The role of *Ureaplasma* spp in neonatal lung disease, activation of the complement system and molecular mechanisms of antibiotic resistance.

A thesis submitted in candidature for the

degree of

DOCTOR OF PHILOSOPHY

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Summary of Thesis:

Ureaplasmas are some of the smallest and simplest free-living organisms known. Little is understood regarding the effects of the complement system upon clearance of these pathogens, but when the immune system fails to control *Ureaplasma* colonization disease, such as chronic lung disease of prematurity (CLD), can occur. Treatment of such *Ureaplasma* infections, especially in neonates, is limited by pathogen and host factors. Treatment can be further compromised by antibiotic resistance.

Firstly this thesis examines an *in vitro* system for determining the bactericidal capacity of a selection of human sera against four representative serovars of *Ureaplasma parvum*. Results showed that the classical activation pathway was essential for the killing of all *U. parvum* serovars, with little effect being attributed to the alternative or lectin pathways. Additionally serovar 3 was shown to be the most serum sensitive isolate.

Secondly the association between presence of *Ureaplasma* and 16S rRNA with development of CLD was examined in a prospective cohort of 192 neonates. Data suggested that presence of *Ureaplasma* as well as 16S rRNA was significantly associated with development of CLD as well as increased levels of inflammatory mediators IL-6 and IL-8.

Finally a retrospective cohort of 61 *Ureaplasma* isolates was examined for resistance to various antibiotics. High level macrolide resistance in isolate UHWO10 resulted from a two amino acid deletion within the L4 ribosomal protein (R66Q67). Tetracycline resistance in isolate HPA23 resulted from the presence of the *tetM* gene while a ciprofloxacin resistance in isolate HPA18 resulted from a D82N substitution within the ParC protein. No differences were found in the GyrA, GyrB or ParE proteins. Comparison of type II topoisomerase genes from all *Ureaplasma* serovars revealed that mutations previously associated with resistance where wrongly identified and were a result of species or serovar specific polymorphisms.

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Abbreviations

5'	5 prime
3'	3 prime
16S rRNA	16S ribosomal RNA
23S rRNA	23S ribosomal RNA
AP	Alternative pathway
APB	Alternative pathway buffer
BAL	Bronchioalveolar lavage
BPD	Bronchopulmonary dysplasia
BSAC	British Society for Antimicrobial Chemotherapy
C4BP	C4-binding protein
CLD	Chronic Lung Disease of prematurity
c.f.u.	Colony forming unit
CNS	Central nervous system
CP	Classical pathway
CSF	Cerebrobral spinal fluid
C. trachomatis	*Chlamydia trachomatis*
E. coli	*Escherichia coli*
ETA	Endotracheal aspirate
GF	Gastric fluid
HPA	Health Protection Agency
ICAM	Intracellular adhesion molecule
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IL-6	Interleukin-6
IL-8	Interleukin-8
Kb	Kilo bases
Kg	Kilo gram
LP	Lectin pathway
LRT	Lower respiratory tract
MAb	Mono-clonal antibody
MAC	Membrane attack complex
MASP	MBL-associated serine proteases
Mb	Mega bases
MBA	Multiple banded antigen
MBL	Mannose binding lectin
M. catarrhalis	*Moraxella catarrhalis*
M. genitalium	*Mycoplasma genitalium*
M. hominis	*Mycoplasma hominis*
M. pneumoniae	*Mycoplasma pneumoniae*
NPA	Nasopharyngeal aspirate
NGU	Non-gonococcal urethritis
PAMP	Pathogen-associated molecular patterns
PAI	Pathogenicity Island
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.05% tween
PCR	Polymerase Chain Reaction
PL	Phospholipase
PRM	Pattern recongnition molecules

RCA	Regulators of complement activation
RDS	Respiratory distress disease
S. aureus	*Staphylococcus aureus*
SNPs	Single nucleotide polymorphism
TK	Thymidine kinase
TX-114	Triton X-114
U. parvum	*Ureaplasma parvum*
Upvmp376	*Ureaplasma* phase-variable membrane protein 376
U. urealyticum	*Ureaplasma urealyticum*
VBS	Veronal buffered saline
VLBW	Very low birth weight

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Chapter 1

Introduction

Thesis overview

In this introduction I aim to summarize the current literature regarding the neonatal pathogens classified within the genus *Ureaplasma*. I will discuss aspects of the organisms basic biology, its role in disease, as well as its susceptibility and resistance to a number of antibiotics. I will also introduce some of the additional key concepts which are found within in this thesis such as chronic lung disease of prematurity as well as an overview of the complement cascade and how these topics relate to *Ureaplasma* and disease.

1.1 *Ureaplasma* spp.

1.1.1 Classification of Ureaplasma spp.

Ureaplasma spp. are taxonomically placed in the phylum *Firmicutes*, class *Mollicutes*, order *Mycoplasmatales*, family *Mycoplasmataceae* and genus *Ureaplasma*. First isolated from men with non-gonococcal urethritis (NGU) (Shepard, 1954), these organisms were originally referred to as T-mycoplasmas due to their tiny colonies produced relative to other mycoplasmas, but due to their ability to utilize urea as an energy source the new genus of *Ureaplasma* was created (Robertson *et al.* 2002). Prior to 2002, the human Ureaplasmas comprised of a single species, *Ureaplasma urealyticum*, which was divided into 2 biovars. Biovar 1, included serovars 1, 3, 6 and 14, whereas biovar 2 included serovars 2, 4, 5, 7, 8, 9, 10, 11, 12 and 13. In 2002, the biovars for *U. urealyticum* were officially reclassified into two distinct human associated species *Ureaplasma parvum* (previously biovar 1) and *U. urealyticum* (previously biovar 2) (Robertson *et al.* 2002). *U. parvum*, with serovar 3 being the species prototype strain, contain the smallest genomes ranging from 0.75 – 0.77 Mb whereas *U. urealyticum* species, with serovar 8 being the species prototype strain, all contain larger genomes ranging from 0.83 – 0.94 Mb.

1.1.2 Characteristics of Ureaplasma spp.

Ureaplasma spp. are small (0.2μm) pleomorphic mucosal pathogens (Figure 1.1) which require sterols for growth and like other members of the class *Mollicutes*, do not possess a cell wall (Robertson *et al.* 2002). They are surrounded by a triple layered membrane with an electron dense outer

surface (Black *et al.* 1972). A property unique among Mycoplasmas is their ability to hydrolyse urea into ammonia. Ammonia which accumulates intracellularly, as a result of urea hydrolysis by the urease enzyme, creates an electrochemical gradient in which ATP is generated. Smith *et al.* showed that this unique property among *Mollicutes* was essential for 95% of energy production by the addition of the urease inhibitor flurofamide which reduced *de novo* ATP synthesis to 5% (Smith *et al.* 1993).

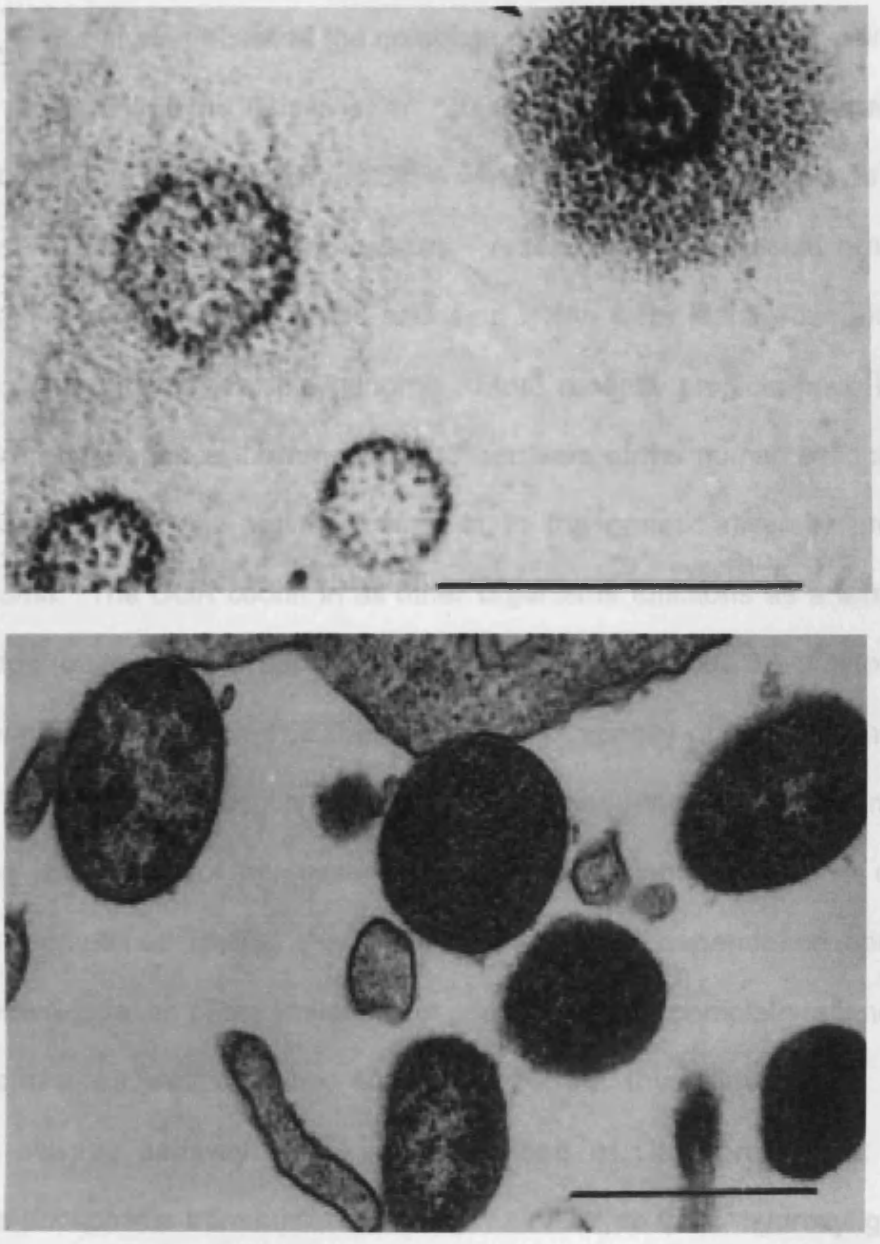


Figure 1.1. Micrographs of *Ureaplasma* spp. (A) Microcolonies of *Ureaplasma* showing urease activity (bar=100μm). (B) Transmission electron micrograph of *Ureaplasma* showing the small size of cells (bar = 0.5μm) (Robertson *et al.* 2002).

