can ascend from the colonised vagina into the upper genital tract even before pregnancy commences (Cassell *et al.,* 1983) or be acquired during passage of the baby through the colonised birth canal (both represent the extremes of vertical transmission) which is reported to occur at a rate of 45- 66% in full-term infants and about 58% in preterm infants (Sanches and Regan,

1987). Most of these intrauterine infections present as chorioamnionitis, which comprise infection and inflammation of the fetal membranes (amnion and chorion), placenta and/or decidua. This represents a substantial risk to the fetal well-being and influences pregnancy outcome and subsequent short and long term neonatal sequel (Romero and Mazor, 1988; Sung, 2010; Viscardi, 2010). Many studies in human pregnancies showed that *Ureaplasma* can be isolated from the amniotic fluid and fetal tissues as early as 16-20 week of pregnancy (Cassell *et al.,* 1986) or even earlier at the time of conception (Kasprzykowska *et al.,* 2014).

The rate of *Ureaplasma* colonisation and subsequent chorioamnionitis is reported by many studies to be inversely proportional to gestational age. Goldenberg and his team in the Alabama preterm birth study reported that 23% of neonates born between 23-32 weeks of gestation had positive umbilical blood cultures for *Ureaplasma* and *Mycoplasma* (Goldenberg *et al.,* 2008). My analysis of these findings would contribute that the earlier the gestational age, the more likely that these organisms would be isolated from the amniotic fluid, placenta, and membranes due to increased developmental insufficiency of the complement system. Beeton *et al.* also reported in 2011 that colonisation of the new born lungs by *Ureaplasma* is higher in preterm infants born between 23-26 weeks than those born near term, which contributed subsequently to greater risk for chronic lung diseases (CLD) development (Beeton, 2011). The most recent meta-analysis of all Ureaplasma studies bares this correlation out showing *Ureaplasma* infection gives an odds ratio of 3.04 for development of CLD in preterm neonates (Lowe *et al.,* 2014).

However, studies determining the association of timing, dose, serovar type and co-infection with other microorganisms have not been possible to examine systematically before now.

7.4 Kinetics of *Ureaplasma* infection in sheep

HPA5 grown from samples collected from the AF of catheterised pregnant sheep showed that *Ureaplasma* titres continued to increase to a peak at 48 hr and then remain relatively stable. This pattern is consistent with the studies performed on pregnant macaque monkeys reported by Novy *et al.* in 2009. If I compare the titres at 5 days for the catheterised animals to the titres from other groups of sheep infected for varying lengths of time (from 1-5 weeks), it would appear that the levels of *Ureaplasma* during chronic infection remain reproducibly stable. Knox *et al.* (2012) also reported that *Ureaplasma* can stay viable for more than 70 days in the amnion and lungs of sheep foetuses (Dand *et al.*, 2012; Knox et al., 2010). Moss *et al.* (2005) also reported the ability to recover *Ureaplasma* from the amniotic and lung fluid of lambs up to 10 weeks after inoculation (Moss *et al.,* 2005). This ability of *Ureaplasma* to overwhelm the continually developing innate immune system and any potential adaptive immune response resulting in colonisation of the host tissue for extended periods could be attributed to a strategy in *Ureaplasma* to adapt dynamically to avoid recognition by the host immune system as will be discussed later on in 7.8.

7.5 The increased incidence of bacteraemia with increasing prematurity

The use of sheep as a non-primate model of human disease is increasing, especially for studies of disease in pregnancy and pregnancy complications. For example, neonatal asphyxia induced by transient occlusion of the umbilical cord before birth has been shown to lead to complement-mediated reperfusion injury and to mimic the intrauterine hypoxia that complicates a proportion of human births (Castillo-Melendez *et al.,* 2013). Further, hypoxic injury following preterm birth and associated newborn respiratory disease is also studied in sheep

models (Wolfson *et al.,* 2012). More recently, sheep models have been developed to investigate intrauterine inflammation induced by injection or infusion of lipopolysaccharides or microbial infection (Dean *et al.*, 2011; Hillman *et al*., 2014; Kemp *et al*., 2011; Maneenil *et al*., 2015; Zhang *et al*., 2012). None of these studies has investigated the contribution of the complement system to the disease pathogenesis or to the role of complement in controlling systemic spread, largely due to the current lack of knowledge on the sheep complement system.

The increased incidence of bacteraemia in younger infected lambs relative to more mature lambs is directly related to the underdeveloped complement function in the fetal sera with increasing prematurity. Adinolfi and Beck (1975) found that after 16 weeks of gestation all complement components were detectable in human fetal serum and then increased subsequently in concentration, nevertheless, newborn infants remain more susceptible to infection especially those born prematurely (Adinolfi and Beck , 1975). This hypo-activity of the complement system and its low haemolytic activity may enhance capacity for microorganism invasion and greater potential susceptibility to infection (Grumach *et al.,* 2014). Fonseca *et al*. reported in 2011 that spontaneous preterm human babies had a 9-fold increased chance of having *Ureaplasma* bacteraemia and he stated that about 12.6% of very low birth weight new born babies (VLBW) with gestational age ≤32 weeks have positive blood culture for *Ureaplasma.* Again the enrichment of *Ureaplasma* bacteraemia likely attributed to the deficient complement activity in this group of babies (Fonseca *et al.*, 2011).