1.1.3 Genome and coding capacity

In 2000, Glass *et al.*, published the complete genome sequence of *U. parvum* serovar 3 ATCC 700790 (Glass *et al.* 2000). The total genome contained 751,719 bp with a G+C content of 25.5% which is typical of *Mollicutes* as well as bacteria with reduced genome sizes. A total of 613 predicted protein-coding genes along with 39 genes encoding RNAs were identified, which in total accounted for 93% of the genome. More recently projects have been undertaken to sequence the remaining 13 serovars of the human associated *Ureaplasmas* which will provide insight in to the genetic diversity among *Ureaplasma*. The UGA codon in all other organisms functions as a terminal stop codon (opal), but in *Ureaplasma* and *Mycoplasma* encode the amino acid tryptophan (Blanchard, 1990). The small coding capacity of mycoplasmas is a result of genome reduction over time. A consequence of such genome reduction is the loss of non-essential genes which can be found in other bacteria, sometimes limiting the organism to obligate dependence upon a host. One illustration of the limitations of gene loss is the complete reliance of ureaplasmas, as well as other *Mollicutes*, on the thymidine kinase (TK) enzyme salvage pathway for *de novo* systhesis of DNA precursors. TK transfers phosphates from nucleosides, such as ATP, to the 5' hydroxyl group of pyrimidine deoxyribonucleoside (Carnrot *et al.* 2003).

1.1.4 Virulence factors of ureaplasmas

1.1.4.1 Urease production

As well as being essential for 95% of the energy production in *Ureaplasma*, urease has also been proposed as a significant virulence factor. The ability of *Ureaplasma* to colonize regions such as the upper urinary tract and cause infective kidney stones is dependent on the ability to hydrolyse urea. Ligon and Kenny found that when trying to raise monoclonal antibodies to viable *Ureaplasma* in mice, death was experienced only 5 minutes after inoculation (Ligon and Kenny, 1991). However, mice which received whole bacteria which were pre-treated with the potent urease inhibitor, flurofamide, were shown to be protected against the urease activity and did not show any sign of adverse symptoms. As a result of these experiments ammonia toxicity was determined to be the cause of death for mice injected with *Ureaplasma* in the absence of inhibitor. The authors discussed that although numbers found in a normal infection would not be as high as those used in the mouse model, it would be likely that local tissue damage would occur thus causing an inflammatory response.

1.1.4.2 *Ureaplasma* phospholipases

It is accepted that there is a significant link between intrauterine infection and preterm birth (Andrews *et al.* 2000). Phospholipase (PL) activity in bacteria has been suggested as a contributing factor to premature birth, by hydrolyzing placental membrane phospholipids, resulting in an increase in free arachidonic acid followed by increase in prostaglandins. The presence of PL

A1, A2 and C activity in *Ureaplasma* was first noticed by De Silva and Quinn (De Silva and Quinn, 1986). Later work by the same group determined that there was great variation in activity of these enzymes between different serovars with PL A2 activity being 3-fold greater in serovar 8 than that of serovars 3 or 4. The authors suggested that this variation was a possible explanation for varied clinical outcomes in patients colonized with these organisms (De Silva and Quinn, 1991). The functional conditions at which the enzymes work optimally also differ between the PL: PL A1's optimal range is between pH 8.5 – 9 whereas PL A2's optimal range is pH 7, notably with 50-100 fold greater activity than that of PL A1 (DeSilva and Quinn, 1999). PL C on the other hand has a more acidic preference with an optimal range of pH 6. Although the enzymes have been studied, the published genome sequence data did not identify any putative orthologous sequences, possibly due to sequence divergence compared to other bacterial species (Glass *et al.* 2000). Non-*ureaplasma* derived PL A2 has been shown to inhibit pulmonary surfactant *in vitro* (Schrama *et al.* 2001) which has led to suggestions that, inhibition of pulmonary surfactant by *Ureaplasma* PL A2 might be a mechanism of causing lung injury (Cultrera *et al.* 2006).

1.1.4.3 *Ureaplasma* IgA protesase

IgA is the most predominant immunoglubulin in mucosal layers, the presence of an IgA proteases suggests an essential virulence factor. Kapatais-Zoumbos K *et al.,* demonstrated that the production of IgA proteases was host specific with human *Ureaplasma* only producing proteases capable of degrading human IgA which was specific for IgA1 and not IgA2 (Kapatais-

Zoumbos *et al.* 1985). This specificity suggests co-evolution between specific *Ureaplasma* species and their host. The specific region within the IgA1 which the enzyme cleaves was determined by Spooner *et al.*, who deduced via N-terminal sequencing that cleavage occurs between proline and threonine between residues 235 and 236 (Spooner *et al.* 1992). The absence of this region in IgA2 explains the enzymes specificity. Interestingly as with the PL discussed earlier, no orthologues for the protease were found within the sequenced genome.

1.1.4.4 Haemolysins

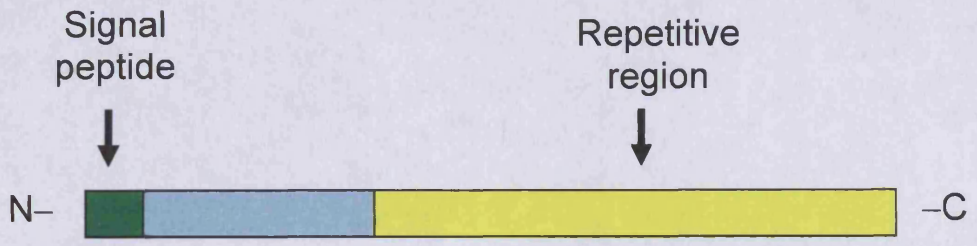
The sequenced SV3 genome contained two hypothesized enzymatic haemolysins. The haemolysin transcribed from the *hlyA* gene is thought to be the predominant of the two with *hlyC* being an orthologue of one found in *M. pneumoniae* (Glass *et al.* 2000). The haemolysin encoded by *hlyA* maybe a significant virulence factor of the *Ureaplasma* as data from mycobacteria demonstrate that the gene is only present in pathogenic species of the genus (Wren *et al.* 1998).

1.1.4.5 The multiple banded antigen

The multiple banded antigen (MBA) of *Ureaplasma* has been proposed as being one of the predominant antigens recognized during infection (Watson *et al.* 1990). As the names suggests immunoblots of *Ureaplasma* probed with monoclonal antibodies (MAbs) produce a laddering "multiple banded" effect and has been proposed to function as an adhesin (Monecke *et al.* 2003;

Manchee and Taylor-Robinson, 1969). Watson *et al.*, showed using sera from infected patients, as well as MAbs, that the antigens contain both serovar specific and pan-serovar cross-reacting epitopes. The protein is composed of N-terminal signal peptide with a hydrophobic core and a lipoprotein lipid attachment site at a cysteine (Zheng *et al.* 1995) whereas the final two thirds of the C-terminal end of the protein is comprised of multiple repeat units. In the case of SV3, the repeats consist of the amino acids GKEQPA. Figure 1.2 shows a generalised structure of the MBA with the C-terminal repeat units for all serovars as described by Kong *et al.*, 2000 (Kong *et al.* 2000).

A.



B.

Species	Serovar	C-terminal repeats
U.p	SV1	QQPGKE
U.p	SV3	QPAGKE
U.p	SV6	PGKE
U.p	SV14	QQPAGKE
U.u	SV2, 5, 8	TKPGSGET
U.u	SV10	TQPGSGST
U.u	SV4, 12, 13	TSPEKPGNGT

Figure 1.2. Structure of the multiple banded antigen of *Ureaplasma* spp. (A) The protein contains an N-terminal signal peptide (green) followed by a species conserved non-repetitive region (blue). Two thirds of the C-terminal consists of repetitive amino acid sequences (Yellow). (B) Amino acid repeats contain differences between serovars (Kong *et al.* 2000). No repeat regions have been detected in SV9, 7 and 11. U.p and U.u denote *U. parvum* and *U. urealyticum*, respectively.

The ability to evade a host immune response is an adaptive strategy employed by a number of pathogenic bacteria. Ureaplasmas have several proposed mechanisms to keep one step ahead of the immune system by varying its surface antigenicity. Phase variation was the initial mechanism of altering the antigenicity of MBA (Zheng *et al.* 1995). Zheng *et al.*, described how a clinical SV3 strain isolated from lung tissue of a new born infant contained two major bands of 53 and 68.5 KDa via immunoblotting. This isolate was sub-cloned and found that clones 1 and 21 expressed the 53 KDa protein and a clones 16 and 19 expressed the 68.5 KDa protein. PCR analysis showed no difference in fragments size of the N-terminal region between clones, but clones 1 and 21 had smaller C-terminal PCR products relative to clones 16 and 19 suggesting deletion of repeat units. A second mechanism of variation results in an on/off switching of the MBA gene. Monecke *et al.*, co-incubated *Ureaplasma* from both species with HeLa cells and/or erythrocytes and compared the immunoblot profiles of parental strains to non-adherent strains (Monecke *et al.* 2003). Unlike previously reported by Zheng *et al.*, (Zheng *et al.* 1995) phase variation did not occur, but instead there was complete loss of antibody reactivity. Total removal of all repeat units was dismissed as the reason for this loss of reactivity due to PCR failing to amplify the non-repetitive region of MBA in the negative mutants. Later work by Zimmerman *et al.*, (Zimmerman *et al.* 2009) proposed this on/off switching of MBA to coincide with respective off/on switching of adjacent Upvmp376 protein. They suggests that the non-repeat region of the MBA can either drive transcription of MBA, or as a result of a recombinant inversion

event, will flip its orientation and drive transcription of the oppositely oriented Upvmp376 protein.

1.1.4.6 *Ureaplasma* pathogenicity island

Genomic analysis has revealed a hypothetical pathogenicity island (PAI) within the genome of *U. parvum* (Momynaliev *et al.* 2007). The 19,943bp region designated UU145-UU170 has a number of characteristics suggesting its role as a PAI which has been acquired horizontally. The researchers proposed this region as a PAI on the following observations: the PAI was not seen in all clinical isolates screened; the PAI has a conserved length; the site at which it is inserted into the genome is directly linked to tRNA genes, which have previously been hypothesized as anchorage points for horizontally transferred elements. The greatest supporting evidence was the presence of genes encoding proteins essential for horizontal transfer such as the in:ripX intergrase-recombinase enzyme and the phage recombinase enzyme. Examination of genes within the element showed adhesin homologues, which may be of significance to virulence. Normally PAI have a skewed G+C content relative to the rest of the host genome, but in this case the G+C contents were very similar. Further comparative genomic analysis showed that the tetranucleotides GCGC and CGCG were not present within the element, but prolific among other regions of the *Ureaplasma* genome.

1.1.5 Detection, quantification and serotyping of Ureaplasma spp.

1.1.5.1 Culture and quantification of *Ureaplasma* spp.

Ureaplasmas are generally regarded as fastidious in their growth requirements. Ammonia production as a result of urea hydrolysis in *Ureaplasma* has provided the foundations to culture based detection methods. The preference of *Ureaplasma* of an acidic culture medium has also been useful for promoting *Ureaplasma* growth relative to other bacteria as well. Using a pH indicator, such as phenol red, it is possible to see a shift in media pH from around 6.5-6.8 to a more neutral pH. This is essential as *Ureaplasma* cultures never reach turbidity due to the toxic nature of the metabolites produced, as well as decreased growth with increasing the pH and depletion of urea, precipitating a rapid death phase. Broth cultures are incubated at 37 ^{o}C in atmospheric conditions with colour change in broth cultures taking 24 – 48 hours depending on the size of the inoculum. Agar plates require 5 % CO_2 and can take 1 – 5 days. A microscope or hand lens is required to examine plates due to the tiny colonies that form. Unlike conventional mycoplasmas, such as *M. hominis*, which produce a "fried-egg" morphology, *Ureaplasma* colonies produce brownish zones as a result of urease activity (Figure 1.1A). When working with liquid cultures it is recommended to make serial dilutions of the inoculum to around 10^{-7} so to dilute any potential inhibitors such as antibiotics, antibodies or built up ammonia. Serial 10-fold dilutions in a micro-titre plate are used to quantify the numbers of *Ureaplasma* in the original specimen as colour changing units

(CCU). The CCU is determined when there is no more colour change from the 10-fold dilutions (usually 48 hours post-innoculation). The final well in which colour change occurred is regarded as 1 CCU. By working back through the dilution gradient it is possible to determine the number of CCU's in the original culture.

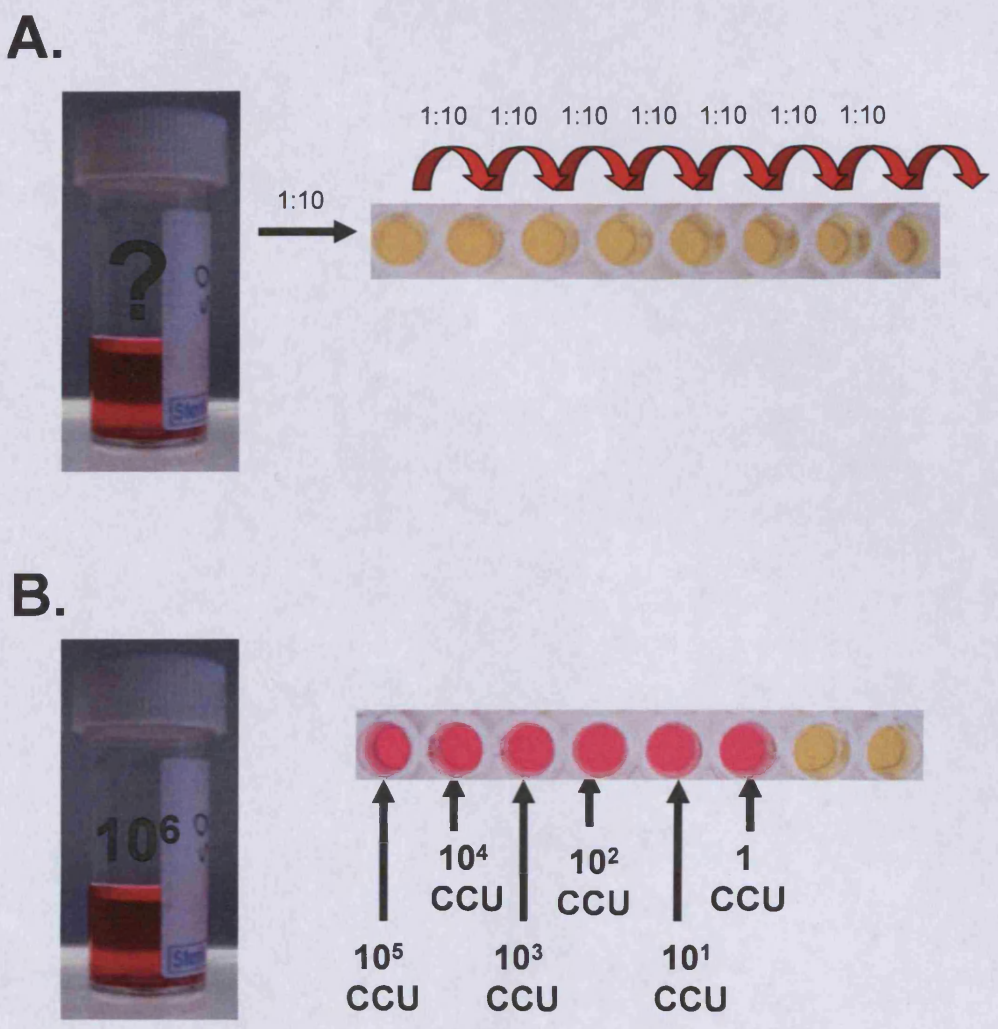


Figure 1.3. The method for determining the number of *Ureaplasma* colour changing units (CCU) in a culture. (A) A sample of unknown CCU is titrated 1:10 in a microtitre plate in *Ureaplasma* specific media. Cultures are then incubated at 37 °C for 48 hours until colour change has ceased (B). The final well which colour change occurs is regarded as 1 CCU. By working back through the dilution gradient it can be concluded that there were 10^6 CCU in the original culture at the time of inoculation.

1.1.5.2 Molecular detection of *Ureaplasma*

PCR has been essential for the detection of many bacterial pathogens with fastidious growth requirements. A number of primers are now available which amplify genes including the urease genes, 16S rRNA gene, 16S-23S intergenic spacer region and the multiple banded antigen (MBA) gene and have been used to associate *Ureaplasma* with arthritic conditions, male and female genitourinary specimens, as well as neonatal respiratory specimens (Colaizy *et al.* 2003). Blanchard *et al.*, (Blanchard *et al.* 1993) designed primers to amplify a 430 bp region of the *ureA* gene. These primers were applied to all 14 serovars and could detect to a level of 1-10 colony forming units (c.f.u.). These primers are selective for *Ureaplasma* and have been tested against other common neonatal pathogens to confirm a high stringency against false positive results.

A highly sensitive method for detecting *Ureaplasma* was also developed by Benstein *et al.* (Benstein *et al.* 2003a; Benstein *et al.* 2003b). The group first developed an *in situ* hybridization assay using a 100 bp biotinylated DNA probe specific for the urease gene of *Ureaplasma* (Benstein *et al.* 2003a). The probe was able to demonstrate that *Ureaplasma* was associated with lung epithelial cells from newborn mice inoculated with *Ureaplasma*. It was also possible to detect *Ureaplasma* inside alveolar macrophages suggesting the organism's prior presence within the lower respiratory tract. This technique was then applied to lung autopsy tissue from fourteen patients, nine of which showed histology consistent with CLD at autopsy. Seven out the nine CLD cases were *Ureaplasma* culture positive (Benstein *et al.* 2003b).

Interestingly, the assay detected the presence of *Ureaplasma* in all the CLD-like tissue samples, including those which were *Ureaplasma* culture negative. These data suggest that the detection rate in many studies may under-represent the true incidence of *Ureaplasma* in CLD patients when using standard culture techniques alone as well as the clearance of bacteria via the immune response.

1.1.5.3 Typing of *Ureaplasma* isolates

Ureaplasmas were first typed using a metabolic inhibition (MI) assay in which anti-sera to a range of isolates were raised in rabbits (Purcell *et al.* 1966). *Ureaplasma* isolates, including the strain used for immunisation, were added to a range of known dilutions of the anti-sera in the presence of guinea pig serum as a source of complement. The absence of colour change was used as confirmation of a positive recognition of the antiserum for that strain and the strength of recognition was determined by titring the effect of the specific antibodies. Draw backs of this methodology was that it only took into account classical "antibody-dependent" complement killing pathway and did not consider any potential effects from the lectin or alternative pathways which are discussed in section 1.4.3 and 1.4.4, respectively. In addition, the assay was run in medium containing urea, a known nucleophilic acceptor which would inhibit the complement activity which the assay was dependent upon.

1.1.5.4 Pulse-field gel electrophoresis (PFGE) for typing *Ureaplasma* isolates

Current characterisation of *Ureaplasma* serovars is largely carried out using PCR based methodologies whereas Pulse-Field Gel Electophoresis (PFGE) had been the gold standard method for typing bacterial isolates. Moser *et al.* examined the ability of PFGE to characterize *Ureaplasma* into individual species and then into separate serovars (Moser *et al.* 2006). Results showed that using restriction enzymes which cut the *Ureaplasma* genome infrequently, such as *Bss*HII, they were able to differentiate between *U. parvum* and *U. urealyticum*. To determine the method's clinical application, five clinical isolates of serovar 6 were compared to that of the *U. parvum* SV6 reference strain. Results showed that variation in banding patterns occurred between clinical isolates compared to the reference strain. Variation in results was also noticed in serovar 8 following serial passages. It was proposed that the rate of mutation within particularly variable regions led to rapid gain and loss of restriction sites, which caused alterations in banding patterns even after a couple of propagation passages of defined strains.