7.6 Bactericidal activity of preterm infant's sera

Neonates particularly those born prematurely are more prone to infection and even term babies are known to have an under-developed immune system relative to adults particularly the complement system whose activity decreases with increasing prematurity (McGreal *et al.,* 2012). All clinical *Ureaplasma* of serovar type 3 were reported by Beeton *et al*. (2012) to be susceptible to seronegative human serum in the absence of anti-*Ureaplasma* antibodies and this killing was mainly mediated by the classical pathway of the complement system. They found that using anti C1q antibodies blocked the complement–dependent *Ureaplasma*-cidal activity of the human serum (Beeton *et al.,* 2012). Therefore, I planned to use the sheep model to investigate the susceptibility of one of the characterised strains from that manuscript (HPA5) to examine susceptibility to fetal and adult sheep sera (both from infected and non-infected animals) and to examine the effect of developmental complement insufficiency on systemic spread of *Ureaplasma in vivo* from the site of experimental injection (amniotic fluid) to the fetal circulation. The *in vivo* bacteraemic status relating to gestational age was consistent with the bactericidal capacity of serum *in vitro,* which increased with gestational age. As with human serum studies, sheep serum bactericidal activity against this strain appears to be mediated mainly through the classical pathway (as no killing is observed when the alternative pathway is isolated with EGTA), unfortunately no reagents are available to test the lectin pathway, or to block C1q in the sheep sera, therefore, further dissection of the calcium-dependent pathways was not possible (as was performed for human sera in Beeton *et al*., 2012). While the classical pathway is expected to mediate *Ureaplasma* killing, failure of complement inhibitor sCR1 to block killing was unexpected, and this failure appears to be species specific as CDX-1135 regulates human, mouse, guinea pig, and rat sera.

Regarding the *Ureaplasma*-cidal efficiency of preterm sheep foetuses, the most immature sera were significantly deficient in bactericidal activity in comparison to adult sheep sera and apart from the prolonged killing curve, the kinetics for both the adult and preterm sheep serum killing were identical. Maximum killing was obtained in the first 10 minutes with all sera and no increase even after prolonged incubation for 4 hours. This also agrees with unpublished data examining term human serum and *Ureaplasma* killing (Abdulla S. thesis, 2012), where she found low *Ureaplasma*-cidal capacity of human cord sera in comparison to adult sera and attributed that to the lower complement components in the cord blood despite transfer of maternal anti-*Ureaplasma* IgG observed by Western blot using the cord sera.

In terms of fetal sheep immune response, less than 50% of the chronically infected (6 week infection) animals developed anti-*Ureaplasma* antibodies that reacted with whole HPA5 antigens separated by SDS-PAGE. Comparing the weak response of those that did develop an immune response, using HPA5 and a serovar 6 isolate (HPA61) identified the leading recognised antigen as the surface-exposed *Ureaplasma* antigen called the multiple banded antigen (MBA). This was confirmed by reactivity of the sera with recombinant MBA expressed by *E.coli*. However, those animals infected for 24 days from 70 days gestation, showed no seroreactivity. When matching the fetal sera anti-*Ureaplasma* reactivity to reactivity with antibodies in the amniotic fluid, they both showed equivalent reactivity reflecting leakage of the antibodies from the fetal sera into the amniotic fluid (data not shown).

The deficiencies in bactericidal activity of the immature sera were consistent with significant bacteraemia being observed in preterm lamb sera, both of which could be explained by the underdeveloped complement activity. This correlation between *Ureaplasma* bactericidal activity and complement function was not influenced by experimental infection, as a range of complement function and bactericidal activity was observed in sera examined *ex vivo* from

uninfected lambs at 95 days gestational age. Hence a normal variation in development of the complement system is observed in this hetrogenous population of Merino sheep. Less variation would likely be observed in defined inbred strains of labouratory animals.

7.7 Characterisation of monoclonal antibodies used for typing different strains of *Ureaplasma* species

Real-time PCR technology can segregate the 14 serovars of *Ureaplasma* from each other as can analysis of 16s rRNA sequence (Xiao *et al.,* 2010; Maniloff, 2002; Robertson *et al.,* 1994; Harasawa & Cassell, 1996) and standard PCR can separate *Ureaplasma* species by amplicon product size by using primers that target a 40 bp insert in the 5' end of *MBA* gene, and strains can be grouped into genotypes by sequencing an internal fragment of the *ure* gene (Knox *et al.,* 1998). These methods represent a range of technical ability for labouratories, from standard PCR, to primer/probe sets and genome sequencing. They all examine nucleic acid variation, and have the advantage of not requiring culture of fastidious organisms. There is no reason; however, why monoclonal antibodies cannot be used to detect surface proteins and even to separate these into species or in some cases serovars. Differences in epitopes are certainly predicted to be able to separate serovars and many labouratories have generated serovar-specific monoclonal antibodies (Watson, *et al*. 1990; Cheng, *et al.* 1994; Zheng, *et al.,* 1995; Zheng, *et al.* 1996).

Screening the MBA protein from a panel of prototype strains of *Ureaplasma*, the only completely sequenced and annotated *U. urealyticum* genome (serovar 10) identified a single MBA protein with the C-terminal repeat TQPGSGST was found to be the best MBA candidates. Some serovars were found to have close homology to serovar 10 N-terminus as serovars 2, 5

and 8 with the expected TKPGSGET repeat as shown in (Table 5.2) with only 3 polymorphisms. The next closest group of homologues comprised serovars 4, 12 and 13 with the expected TSPEKPGNGT repeat. MBA homologues with no identifiable repeat in the C-terminus were found for two serovar 7 MBA genes, as well as putative genes for serovar 2, 11 and 13. The potential repeat of GAQSGNGSGSASGS was identified for serovar 11 in an MBA homologue, but the potential MBA protein with this repeat for serovar 7 does not retain high homology for the N-terminal sequence. Furthermore, two different MBA putative proteins were identified for serovar 10 (ACI60338.1 and AAD02692.2) were found in the database with 100% identity, both with the expected TQPGSGST repeat. However, variants (other than the validated annotated serovar 10 genome) of the *U. urealyticum* may be due to errors in the shotgun genome sequencing data.

Using these prototype strains, MBA proteins from *U. parvum* can be visualised by western blot by probing the membranes with a panel monoclonal antibodies previously published by Watson *et al.*, (1990) (Table 5.3). Comparing our findings with the previously published (Watson *et al.*, 1990), mAbs behaved mostly as expected: 5B1.1 detected only serovars 3 and 14, 10C6.6 detected only serovar 3 and 8A1.2 did not react with any of the *U. parvum* MBAs, but did uniquely identify *U. urealyticum* serovar 10. Three of the commercial mAbs detected all the *U. parvum* MBA species equivalently (6522, 6523 and 2B11), performing better than the previously published mAb 8B5.2.

Echahidi and colleagues found that there is a cross reactivity between SV3 and SV14 and also between SV1 and SV6 (Echahidi, *et al.* 2000). Monoclonal antibody 5B1.1 and 6525 recognized both serovars 3 and 14, while 2G9 only recognised SV1 and SV6, and mAb 3B1.5 reacted strongly with serotype 3 with minimal cross-reaction with other serovars. Cross-reactivity between serovars, or in my case cross-reactivity across all *U. parvum* and *U. urealyticum*

by 6522, cannot be attributed to the reported expectation of high frequency horizontal genes transfer which occur in clinical isolates (Paralanov, *et al.* 2012). Furthermore, Thirkell *et al.,* (1989) generated mAb UU8/39, by immunising mice with serovar 8, which recognized the 16- and 17-kDa surface-expressed polypeptides in all serovars of *Ureaplasma,* but the 17-kDa polypeptide was only present in serotypes of the smaller serocluster B (serovar 1, 3, 6, and 14 *Ureaplasma*). The lack of variation in isoform mass for this protein signifies that these proteins are not MBA and therefore are likely not the basis of "serovar" specificity. However, the mAb UU8/29 reported in the same manuscript could identify a 96 kDa band which is the first serovar-specific mAb for SV8 (Thirkell *et al.,* 1989). *Ureaplasma* isolates showed a different immunoblot patterns and in some cases even different from each other as well as from some of the reference strains, signifying a high rate of antigenic variation among *Ureaplasma* strains (Teng *et al.*, 1994 ; Zheng *et al.,* 1992).

In 2008, Vancutsem and his colleagues succeeded in generating a recombinant MBA antigen of Ureaplasma SV3 and SV6 (rMBAs) by using a PCR method then cloning it into an expression vector plasmid and he proved the usefulness of this (rMBAs) in testing the sera of women with *Ureaplasma* infection for seroreactivity (Vancutsem *et al.* 2008). I also succeeded generating a recombinant SV3 MBA form created solely by gene synthesis of a codon-optimised predicted protein sequence with a histidine tag, complete with promoter (also from Ureaplasma – house-keeping gene EF-tu) that was expressed in *E. coli* and subsequently used for Western blot and immunohistochemistry testing of antibodies.