1.1.5.5 PCR and sequencing for typing *Ureaplasma*

One of the most accurate and rapid methods of determining the species of *Ureaplasma* within a clinical sample was developed by Teng *et al.*, (Teng *et al.* 1994). Primer set UM-1 amplified the 5' region of the MBA gene to exploiting the reduced coding capacity of *U. parvum* serovars relative to *U. urealyticum* therefore producing differential sized PCR products of 403 bp and

448 bp, respectively. By direct sequencing the UM-1 PCR product it is further possible to determine the SV of *U. parvum* isolates by comparison of -54 to -56 regions and the -82 to -84 regions, unfortunately due to sequence conservation among *U. urealyticum*, serovar determination in the region is not possible. By sequencing the whole 5' and partial repeat region of the 3' region of the MBA from all 14 serovars Kong *et al.*, (Kong *et al.* 2000) showed that *U. urealyticum* could be divided into five different MBA genotypes. Genotype A consisted of serovars 2, 5 and 8; genotype B serovar 10, genotype C 4, 12 and 13; genotype D serovar 9 and genotype E with serovars 7 and 11.

1.1.5.6 Real-time PCR detection for *Ureaplasma*

A highly sensitive method for detecting bacteria with fastidious growth requirements, such as *Ureaplasma*, is real-time PCR. A number of studies have examined the use of this technique to detect *Ureaplasma* in a variety of clinical samples. Yi *et al.*, designed primers against the urease gene which allowed the simultaneous detection as well as speciation of *Ureaplasma* in two reference strains as well as confirming 42 strains which the species had been determined by PCR and sequencing (Yi *et al.* 2005). The investigators subsequently examined 87 clinical specimens and confirmed that the method was more sensitive than direct culture. A similar study published in the same year by Mallard *et al.*, again used the urease gene as a target for a real-time probe (Mallard *et al.* 2005). In contrast to the study by Li *et al.*, the probes were more rigorously tested against a range of sixteen clinically relevant bacteria including seven urease-producing species. Results showed that the

probes were specific for the *Ureaplasma* urease as well as having a sensitivity of detecting five copies of the gene per reaction. As only a single copy of the *ureB* gene has been identified in the sequenced genome of SV3, this would correspond to five bacteria per reaction (Glass *et al.*, 2000).

1.1.6 Ureaplasma and disease

Ureaplasmas were first identified as a human pathogen in 1954, when it was isolated from the urethra of a male patient with NGU (Shepard, 1954). Since the initial report, *Ureaplasma* infection has been linked to NGU (Horner *et al.* 2001), infertility (Reichart *et al.* 2001), infectious kidney stones (Hedelin *et al.* 1984), arthritis in hypogammaglobulinemic patients (Frangogiannis and Cate, 1998; Furr *et al.* 1994; Taylor-Robinson *et al.* 1985), pelvic inflammatory disease (Abele-Horn *et al.* 1997a), meningitis in adults (Geissdorfer *et al.* 2008), kidney abscess in transplant patients (Eilers *et al.* 2007), pericarditis (Tarrant *et al.* 2009) as well as multiple diseases in neonates (Waites *et al.* 2005).

1.1.6.1 *Ureaplasma* and disease in adults

After the first link between *Ureaplasma* and NGU in the 1950's, studies have continued to examine this association. One study providing evidence for the role of *Ureaplasma* to induce NGU was carried out by D. Taylor-Robinson and G. W. Csonka who self-inoculated themselves with two clinically isolated SV5 strains (Taylor-Robinson *et al.* 1977). The first volunteer showed signs of dysuria following inoculation, but following treatment with tetracycline cleared the infection and symptoms resolved. The second volunteer also developed symptoms of NGU, but additionally urinary threads were noticed in the urine. The volunteer responded to treatment, but threads persisted. Seminal fluid showed that the prostate was infected and was contributing to the urinary threads.

Horner *et al.*, (Horner *et al.* 2001) tested 114 patients with acute NGU for *Chlamydia trachomatis*, *Mycoplasma genitalium* and *Ureaplasma* spp and compared these with 64 NGU negative controls. They found that after multivariate analysis controlling for *C. trachomatis*, *M. genitalium*, and sexual lifestyle *Ureaplasma* were not associated with acute NGU, whereas on univarite analysis they were. The authors suggested that although in their study *Ureaplasma* was not associated with acute NGU, possibly due to the high carriage rate in the sexually active control group, it does cause disease in a subset of patients. They proposed that infection with symptoms is caused on initial exposure, but patients develop tolerance over time which then results in the high carriage rate. An additional interesting finding was that detection of *Ureaplasma* in the control group was associated with a recent change in sexual partner. Of the 114 acute NGU patients, 86 men re-attended the clinic within 10 to 92 days post treatment with either doxycycline or erythromycin, in which *Ureaplasma* detection was then significantly associated with chronic NGU in patients which were symptomatic. This may additionally suggest a high rate of antibiotic resistance within this population.

Brown *et al.*, (Brown *et al.* 1983) showed a differential serological response between *Ureaplasma* positive NGU patients and *Ureaplasma* positive healthy controls by developing an enzyme linked immunosorbant assay (ELISA) for the detection of IgG, IgM and IgA in acute and convalescent phase sera. Antibody titres were significantly higher in culture positive NGU patients relative to that of culture positive healthy controls. A further interesting result was the degree of change in each antibody titre. *Ureaplasma* positive control

patients had a significantly lower change in antibody titre over a four to six week time period relative to *Ureaplasma* positive NGU patients. The authors suggested that the presence of antibody alone was not useful in determining a relationship between infection and disease, but perhaps the degree of change in titre would be a more valid measure to correlate disease with infection. One draw back of the assay was the inability to differentiate between which serovars the antibody response was raised to, which is most likely a result of the cross reactive epitopes in the MBA which were described earlier.

1.1.6.2 *Ureaplasma* and its role in chorioamnionitis and preterm birth

The amniotic sac which surrounds the developing fetus consists of an outer and inner membrane known as the chorion and amnion, respectively. Inflammation of this region, chorioamnionitis, has been linked to spontaneous abortion, preterm birth as well as development of chronic lung disease (CLD) which will be discussed further in section 1.2.4. *Ureaplasma* are believed to be one of the leading causes of such inflammation due to being the most commonly isolated organism from mothers with the condition (Cassell *et al.* 1986; Yoon *et al.* 1998). Amniocentesis samples have shown that colonisation of the chorioaminon by *Ureaplasma* can occur in the presence of intact membranes as early as the second trimester at weeks 15-20 (Cassell *et al.* 1983; Gerber *et al.* 2003; Perni *et al.* 2004). Abele-Horn *et al.* showed that from 170 mothers positive for *Ureaplasma* colonisation 10% experienced chorioamnionitis compared to none in the *Ureaplasma* negative group. Additionally 35% of the *Ureaplasma* positive group had premature rupture of

membranes compared to 12% in the control group and 41% delivered prematurely compared to only 10% (Abele-Horn *et al.* 1997b). Intrauterine infections are a significant risk factor in preterm delivery. The presence of bacteria induces an inflammatory response resulting in the production of prostaglandins giving uterine contractions, cervical dilations and membrane rupture (Waites *et al.* 2009). Due to the strong evidence associating *Ureaplasma* with chorioamnionitis, it is not unreasonable to implicate *Ureaplasma* in the role of preterm birth.

1.1.6.3 *Ureaplasma* and disease states of neonates

Ureaplasmas can be transmitted vertically from mother to child via a number of routes including ascending the genital tract, haematogenously, and contraction from the birth canal during birth. Rates of maternal transmission of *Ureaplasma* are strongly inversely correlated with birth weight and prematurity with infants born during the second trimester weighing less than 1,500 g having the highest rate (Chua *et al.* 1998; Izraeli *et al.* 1991). Chua *et al.* (Chua *et al.* 1998) determined the rate of transmission from mothers with cervical colonisation of *Ureaplasma* to their child was 88.2%. This data confirms that of earlier studies by Izraeli *et al.* who had found rates of transmission to be 80% (Izraeli *et al.* 1991). A number of conditions in neonates have been associated with *Ureaplasma* infection following transmission from the mother which include meningitis, systemic infection, pneumonia and CLD .

1.1.6.4 Central nervous system and systemic *Ureaplasma* infections in neonates

Rates of systemic bacterial infection has been predicted to be 4-fold higher in neonates less then 2.5 Kg relative to those of a greater birth weight. Group B *Streptococci* and *Escherichia coli* are known as leading infectious agents of the central nervous system (CNS) in this patient group, but since the mid-1980s a body of evidence has been growing to support the idea of mycoplasmas as a causes of systemic (circulating) infections, as well as presence in the cerebrospinal fluid (CSF) coincident with meningitis and brain abscesses (Rao *et al.* 2002). *Mycoplasma* presence in neonatal has been hypothesised to originate from lung colonisation which spreads to the CSF via a haematogenous route. Two sets of data support this hypothesis: Firstly, a study of 19 infants with positive *Ureaplasma* endotracheal aspirates (ETA) were examined for systemic infection. 26% of blood cultures were positive for the organism, demonstrating a link between lung colonization and systemic infection (Cassell *et al.* 1988). Secondly, a study by Waites *et al.* showed *Ureaplasma* was isolated from the lower respiratory tract of half of patients with a positive CSF culture and the authors proposed that the lungs were the original source of the infection (Waites *et al.* 1988).

In 1988, Waites *et al.* studied CSF from 100 predominately preterm neonates, 8% were positive for *Ureaplasma* and 5% were positive for *Mycoplasma hominis* with suggesting that *Mollicutes* were most common organisms isolated from the CSF of very low birth weight preterm infants (VLBW) infants (Waites *et al.* 1988). A second study by Waites *et al.* in 1990 again looked

for *Ureaplasma* and *M. hominis* in 318 infants born near to or at term (Waites *et al.* 1990). *M. hominis* was found in 9 patients where as *Ureaplasma* was found in 5. From the total of 14 patients with positive cultures, 5 cleared spontaneously without treatment and one infected infant died with evidence of meningitis. Spontaneous clearance of *Ureaplasma* has also been documented in other studies (Neal *et al.* 1994). A case report describes the clearance *Ureaplasma* from the CSF of on 28 week neonate using chloramphenicol following the isolation of an erythromycin-resistant strain (Hentschel *et al.* 1993).

The true rate at which *Ureaplasma* and other mycoplasmas are isolated from neonatal CSF may be under-reported as a result of not being detected by Gram stains, as well as not being detected by routine bacterial culture. For this reason, it has been recommended that examination for mycoplasmas in the CSF should be carried out in all neonatal samples which are negative upon Gram stain examination and/or culture with pleocytosis, hydrocephalus or radiographic signs of CNS infection (Waites *et al.* 1993).