The future use of mAb in *Ureaplasma* infection diagnostics would depend on whether detection of all *Ureaplasma* species was required, or if differential determination of *U. parvum* versus *U. urealyticum* was required (or if specific serovars are subsequently found to be more pathogenic than others). Use of mAb as the basis of lateral flow methods (like pregnancy

tests) or bead agglutination (like many serological tests) would need to further investigate if these mAb recognise native proteins on *Ureaplasma* species. Furthermore, the use of mAb may be developed for therapeutics, as preliminary data in sheep show that inducing a strong fetal immune response against the MBA results in decreased amniotic fluid clearance. Other mAb that have been humanised have been used as therapeutics in the past as well.

7.8 Evolution of *Ureaplasma* SV3 during *in utero* infection

Although *Ureaplasma* species long thought to be harmless commensal microbe of the mucosal epithelium of the human urogenital tract, they are also endowed with the ability to be transmitted both horizontally during sexual intercourse and vertically during gestation (up to 90% vertical transmission rate)(Abele-Horn, *et al.* 1997; Behnam *et al.,* 2014) where it can exist asymptomatically inside the amniotic fluid for extended periods and cause subclinical infection in spite of its extreme susceptibility to fetal serum and may end in devastating reproductive outcome (Waites *et al.*, 2006; Cassell *et al.*, 1986; Viscardi *et al.*, 2010; Alfa *et al.*, 1995). Once inside the host, it will be confronted by the immune response of the host: both the immediate innate response and later by the adaptive immunity. In spite of that, *Ureaplasma* can remain viable, colonise and replicate inside the host tissues. Experimental *in utero* infection in sheep with *U. parvum* has been used to investigate the pathogenicity of these bacteria which is associated with induction of preterm birth and the ability of *Ureaplasma* to escape host immune response during its colonisation in the uterus has been challenged by the scientists for many years. *Ureaplasma* has many virulence factors to overcome the host immune response like IgA protease, phospholipase A and C, urease as well as the hydrogen peroxide-generating activity, which help the organism to overcome host defence and enable it to hydrolyse and invade host tissue (Viscardi *et al.* 2012; Kallapur *et al.* 2013). Yet, the

most important immunodominant antigen in *Ureaplasma* is a surface- exposed lipoprotein referred to as the MBA. This lipoprotein has two domains: the variable part consisting of a number of uniform repeating units and a conserved part consisting of a signal peptide and a membrane –anchor signal. These domains represent the serovar-specific and cross reactive epitopes, respectively (Teng *et al.*, 1994; Viscardi, 2012; Monecke *et al.,* 2003). In all 14 *Ureaplasma* serovars, the gene which encodes MBA (*MBA* gene) consist of a conserved 5' nucleotide sequence and a variable 3' region, and proceeds to produce a molecule which consists of both a conserved N-terminal intracellular domain (later cleaved and replaced with a lipid anchor) and variable C-terminal extracellular domain respectively. This variable extracellular domain is defined by a varying copy number of a tandem amino acid sequence (QPAGKE in *Ureaplasma* SV3), and is the primary immunogenic antigen of *Ureaplasma* cells (Zheng *et al.,* 1995). The *MBA* gene can undergo continual antigenic variation either by changing the number repeats by expansion of the multiple C-terminal short amino acid (QPAGKE) repeats (size variation) or by synchronised switching on some genes and switching off other genes (phase variation) when struggling against an immunological pressure to help *Ureaplasma* to evade the host immune response (Paralanov *et al.,* 2012) and to allow *Ureaplasma* to persist for longer duration and promote host adaptation (Monecke *et al.,* 2003; Zimmerman *et al.,* 2009; Viscardi, 2012; Zimmerman *et al.* 2014).

7.8.1 Susceptibility of *Ureaplasma* isolates to sheep sera

The susceptibility of *Ureaplasma* serovar 3 to fetal lamb sera compared to the matched maternal and to adult seronegative sera from non-pregnant sheep was investigated and I found that *Ureaplasma parvum* SV3 (HPA5) was very sensitive to non-immune sheep sera, while an unrelated clinical isolate, HPA61 strain of SV6, was identified as completely resistant to

non-immune sheep sera. Comparing sensitivity of original HPA5 to HPA5 recovered from experimentally infected sheep of differing lengths of infection, demonstrated that the original HPA5 and isolates recovered one week post-infection were equally sensitive to non-immune adult sera even at concentration of 6.25%. I demonstrated that the adaption is not simply a rapid selection and takes time to occur. Isolates recovered following a 3 week infection, however, started to become serum resistant to fetal sheep sera. However, by 5 weeks post-infection HPA5 had completely adapted and was resistant to fetal sheep sera and was starting to become resistant to adult sheep sera.

Additionally, some isolates recovered 24-42 days post infection in sheep gained serum resistance to seronegative human sera, which could not be induced by repeated sub-lytic challenge (Aboklaish thesis, 2014). This confirms that the longer the time the *Ureaplasma* remained *in utero*, the more it changed phenotypically. Genomic sequencing of the DNA of the above isolates was compared to a reference strain of SV3 (American Type Culture Collection (ATCC), strain 700970) and will be discussed below in 7.8.2.