1.1.6.5 *Ureaplasma* as a causative agent of neonatal pneumonia

One of the earliest bodies of evidence for the causative role of *Ureaplasma* in neonatal pneumonia came from a study of 290 neonatal deaths occurring from congenital pneumonia. *Ureaplasma* was found in 23 of the 290 samples examined, with all but two from still born births. The authors suggested that an infection *in utero* resulting from transition of the bacteria through intact foetal membranes (Tafari *et al.* 1976). A case report commented on the

isolation of *Ureaplasma* from a neonate who died from pneumonia shortly after birth. A significant amount of lung inflammation and hyaline membrane disease (and early pseudonym for respiratory distress syndrome) was documented in post-mortem samples giving further evidence for the role of *Ureaplasma* in the onset of pneumonia (Brus *et al.* 1991). Pacifico *et al.*, examined 94 infants and 53% of *Ureaplasma*-positive infants showed radiographic signs of pneumonia relative to 21% of those with a *Ureaplasma* negative culture. The study concluded that VLBW infants colonised with *Ureaplasma* were twice as likely to develop pneumonia than non-colonised infants (Pacifico *et al.* 1997). Rudd and colleagues found that two separate strains of *Ureaplasma* isolated from infants with pneumonia were able to cause pneumonia in newborn mice, but those who were 2 weeks old were less susceptible, suggesting an age-dependent risk factor (Rudd *et al.* 1989).

Several investigators have suggested a link between congenital pneumonia resulting from *Ureaplasma* and the onset of chronic lung disease of prematurity (CLD). Cassell *et al.* suggested that *Ureaplasma* may lead to CLD by initially causing neonatal pneumonia. If the pneumonia goes undetected, then oxygen requirement will likely increase thus setting up further conditions which will increase the risk of developing CLD (Cassell *et al.* 1993).

1.1.6.6 Differential pathogenicity of *Ureaplasma*

Onset of infectious disease may not only be dependent upon the genus or species of the organism present, but in some cases the serotype, as with

Haemophilus influenzae serotype B can which can account for around 95% of *H. influenzae* invasive disease in children (van Alphen and Bijlmer, 1990). It has been proposed that this species or serovar association with disease occurs in *Ureaplasma* accounting for why some neonates develop disease, but results are far from conclusive. An argument for species specific pathogenicity was offered by Deguchi *et al.* who compared the incidence of each species between 572 male patients with NGU and a control group of 141 men with no signs of urethritis. Results showed that numbers of patients with *U. urealyticum* were significantly higher in patients with NGU (15.8%) than those of the control group (7.8%), whereas there was no significant difference between NGU patients and the control group with *U. parvum*. The authors concluded that the low levels of *U. parvum* may reflect colonization rather than infection (Deguchi *et al.* 2004). Katz *et al.* examined whether there was a link between colonization with *U. parvum* or *U. urealyticum* and clinical outcome in a neonatal population (Katz *et al.* 2005). Results showed that *U. parvum* was the most commonly isolated from ETA from neonates, but no link was seen between the isolation of a single species and adverse clinical outcome. A significant number of infants colonized with both species developed CLD relative to those who did not develop the condition. Alternatively, Cultrera *et al.* found that from a study of 50 infants, 24 with and 26 without respiratory distress syndrome (RDS), only infants colonized with *U. parvum* developed RDS whereas a number without RDS had *U. urealyticum* (Cultrera *et al.* 2006). The significance of both these studies is questionable due to the low numbers of individuals therefore a larger study size is needed to determine a true role. A study of invasive isolates was carried out by

Zheng *et al.*, (Zheng *et al.* 1992) who used MAbs to look at serovar differences in invasive disease from ten CSF and three blood isolates from varying neonates. They found that of the CSF isolates, 1 isolate was an SV1, 2 were SV3, 1 was SV6, 1 was SV 8 and 2 were SV10 and 3 unknown. For the *Ureaplasma* isolated from blood, 2 out of the 3 were unknown, while the remaining isolate was found to be SV3. This suggests that there was no difference between serovars, but virulence may lie in the ability of the organism to vary its surface antigens. As mentioned earlier, this study by Zheng *et al.* demonstrated that *Ureaplasma* have the ability to vary their antigen expression and this could ultimately be more significant in disease progression rather than serovar (Zheng *et al.* 1992).

From these results no conclusive agreement has been reached. Results have varied between studies, cross reactivity of antibodies remains poorly defined and studies are always underpowered due to small study group sizes. To ascertain a potential role of either a specific species or serovar in pathogenicity, more serovar specific reagents and larger well controlled studies are required.

1.2 Chronic lung disease of prematurity

1.2.1 Chronic lung disease of premetuirty; an overview

CLD, also known as bronchopulmonary dysplasia (BPD), is a disease primarily seen in preterm neonates resulting from a number of aetiologies. It was first defined in 1967 by Northway *et al.*, who noted that preterm infants who had received mechanical ventilation and oxygen therapy had significant lung injury (Northway *et al.* 1967). Northway originally defined the disease as a chronic lung disease that affected preterm infants with respiratory distress syndrome (RDS). Since the initial definition, a number of modifications have been made to better describe the disease, such as the need for continued oxygen dependence over 28 days. Additional modifications included correction for gestational age after 36 weeks, or 28 days of ventilation along with characteristic chest x-rays. A study examining infants born extremely prematurely (before 26 weeks gestational age) found that at six years of age 13 % had developed cerebral palsy and 13 % had severe sensory impairment (Costeloe, 2006), suggesting up to a quarter of this group will have life long sequelae.

1.2.2 The changing aetiology and clinical presentation of CLD

The epidemiology of the disease has also evolved since Northway's first observations. Initially CLD was common in neonates who were born at 30-32 weeks gestation, which was the limit of survival at that time. As surfactant therapy, better ventilation techniques and increasing palliative care methods have evolved over the last 20 years, the trend has now been skewed towards CLD being primarily a disease of VLBW. Sixty-seven percent of neonates

with birth weights between 500 to 750 g develop CLD, compared to only 1% of those weighing 1,250 to 2,500 g (Bancalari *et al.* 2003). A variation in the presentation of CLD between the late 1960s and that of today has resulted in the idea of "old" and "new" CLD especially after the introduction of antenatal steroids and postnatal surfactant treatment. New CLD differs from old in causation as well as resulting lung pathology. Old CLD was characterized by smooth muscle hyperplasia, extensive fibrosis and inflammation within the lung, all of which were related to harsh ventilation strategies including barotrauma, volutrauma and oxygen toxicity. As therapeutic strategies for preterm infants changed, such as administration of surfactants (so to reduce surface tension and allowing inflation of surfactant deprived lungs), administration of antenatal glucocortoicoids and better, less harsh ventilation techniques were used the numbers of larger birth weight preterms with CLD began to decrease. As a result of the improved therapeutic strategies, survival rates for more premature infants also increased, which subsequently altered the pathological hallmarks and underlying mechanisms of CLD ("new CLD"). The new form is primarily seen in extremely preterm infants, born at 24-26 weeks, and infrequently in the larger preterms, in which CLD had been previously present (Coalson, 2006). Histological data from autopsied lung tissue of extreme preterm infants with CLD noted a shift in the histology towards large and simplified alveoli which were decreased in number, had variable interstitial fibroproliferation and variable smooth muscle hyperplasia (Figure 1.3) (Coalson, 2003). New CLD is due to the highly immature status of the neonatal lung as infants were born during the late canlicular and early saccular stage of lung development. At this early stage of development,

alveolarisation (the formation of terminal saccules and production of surfactant) has yet to begin, compared with those born at a later gestational age. A lack of alveolarisation, which begins at 32 weeks, means that the lung is not prepared for gaseous exchange (Coalson, 2003). Unlike old CLD, a number of infants that develop the new form do not present with initial respiratory complications, such as RDS. It has been hypothesized that an *in utero* infection, such as chorioamnionitits, may promote lung maturation through induction of natural corticosteroids, but limit the lung for future full development (Watterberg *et al.* 1996).

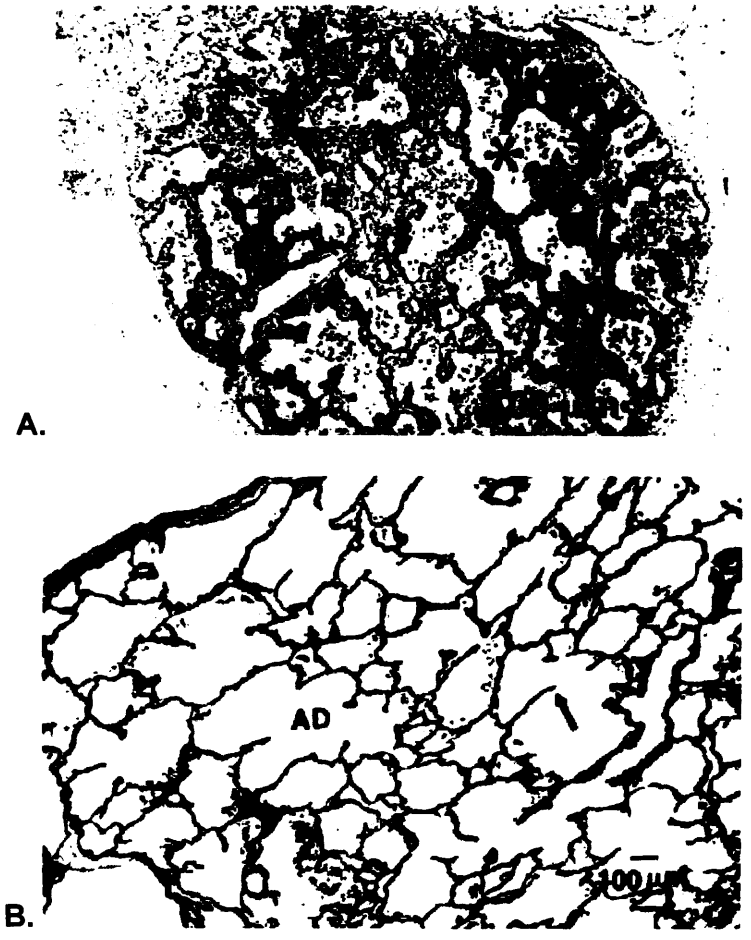


Figure 1.4. Comparison of lung biopsy between neonatal lungs with old (A) and new (B) CLD. (A) Lung biopsy from a 26 week old neonate who did not receive antenatal surfactant. The (*) denotes an area of dilated alveoli. (B) Lung biopsy from a 28 week gestation infant who received prenatal steroids and postnatal steroids. AD denotes alveolar ducts with alveolar structure (arrow). (Coalson, 2003).