The slow adaptation of *Ureaplasma in utero* supports the hypothesis of Citti *et al.* (2010) that it is the delayed, adaptive immune response which acts as the selection pressure driving MBA diversification. Interestingly, it is worth noting that increasing MBA size and diversity, does not accompany a significant immune response from the sheep, and size of the MBA mass appears to be consistently driven to an increase, which would be expected to result in increased immunogenicity, with only the largest isoforms being apparent by 5-6 weeks post-infection *in utero*. As of yet, no *in vivo* recovered isolate has displayed phase variation (shut-off) of MBA expression, although *in vitro* this is a repeated phenomenon that accompanies sub-culture in the presence of polyclonal anti-MBA (Zimmerman *et al.* 2009; Samantha *et al., 2012*).

7.8.2 Genomic adaptation of HPA5

The genome of HPA5 was annotated against the 700970 reference strain with a genome size of 751,719 bp [American Type Culture Collection (ATCC)] downloaded from GenBank (GenBank accession number NC_002162). Automated annotation of the completed genomic sequence of HPA5 (Spiller and Beeton, unpublished data) revealed that HPA5 was considerably smaller (688,770 bp), due to large areas of gene deletion resulting in a loss of 50 genes relative to the prototype strain. Using the Geneious 8.0.5 analysis software the assembled pieces of sequenced genomic DNA from recovered isolates were compared to the fully annotated genome for the original strain HPA5, very few conserved genomic differences were observed.The most notable alterations were 17 SNPs between 2 copies of the ammonium transporter genes *amt*1 and *amt*2, which should have no role in complement resistance. There were also very few other individual or small group SNPs across the entire genome, despite having undergone approximately 800+ generations (generation time for *Ureaplasma* is approximately 1 hour), indicating a robust proof-reading mechanism and lack of spontaneous intragenomic recombination. Regions of repeats of greater than 150 bp are not visible to genomic sequencing by Illumina technology due to the underlying chemistry and the leading unique characteristic of the MBA is the variation in 18 bp repeats at the C-terminus. Variation in the MBA protein was already apparent from the Western blot analysis; therefore, examination of the MBA gene had to be performed separately using primers, standard PCR and sequencing of the amplicons. The original HPA5 sequence was found to have 17 repeats of the QPAGKE motif; while the recovered strains were found to have significantly more. Some of which were too large to amplify clearly as the PCR products exceeded 2 kb for the largest isoforms of *MBA* gene. There was an apparent correlation in increased MBA protein mass in serum resistant strains recovered after 5 weeks infection as well as 14.231 isolated from the plasma of a 125 dGA lamb, which was absent in the serum sensitive strain from lamb 14.115 which had

retained the original MBA size. However, delivery of the *MBA* from serum resistant 13.136b1 to the original HPA5 strain, resulting in dual MBA isoform expression, did not result in a transfer of serum resistance. The alternative possibility that should be investigated in the future is that the shorter MBA isoform is dominant and mediates sensitivity to complement. The next experiment should deliver the original *MBA* from HPA5 to the resistant strain 13.136b1 to see if serum sensitivity is rescued as a phenotype. Failing that, there are the *amt*1 and *amt*2 alterations as well as the UU046 gene SNPs, although these latter gene variations are synonymous and would not result in amino acid alteration in the mature protein. This did not turn out to be an easily provable hypothesis, despite all of the supporting evidence appearing to be very clear. The mechanism of how the repeat unit consistently increases in the absence of other genomic alterations is also unknown. In humans, increasing repeat sizes associated with pathology, including Huntington's disease, are well known. In a review by Pearson (2005), examining the underlying mechanisms of repeat stability, they state: "Hairpins and slipped structures occur at DNA sequences with direct repeat (DR) symmetry. The cellular processes of replication and transcription cause unwinding of the duplex that renders the DNA single-stranded, thus giving the repeat sequences the opportunity to fold back and form alternative base pairs within the same DNA strand (Pearson *et al.,* 2005). The repeat sequences that have been associated with neurological diseases, in particular, are prone to slipped-mispairing and hairpin formation during replication and transcription. The formation of these structures can presumably lead to expansions and deletions of the repeating tract because of the stability of the misaligned intermediates. These authors also go on to show that these mechanisms are present in bacteria, but they concentrate on CAG or CTG repeats. The repeat region of *MBA* does have a CAGCAG motif as part of the 18 bp repeat, but most of the models refer to long tracks of CAG repeats required to form hairpins. Whether CAGCAGN_{12}CAGCAG would suffice is impossible to speculate. Interestingly, there is also a CAGCAGCAG

motif in both *amt1* and *amt2*, but alterations to these genes are not due to repeat length alteration – simply SNP (Panigrahi *et al.,* 2005).