1.2.3 Animal models of CLD

Animal models have significantly aided understanding the pathogenesis of CLD. Although not a direct representation of lung development and injury as seen in humans, models have allowed a mechanistic approach to elucidating contributing factors to the disease. To date there is no single animal model which mimics all risk factors contributing to CLD, but the ability to induce lung inflammation in offspring following experimental preterm birth, even through induced chorioamionitis, have been created (Coalson, 2006). Baboon models have been developed to represent the old CLD model by delivering young baboons at 140 days gestation (76% of normal gestation) representing samples from the saccular to alveolar stages of development. Further similarities to old style CLD were made by administration hyperoxic conditions via 100% oxygen while not administering antenatal surfactant (Coalson, 2006). The results from these studies demonstrated, as in the human form of CLD, levels of inflammatory cytokines were up-regulated in result of hyperoxic conditions. A lamb model developed by Bland and colleagues used lambs delivered at 124 $\pm$ 3 days to represent extremely premature human infants at 24 to 26 weeks who were in the saccular stage of development (Bland *et al.* 2000). Lambs were intubated for 3 to 4 weeks with results which combined pathology of both old and new CLD with muscle hyperplasia and large-simplified alveoli, respectively.

1.2.4 Association between Ureaplasma and CLD

During the late 1980s a number of independent studies found a significant link between neonates colonized with the *Ureaplasma* and the development of

CLD (Cassell *et al.* 1988; Sanchez and Regan 1988; Wang *et al.* 1988). The studies by Sanchez and Regan as well as Wang *et al.*, concluded that oxygen toxicity and other ventilation related traumas alone could not account for CLD, thus *Ureaplasma* had a definite role in CLD development. An additional finding by Cassell *et al.* showed infants weighing <1,000 g had a two-fold increase of mortality, when colonisation of *Ureaplasma* was identified. The results from these studies provided crucial information that VLBW infants were a high risk group for colonization by *Ureaplasma* and developing CLD. Additional supporting information came from Payne and colleagues in 1993 who examined nasopharyngeal aspirates (NPA) from infants born <1,251 g (Payne *et al.* 1993). Results showed that neonates with evidence of *Ureaplasma* colonisation had an increased (1.66-fold) relative risk for developing CLD compared to non-colonised neonates. This study provided useful information regarding the mode of vertical transmission of *Ureaplasma* from the mother to child. Infants born vaginally had a 4.5-fold increased chance of colonization relative to those born by caesarean section. This was likely due to contraction of bacteria during passage of the neonate through the birth canal. Payne *et al.* also commented on the sensitivity of sample type for detection of *Ureaplasma.* Positive samples from NPA correlated poorly with ETA and that site of specimen effected rates of colonization. They determined that ET samples were the best for examining colonization of lower respiratory tract.

Abele-Horn *et al.* in 1997 found in a study of 253 pregnant women (170 *Ureaplasma*-positive, 83 *Ureaplasma* negative) that the risk of developing

CLD was 17% vs. 4% in infants <1,500 g, respectively (Abele-Horn *et al.* 1997b). The study also showed that there was a significant relationship between *Ureaplasma* colonization and premature rupture of membrane (12% vs. 35%; $p < 0.001$), preterm birth (10% vs. 41%; $p < 0.001$) as well as chorioamnionitis (0% vs. 10%; $p < 0.05$).

It has been argued that determining the role of *Ureaplasma* in development of CLD has been hampered by studies containing low numbers of neonates. For this reason a number of meta-analyses have been performed to collate the data from the numerous studies and examine the association of *Ureaplasma* and CLD. In 1995, Wang demonstrated an overall relative risk of 1.72 for collective investigations for likelihood of colonized patients developing CLD (Wang *et al.* 1995). A second meta-analysis examined the data from 23 studies consisting of 2216 infants defined as having CLD at 28 post natal days and 751 infants defined as having CLD at 36 weeks post-menstrual age. There was a significant association between *Ureaplasma* and CLD in both defining criteria (Schelonka *et al.* 2005).

1.2.5 Inflammatory potential of Ureaplasma and relationship to cytokines and CLD

The link between inflammatory markers and development of CLD was demonstrated by Yoon *et al.* who examined the correlation between CLD and amniotic cytokine levels in a study of 69 neonates (Yoon *et al.* 1997). Those who were born from mothers with high median amniotic fluid levels of IL-6, TNF-α, IL-1β and IL-8 were significantly more likely to develop CLD than

those who did not. With evidence such as this, researchers have looked at the inflammatory potential of *Ureaplasma*. The ability of *Ureaplasma* to induce an inflammatory response has been proven through a number of clinical, cell line and animal model studies which have been related back to development of CLD. Baier *et al.* found that *Ureaplasma* could stimulate other inflammatory mediators such as MCP-1 and IL-8 which were significantly elevated in CLD patients, and the levels of these cytokines were significantly increased in ETA were from *Ureaplasma* positive patients, relative to those who were negative (Baier *et al.* 2001). A further study by Kotecha *et al.*, examined a greater range of inflammatory markers, as well as levels of pulmonary neutrophils and alveolar macrophages. Levels of IL-1beta, IL-6, IL-8, soluble intercellular adhesion molecule-1 (ICAM), pulmonary neutrophils and alveolar macrophages were all significantly increased in *Ureaplasma* positive patients (Kotecha *et al.* 2004). These results of increased leukocyte counts were similar to those found by Viscardi *et al.*, who looked at autopsy sections from infants who had died resulting from both *Ureaplasma* induced and non-*Ureaplasma* induced pneumonia. Absolute neutrophil counts were 3-fold higher in *Ureaplasma* induced pneumonia than in pneumonia caused by other pathogens, but there was also a predominance of alveolar macrophages relative to neutrophils in *ureaplasma*-infected patients (Viscardi *et al.* 2002). This predominance of macrophages may be partially due to the ability of *Ureaplasma* to stimulate MCP-1 release.

1.2.6 Inflammatory response to Ureaplasma in cell line studies

In vitro analysis of relevant cell lines under defined conditions mimicking *Ureaplasma* colonisation have also provided useful information. Although not a full biological system, these systems have been successfully used to examine inflammatory regulators in response to *Ureaplasma* infection, dissecting the potential contribution of each to *in vivo* development of CLD in the presence of *Ureaplasma*. Stancombe *et al.* showed that *Ureaplasma* can cause significant production of IL-6 and IL-8 from pulmonary fibroblast *in vitro*, which supports the findings of previous clinical studies (Stancombe *et al.* 1993). As well as fibroblasts, *in vitro* culture data has shown that macrophage cell lines, rat alveolar macrophage and macrophage from ETA all respond in similar ways to *Ureaplasma*. Levels of TNF-alpha and IL-6 have been reported to increase from 14 to 84% and 46 to 268%, respectively, as a result of *Ureaplasma* antigen (Li *et al.* 2000).

1.2.7 Animal models of Ureaplasma infection and CLD

A range of animal models for *Ureaplasma* infection have been developed in varying host species under differing experimental disease states. Early work by Furr and Taylor-Robinson found it was only possible to establish experimental vaginal *Ureaplasma* colonisation in mice with human isolates when mice had been pre-treated with oestradiol (Furr and Taylor-Robinson, 1989). This work was later expanded to examine variation in colonisation between *Ureaplasma* serovars 5, 8 and 10 (Taylor-Robinson and Furr, 2002). Data showed that BALB/c mice again had to be treated with oestradiol for initial colonization to occur with any of these serovars. Serovar 8 yielded the

highest rate of colonization (100% colonization) and longest period of colonization at 84 days, with 6 mice (60%) still colonized at 158 days. Two out of the 10 mice experienced spread from the vagina to the right ovary in one case and the liver and kidney in another. Colonisation was less persistent in strains 5 and 10, with 4/10 and 7/10 mice colonised, respectively.

Mouse models of pneumonia have also been developed to understand the role of host response to *Ureaplasma,* as well as providing supporting evidence for the organism's role in development of lung disease. Viscardi *et al.,* described three distinct stages of *Ureaplasma* infection in the lung, firstly acute inflammation in days 1-2, followed by resolution of acute inflammation at days 3-7 and finally chronic lung inflammation and lung injury in days 14-28 (Viscardi *et al.* 2002). A major histopathological finding was the loss of ciliated respiratory epithelium. It was hypothesized that the loss of cilia was a result of *Ureaplasma* adhesion followed by release of toxic compounds from the organism such as ammonia, hydrogen peroxide or hemolysins which may result in predisposition to secondary bacterial infections. An earlier study looked at the effects of both *Ureaplasma* pneumonia in newborn mice and the effects of hyperoxia: 80% oxygen was applied to new born mice which had been inoculated with serovar 10. Results suggested that high levels of oxygen resulted in persistence of *Ureaplasma*, as well as increasing the inflammatory response (Crouse *et al.* 1990).

A series of baboon models of *Ureaplasma* infection have also been developed. In 1993, Walsh *et al.* (Walsh *et al.* 1993) described a primate

model of *Ureaplasma* infection in premature infants. Three premature baboons delivered at 138-141 days (term 180), which represent human characteristics of delivery at 30-32 weeks, were exposed to *Ureaplasma* and maintained at 100% oxygen throughout the study. Two of the infected animals developed pathological findings of acute bronchitis which was absent in animals of the control group. A second baboon model of *Ureaplasma* infection examined the effects of *Ureaplasma* upon lung pathology following intra-uterine infection. Infant baboons in the exposed group showed persistent levels of IL-6 and IL-8 in ETA samples and reduced lung function relative to controls (Yoder *et al.* 2003). A more recent baboon model of *Ureaplasma* lung infection proved that infection resulted in increased IL-1-beta, extensive fibrosis and higher concentrations of TGFbeta1 (Viscardi *et al.* 2006).