Studies concerning human genetic differences: mutations and polymorphisms leading to alterations in complement activity affecting individual susceptibility to *Ureaplasma* were carried on in patients with acquired immunodeficiency syndrom (AIDS) who proved to have increased susceptibility to facultative anaerobes like *Mycoplasma*, AIDS-associated Mycoplasma. Equally, further studies investigated the role of polymorphic genes in pregnancy outcome and they reported some inter-individual differences among white, black, and Hispanic pregnant women in allelic carriage of immune system-related genes like interleukin-1 receptor antagonist (IL-1ra)gene(which binds to IL-1 receptors and inhibits the binding of IL-1α and IL-1β) and IL-4 –590C homozygosity were 4-fold less common in blacks than in whites or Hispanics (Daniel *et al.,*2004; Witkin *et al.,*2002). Another molecular study was done by *Van der Schee et al.,*in 2001 confirmed increased susceptibility to infection by *U. urealyticum* in human with genetic polymorphism in the interleukin-1 receptor antagonist(IL-1RA)gene (Van der Schee *et al.,*2001).

Additionally, whole-exome sequencing was used to test the association of polymorphisms in immune regulatory genes on the response of the host to infection and subsequent PTL which mandate potential genetic screening in nuclear families to start appropriate interference methods to prevent prematurity. McElroy and his team examined the six genes in the complement and coagulation pathways; complement factor H (CFH), complement regulator 1(CR1), coagulation factor XIII (FXIII), coagulation factor V(F5), CR2, and the human complement component C4b-binding protein. (C4BPA) and found that 50% of all the exoms hide at least one novel missense variants (McElroy *etal*. 2013).

8 Summary

In this study I focused on the reason why preterm babies are more liable to acquire *Ureaplasma* (a bacteria from class *Mollicutes*) infection *in utero* or after birth and how can this bacteria adapt to the host in spite of its extreme sensitivity to human and sheep sera.

Using an experimental infection model of pregnant sheep, I found the following:

- The functional complement activity of preterm neonatal sheep sera is low in comparison to matched adult sheep however; its potency increases with increasing gestational age and that maternal sheep antibody were incapable of crossing the placenta. This latter observation means that any immune response observed in fetal sheep and other ruminants must reflect the fetal immune response alone.

- The most potent complement inhibitor for human sera, CDX-1135, a recombinant form of sCR1 (soluble human complement receptor 1) was capable of inhibiting rat sera and human sera, but had negligible inhibition of sheep sera.

- The increased incidence of bacteraemia in younger infected lambs relative to more mature lambs is directly related to the degree of complement system development: a low haemolytic activity in the fetal sera at earlier gestational ages correlated to diminished bactericidal activity.

- The presence of Ureaplasma bacteraemia *in vivo* corresponded to gestational age and was consistent with the individual bactericidal capacity of serum from those animals *in vitro.*

- As with human serum studies, sheep serum bactericidal activity against *Ureaplasma* was calcium dependent, as EGTA removed all haemolytic and bactericidal activity. However, the contribution of classical and lectin pathways could not be further separated due to a lack of reagents specific for sheep complement components.

- Monoclonal antibodies (mAbs) specific for *Ureaplasma* surface protein MBA protein can differentiate between *U. parvum* and *U. urealyticum,* and in some cases identify specific serovars. While these reagents work extensively by Western blot analysis and can be used in immunohistochemistry, the epitopes for the antibodies have not been identified and capacity for their use against native antigens has not been conclusive.

- A recombinant serovar 3 MBA form was created solely by gene synthesis which confirmed the specificity of the panel of monoclonal antibodies.

- The levels of *Ureaplasma* during chronic infection remain reproducibly stable.

- *Ureaplasma* adapts to sheep during intrauterine infection, but required a few weeks to achieve distinctive phenotypic alterations. By 5 weeks post-infection, HPA5 strain had completely adapted and was resistant to fetal sheep sera and was starting to become resistant to adult sheep sera. This adaptation occurred in the absence of specific anti-Ureaplasma antibodyin serum and coincided with increased MBA isoform mass.

- By 5 weeks post-infection all low mass MBA isoform strains had disappeared, and increased MBA isoform mass accompanied the acquisition of resistance to sheep and human sera killing.

- Besides increasing numbers of AGKEQP repeats in the *MBA* gene as the underlying mechanism of increased MBA isoform mass, very few conserved genomic differences

were observed comparing the whole genome sequences from the recovered *Ureaplasma* strains 5 weeks post-infection. The only other notable conserved genetic alteration in 9 recovered strains was 17 SNPs between 2 copies of the ammonium transporter genes *amt1* and *amt2*, which is not expected to play a role in complement resistance.

- Transposon delivery of a second longer isoform of *MBA* gene to the original HPA5 strain, with successful expression, did not transfer serum resistance. This suggests that the presence of the shorter isoform, even in the presence of larger MBA isoforms, may have a dominant determination for complement sensitivity.