1.2.8. Infection and development of CLD

Although *Ureaplasma* have been the primary organism associated with the development of CLD some investigators have looked at the role of other bacterial species. Lahra *et al.*, examined a cohort of nearly 800 preterm infants over a twelve year period and found that neonatal sepsis was strongly associated with CLD (Lahra *et al.* 2009). Miralles *et al.*, utilized molecular methods for identifying the presence of bacteria using PCR primers specific for the highly conserved region of the bacterial 16S rRNA gene. By examining extracted DNA from BAL samples from 41 preterm infants, they to demonstrated a link between infection and CLD (Miralles *et al.* 2005).

1.3 The complement system

1.3.1 Function of the complement system

The complement system is a major first line defence against invading pathogens. Composed of more than 30 soluble and surface associated proteins, the complement system additionally functions to clear apoptotic cells as well as bridging the gap between the innate and adaptive immune systems (Walport, 2001). Three pathways exist to activate the complement cascade in response to differing initiators, but converge at the hydolysis of central C3 component by their respective pathway C3 convertases. From the central C3 conversion, all pathways elicit killing of pathogens either through the formation of the terminal membrane attack complex (MAC), resulting in cell lysis, or through phagocytosis of complement opsonised cells. These pathways are represented in Figure 1.5.

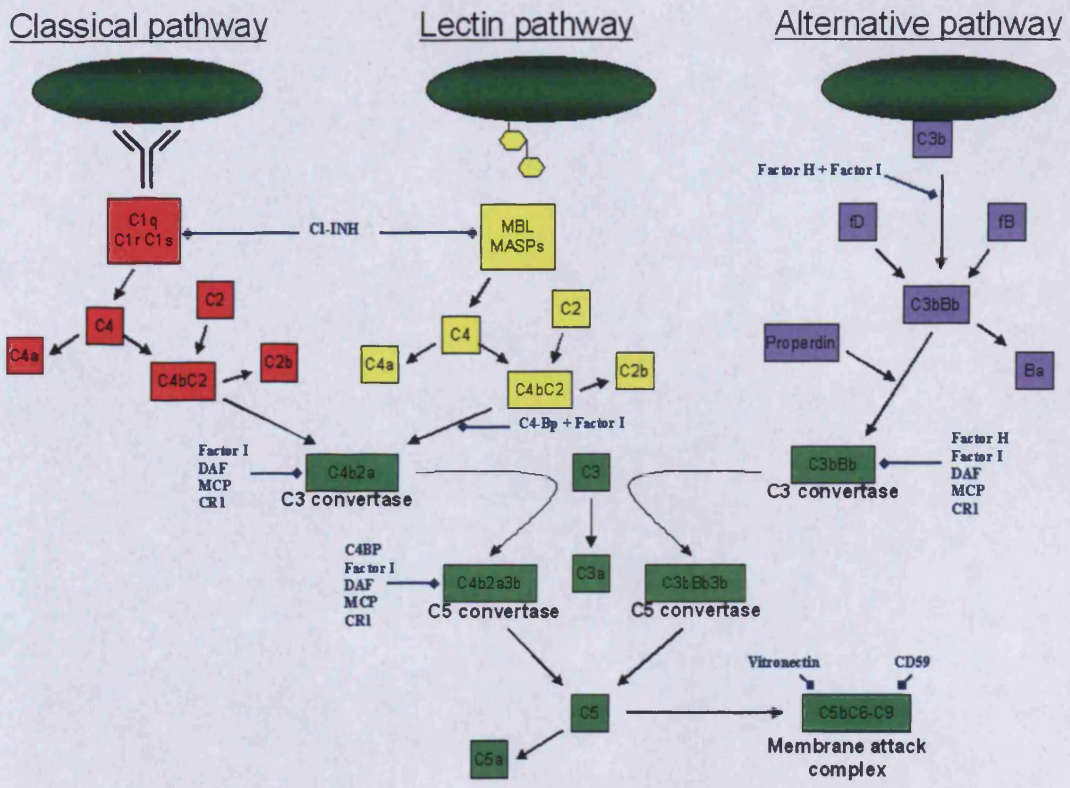


Figure 1.5. An overview of the three activation pathways of the complement system. The complement system can be activated by three independent activation pathways. The classical pathway is activated by an antigen/antibody complex; the lectin pathways is activated by pattern recognition molecules such as mannose binding lectin, binding to pathogen specific carbohydrates; the alternative pathway is activated by the spontaneous hydrolysis of C3. All pathways converge at the conversion of C3 in to C3a and C3b. The cascade continues in the final formation of the membrane attack complex leading to cell lysis. The complement systems is tightly regulated by a number of regulators (blue lettering)

1.3.2 The classical pathway

The first pathway to be discovered was the classical complement pathway (CP). It was found to be a heat-labile component of serum which acted to "complement" the antibody killing of bacteria. Initiation of the pathway begins when the primary component of the pathway, C1q, and its additional bound tetramer of two C1r and two C1s molecules, bind to cell associated IgG or IgM. This binding causes the auto-activation of C1r which in turn activates the serine protease C1s causing cleavage of C4 into the fragments C4b, which covalently binds to the activator cell, and C4a which is released where it exhibits pro-inflammatory effects. C2 binds to the cell bound C4b, which also becomes cleaved by C1s to produce the split products C2a and C2b. C2b is released, whereas C2a remains bound to C4b complex to form (C2aC4b) the C3 convertase of the classical pathway. C4b has no enzymatic role in the C3 convertase but acts as an anchor for C2b where the enzymatic activity lies.

1.3.3 The lectin pathway

The lectin pathway (LP) is the most recently discovered of the complement pathways and varies when compared to the CP by being independent of antibody binding (Turner, 2003). Activation results from pattern recognition molecules (PRM) such as mannose binding lectin (MBL) or ficolins binding to pathogen-associated molecular patterns (PAMP) found only on "foreign" cells. These include simple and complex carbohydrates with 3- and 4-OH groups across the equatorial plane of the sugar (Weis *et al.* 1992). Like the C1q,r,s complex, MBL exists in complex with serine proteases known as MBL-

associated serine proteases (MASP) MASP-1, MASP-2, MASP-3 and Map19. Structurally and functionally similar to C1r and C1s, MASP-2 acts like C1r in that it auto-activates upon MBL binding to a surface, but functions like C1s in that it cleaves C4 and C2 into their respective split products. MASP-1 does not activate C4, but direct cleavage of C3 has been suggested (Matsushita and Fujita, 1995).

1.3.4 The alternative pathway

The alternative pathway, unlike the CP and LP, is activated by spontaneous hydrolysis of C3 and subsequent deposition of cleaved C3 to cells. An additional difference is the nomenclature used with components of the AP commonly referred to as factors. Deposited C3b is bound by factor B, a homologue protein to C2, which is then cleaved by factor D to give C3bBb or the alternative pathway C3 convertase. This complex is stabilised by properdin (also known as factor P). The absence of polyanionic structures, such as sialic acid, on microbial cells results in a failure to recruit the powerful complement regulator factor H and the C3 convertase amplifies unregulated resulting in substantial deposition of C3b and membrane attack complex formation. Failure to recruit complement regulators is one of the important mechanisms for innate immune discrimination between self and foreign invading microbes.

1.3.5 Terminal complement pathway and formation of the membrane attack complex (MAC)

Cleaved C3 then binds to either C3b or C4b of the AP or CP/LP C3 convertases, respectively, acting as a receptor of for C5 in the newly formed C5 convertase complex (i.e. C3bBb3b or C4b2a3b), that activates C5 through cleavage to C5a and C5b. This is the first step of the terminal lytic pathway and the last point of which cleavage is required for complement component recruitment. The end result being the formation of the membrane attack complex (MAC) which constitutes the lytic component of the complement pathway. Non covalent sequential addition of C6, C7, C8 molecules to the C5b molecule, results in increasing hydrophobicity of the complex, driving it further into the activating membrane, then up to 12 C9 molecules generate a amphipathic pore that disrupts the osmotic balance. There is some suggestion that C5b678 is sufficiently "leaky" to cause lysis in sufficient numbers.

1.3.6 Regulators of complement activation (RCA)

Due to the potential destructive capabilities of the complement system measures are needed to prevent damage upon host cells. Regulators of complement activation (RCA) are a series of either bound or circulating proteins which keep the complement system in order at numerous check points of the cascade and aid in differentiating self from non-self cells. Using factor H (fH) as an example of an RCA, it functions by regulating the AP in two ways. Firstly it acts as a cofactor for factor I cleavage of C3b to produce inactive iC3b, disabling the ability to form a functional C3 convertase.

Secondly it accelerates the dissociation of Bb from C3b limiting the amount of further C3b produced. Auto-immune diseases such as atypical haemolytic uremic syndrome (aHUS) result from genetic abnormalities in genes encoding these regulators, such as fH, leading to reduced or failed regulation of complement activation at the filtering surface of the kidneys (Atkinson and Goodship, 2007).

1.3.7 The complement system of neonates

As newborn infants do not have previous exposure to immunogens and their adaptive immune system is sluggish and under-developed, their innate immunity plays a large role in the defence against infection. The complement system in neonates is also immature relative to adults; therefore, may predispose to infection (with increasing immaturity and infection predisposition being inversely proportional to gestational age). Drossou *et al.*, 1995 looked at levels of opsonins IgG, IgG subclasses, IgM, C3 and C4 in neonates of three groups of neonates 26-30 weeks, 31-37 weeks and 37-39 weeks (Drossou *et al.* 1995). They found that the levels of opsonin were dependent on the infants postnatal age as well as gestational age. The levels of opsonins were half to two thirds that of healthy adults, but levels reached that of adults by third or sixth month of life.

Studies examining amounts of C2 and C4 in preterm infants found levels of 62-85% and 42-73% of adult values (Sonntag *et al.* 1998; Miyano *et al.* 1996; Drew and Arroyave, 1980; Wolach *et al.* 1994), but with levels increasing to the adult range by 1-6 months of age. Factor B and properdin of

the AP are also reduced in neonates at 27-64% and 13-72%, respectively (Wolach *et al.* 1997; Drew and Arroyave, 1980). It is only C7 of the terminal pathway which does not seem to be dramatically deficient in either term or preterm infants with ranges between 95 – 120% and 73 – 75% (Wolach *et al.* 1997; Johnson *et al.* 1983). It seems that although levels of complement components are deficient there is no effect upon the ability on activation. Hogasen *et al.* demonstrated that in preterm infants who were born from mothers with PPROM activation products C3bBbP and SC5b-9 were 1.5 and 2 times greater than that of healthy term infants (Hogasen *et al.* 2000).