Future recommendations,

Identifying pregnant women at risk of PTB: the most trustworthy method is detecting increased pro-inflammatory cytokine levels in the amniotic fluid of women highly colonised with *Ureaplasma* (detected as microbial load in their vagina and cervix) represent those who are probably at highest risk to deliver prematurely. However, this method is invasive and may cause complications later on therefore is unlikely to be accepted as a screening method even with the advent of therapeutic strategies. Other methods in the future may involve use of characterised anti-MBA mAbs, especially if an association for a particular species or serovar can be determined. Alternatively, these mAbs may form the basis of diagnostics to determine such an association. Equally, further studies regarding the number and size of MBA isoforms, irrespective of the species or serovar should be examined, as this has been highlighted in previous sheep studies (Knox *et al.,* 2010) and the mAb based Western blot analysis is the only method of investigating this appropriately. Another future suggestion is to use the synthetic recombenant *MBA* gene instead of the whole *Ureaplasma* protein to confirm that polyclonal

antibodies in serum are picking up MBA .Probably the most important future experiment would be to determine if delivering the short MBA form to a serum-resistant recovered strain of HPA5, returns the strain to serum susceptibility, then the role of MBA as a sole contributor to pathogenicity could finally be ascertained to determine most suitable therapeutic targets in animal models.

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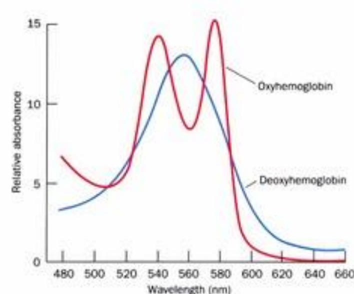
Appendixes

Appendix I: Supplementary methods for chapter 3

Improved measurement of CH50 assay. –This method for measuring the lysis of target erythrocytes by complement depends on increasing transmission of light through the solution of erythrocytes rather than measuring the release of haemoglobin. Other methods that rely on measurement of released haemoglobin have to take into consideration the amounts of haemoglobin that are already in partially haemolysed samples and require centrifugation of unlysed cells with transfer of supernatant to be measured. Certainly repeated measurements on the same samples following different incubation times (as we utilise in this thesis) would not be possible with these methods. We utilise a 96-well plate reader set at 595 nm to measure the obstruction of light by the intact erythrocytes. This is a common filter set utilised for many chromogenic protein measurement assays and is at the very edge of the absorbance spectra for released haemoglobin (image copied from quizlet.com/7051703/bc-ch-7-hemoglobin-and-myoglobin-flash-cards/)



We set up our assays in a 96-well plate, as follows:

A1/A2: 50 µl 0.1% sodium dodecylsulphate in water (for total lysis background subtraction)

B1-H12: 50 µl complement fixation buffer (Oxoid ltd. Basingstoke, UK)

Then add 50 µl test sera in triplicate columns (B1-B3, B4-B6, B7-B9, and B10-B12), then transfer 50 µl from each well in B to the well directly below in C (2-fold dilution), repeat to row G, and then discard 50 µl. The H row is left as a no complement/no lysis control for calculation of percent lysis.

Add 50 µl of approximately 1% target erythrocytes (sensitised or unsensitised) to A1-A2 and B1-H1 and incubate at 37°C. At various time points (5, 10, 20, 30, 60, 90, and 120 min) read the absorbance of the plate at 595 nm utilising the blank correction option of the MRX Revelation software to subtract the average absorbance from total lysis control wells A1 and A2 from the rest of the plate.

At each time point calculate the percent remaining target using the following formula:

% remaining target = (Bn / Hn) x100, where n is the column number. Percent lysis is then calculated = 100 - % remaining target.

Demonstration data set (abs at 595 nm)

		1	2	3
Serum concentration	A	blank	blank	
50%	B	0.000	0.000	0.000
25%	C	0.000	0.045	0.001
12.50%	D	0.127	0.136	0.079
6.25%	E	0.622	0.585	0.612
3.12%	F	0.749	0.760	0.795
0.16%	G	0.777	0.786	0.789
0%	H	0.795	0.799	0.797

Calculate % target remaining after incubation at 37°C

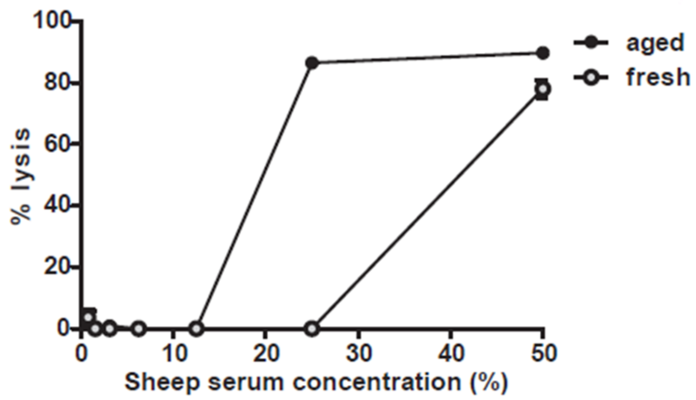
	1	2	3
A	blank	blank	
B	100 x (0/0.795)	100 x (0/0.799)	100 x (0/0.797)
C	100 x (0/0.795)	100 x (0.045/0.799)	100 x (0.001/0.797)
D	100 x (0.127/0.795)	100 x (0.136/0.799)	100 x (0.079/0.797)
E	100 x (0.622/0.795)	100 x (0.585/0.799)	100 x (0.612/0.797)
F	100 x (0.749/0.795)	100 x (0.760/0.799)	100 x (0.795/0.797)
G	100 x (0.777/0.795)	100 x (0.786/0.799)	100 x (0.789/0.797)
H	100 x (0.795/0.795)	100 x (0.799/0.799)	100 x (0.797/0.797)

Calculate % lysis from above:

	1	2	3
A	blank	blank	
B	100 - 0 = 100%	100 - 0 = 100%	100 - 0 = 100%
C	100 - 0 = 100%	100 - 5.6 = 94.4%	100 - 0.1 = 99.9%
D	100 - 16.0 = 84.0%	100 - 17.0 = 83.0%	100 - 9.9 = 90.1%
E	100 - 78.2 = 21.8%	100 - 73.2 = 26.8%	100 - 76.8 = 23.2%
F	100 - 94.2 = 5.8%	100 - 95.1 = 4.9%	100 - 99.7 = 0.3%
G	100 - 97.7 = 2.3%	100 - 98.4 = 1.6%	100 - 99.0 = 1.0%
H	100 - 100 = 0%	100 - 0 = 0%	100 - 0 = 0%

Mean and standard deviation (or standard error of the mean) can then be calculated for each serum concentration from these triplicates.

Supplementary figure 1 chapter 3



Lysis of rabbit antibody pre-sensitised human erythrocytes by adult sheep sera at increasing concentrations after 60 min incubation at 37 C. Cells were prepared in parallel except that "fresh" erythrocytes were obtained 24 hours before the experiment and the "aged" cells had been kept at 4 C for 4 weeks prior to use in the assay.

Appendix II: Abstracts and Publications from this work

Puplished paper “Comparison of Complement Activity in Adult and Preterm Sheep Serum”.

Shatha, A., Matthew, W., Matthew, K., Payne, S., Suhas G., Kallapur., S., Stock,H.J., Marsh,C., Alan H. J., Newnham, J. P. and Spiller,B.O. **published manuscript** in the *American Journal of Reproductive Immunology* 73 (2015) 232–241.

Problem

Functional complement activity is routinely measured utilizing rabbit antibody-sensitized sheep erythrocytes. Due to complement inhibitor expression on erythrocytes, the development of an alternative method to measure complement function in sheep serum was required.

Method of study

Several species of target erythrocyte and sensitizing antibody were investigated for improved measurement of complement function testing.

Results and conclusion

Guinea pig erythrocytes were identified as the optimal target, although sensitizing them with rabbit antiguinea pig erythrocyte antibody did not enhance the lysis by maternal sheep serum. In contrast, preterm neonatal sheep serum was unable to efficiently lyse guinea pig erythrocytes unless pre-sensitized with antibody. Further investigation revealed that maternal serum contained high levels of antibodies that cross-reacted with guinea pig and rabbit erythrocytes, while no cross-reacting antierythrocyte antibodies were found in preterm neonatal serum. Therefore, unlike primates, rabbits, and guinea pigs, no transplacental transfer of maternal IgG to foetal sheep occurs. Use of exogenous complement regulators is often used to dissect the contribution of complement to disease pathogenesis; however, we found that while full-length soluble human complement receptor 1 (sCR1, CDX-1135) was able to inhibit lysis of guinea pig erythrocytes by human and rat serum, no inhibition of sheep serum could be observed. Investigation of complement contribution to disease pathogenesis in the future will require the identification of an inhibitor that is effective against sheep complement.

II- Poster to Postgraduate Research Day 2012, 27th Annual Life Sciences 16th November. "Bacterial evolution of *Ureaplasma parvum* during *in utero* infection in pregnant sheep" by Shatha, A., Matthew, W., Matthew, K., Payne, S., Suhas, G., Kallapur, S., Stock,H.J., Marsh,C., Alan, H. J., Newnham,J.P. and Spiller,B.O.

III -Power point presentation "Bacterial evolution of *Ureaplasma* parvum during *in utero* infection in pregnant sheep"presented in Welsh Microbiological Association(WMA) SUMMER MEETING 20th – 21st June 2014 in the Village Hotel, Swansea. .

1V- Poster presentation to MITReG Annual Event in the 19th January 2015 for which I was awarded the **first prize**. "Bacterial evolution of *Ureaplasma parvum* during in utero infection in pregnant sheep" by Shatha, A., Matthew, W., Matthew, K., Payne, S., Suhas, G., Kallapur, S., Stock, H.J., Marsh, C., Alan, H. J.,Newnham, J.P. and Spiller, B.O.