As there is no gestational age effect of upon complement activation, appropriate levels of free and bound complement regulators are crucial. Consistent ranges of C4-binding protein (C4BP) have been found in term neonates at 18-28% (Malm *et al.* 1988; Sthoeger *et al.* 1989) whereas ranges from 61-75% of factor H have been found in term infants (Davis *et al.* 1979; Johnson *et al.* 1983). These reduced levels of regulators in the presence of unaffected activation can potentially give rise to damage to self.

1.3.8 Complement and its role in Ureaplasma infection

A limited body of literature is available on the role of complement in the clearance of *Ureaplasma* infections. As discussed in section 1.1.4.5, an antibody response is raised in patients infected with *Ureaplasma* suggesting a role in clearance of infection (Brown *et al.* 1983). A single study by Benstein *et al.*, examined the ability of MBL to bind the surface of *Ureaplasma* (Benstein *et al.* 2004). They demonstrated binding of human MBL to

Ureaplasma, but did not provide any data regarding a functional role. To date, no report has commented on the role of the alternative pathway in clearance of *Ureaplasma*. A single report suggested the ability of C1q to bind directly to *Ureaplasma* as evidenced by the increased rate of C1r and C1s activation relative to spontaneous cleavage in the absence of *Ureaplasma*. Additional findings from this study suggested an inability of complement alone to result in killing, but these results utilised non-standardised methods and the methodology and results were not as conclusive as the conclusions that were drawn.

1.4 Antimicrobial therapy against *Ureaplasma*

1.4.1. Intrinsic antibiotic resistance in Ureaplasma and therapeutic considerations

A number of physiological factors of *Ureaplasma* provide intrinsic resistance to a number of clinically available antibiotics, which include cell wall inhibitors such as the β-lactams and glycopeptides due to the lack of a cell wall, RNA polymerase targeting rifampins due to mutation in the beta-subunit of RNA polymerase (Gaurivaud *et al.* 1996), folic acid synthesizing sulphonamides and trimethoprim, as well as a the protein synthesis inhibiting lincosamides (Waites *et al.* 2005). Aminoglycoside antibiotics, which are routinely used to treat Gram-negative sepsis in neonates, have unreliable activity against *Ureaplasma*. Cassell *et al.*, commented on the ability to isolate *Ureaplasma* from CSF, blood culture and LRT in neonates following greater than three days of treatment with gentamicin (Cassell *et al.* 1993). A further protein synthesis targeting antibiotic, like the aminoglycosides, is chloramphenicol. Chloramphenicol generally has good activity against *Ureaplasma* as seen in a number of clinical case-studies (Geissdorfer *et al.* 2008; Hentschel et al. 1993) as well as *in vitro* data on susceptibility test (Matlow *et al.* 1998; Waites 1992), but the drug is not routinely used in treatment and is reserved for cases of antibiotic resistant strains. For these reasons stated above therapy is restricted to a small number of agents including the tetracyclines, macrolides and fluoroquinolones. Further complications for therapy arise in neonates who are colonized with *Ureaplasma*. Tetracycline use in neonates is restricted due to effects of bone toxicity and deposition within calcifying tissue, but has been reported in a limited number of CSF infections (Rao *et al.*

2002; Waites *et al.* 1993). As quinolone usage in neonates is additionally restricted due to effects of cartilage development, the macrolides are the preferred drug of choice

1.4.2. The macrolides

1.4.2.1 Macrolide structure and mode of action

The macrolide family of antibiotics are of great clinical significance in many patients groups, including paediatrics, but their use is becoming compromised due to increasing numbers of antibiotic resistant organisms (Jacobs and Johnson, 2003). Erythromycin, the first clinically available macrolide, as well as clariythromycin are composed of a 14 membered lactone rings where as the newer azithromycin is composed of a 15 membered ring (Figure 1.6). In addition to being antibacterial, they also exhibit anti-inflammatory properties which have been utlized in managing conditions such as cystic fibrosis and asthma, which could be potentially used in the management of CLD (Jaffe and Bush, 2001).

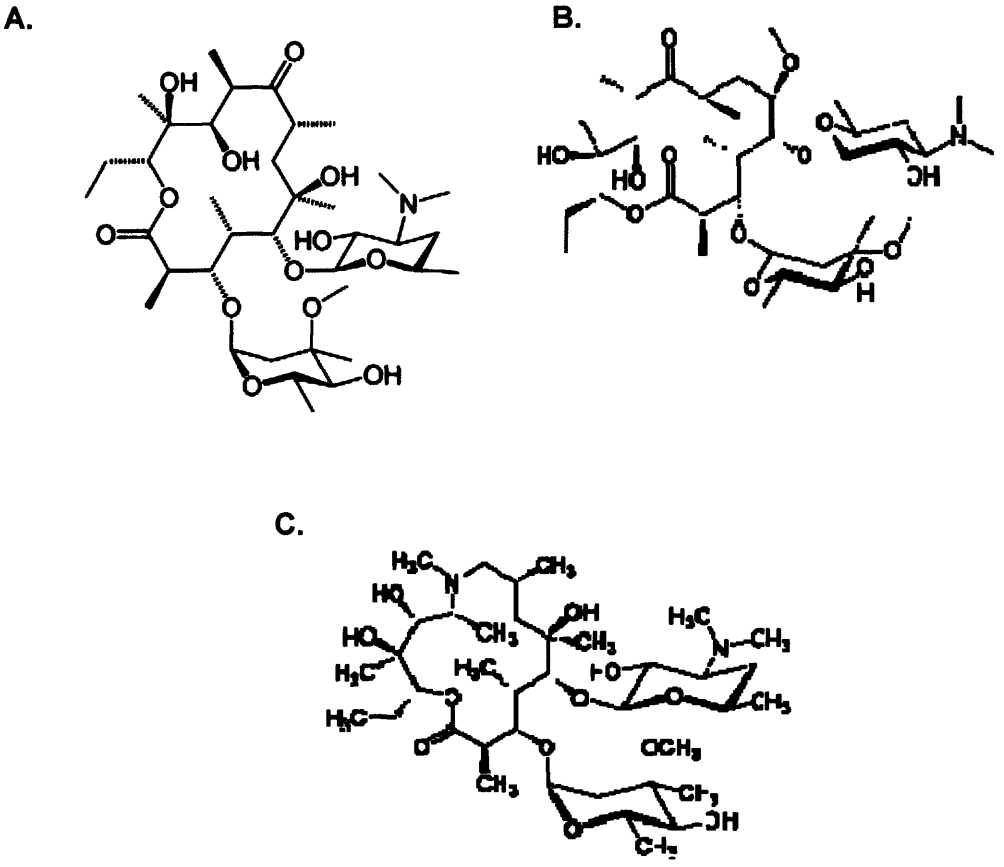


Figure 1.6. Structures of the macrolide antibiotics used in the study. A Erythromycin, B Clarithromycin, C Azithromycin.

Macrolides inhibit protein synthesis by binding within the exit tunnel of the 50s ribosomal subunit, therefore preventing elongation of the peptide chain (Vester and Douthwaite, 2001). Macrolide binding to the ribosome has been mapped to the 50s ribosomal subunit, but more specifically to the 23S ribosomal RNA (rRNA). Binding results primarily from hydrogen bonds forming between the C5 sugars of the drug and the specific nucleotides A2058 and A2059 within domain V of the peptidyl loop.

1.4.2.2 Mechanism of macrolide resistance in *Ureaplasma* spp.

Macrolide resistance can result from either target site modification, which can be mutational or post-transcriptional or by active efflux. A trend is clear between the number of ribosomal operons within the bacterial genome and methods by which bacteria exhibit macrolide resistance (Vester and Douthwaite, 2001). Bacteria with 1 to 3 ribosomal operons such as the *Mycoplasma pneumoniae*, *Helicobacter pylori* and *Propionibacterium acnes* confer resistance via mutations within domain V of their 23S rRNA (Figure 1.7), whereas bacteria with 4 to 9 operons such as *Neisseria gonorrhoeae* and *Clostridium perfringens* mediate resistance via methylation and/or efflux pumps (Table 1.1).

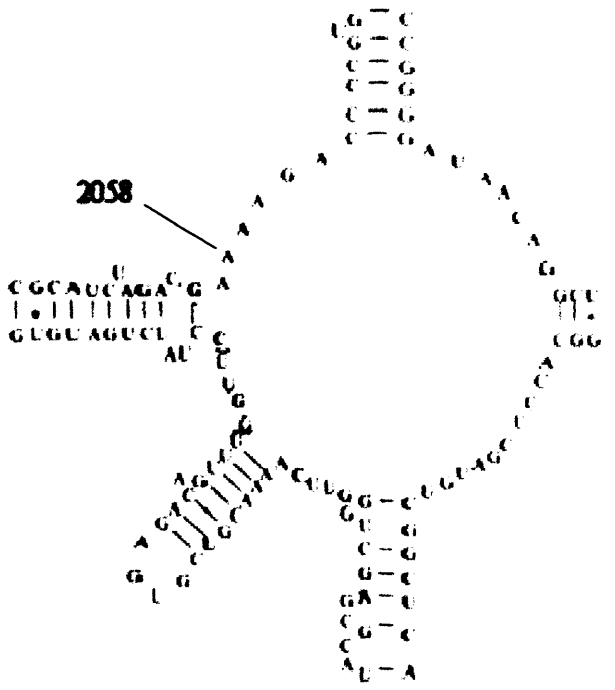


Figure 1.7. An indication of the predominant nucleotide at which mutations can give rise to macrolide resistance. Mutations in domain V of the pepdityl-transferase loop of the 23S rRNA can give macrolide resistance with mutation at position 2058 yielding high levels of resistance. Image adapted from Pereyre *et al.* (Pereyre *et al.* 2006).

Organism	Mechanism	No. of rRNA operons
Mycoplasma pneumoniae	23S RNA mutation	1
Mycobacterium avium	23S RNA mutation	1
Mycobacterium intracellulare	23S RNA mutation	1
Helicobacter pylori	23S RNA mutation	2
Treponema pallidum	23S RNA mutation	2
Propionibacterium acnes	23S RNA mutation	3
Streptococcus pneumoniae	23S RNA mutation	4
Neisseria gonorrhoeae	Methylation & efflux	4
Staphylococcus aureus	Methylation	6
Clostridium perfringens	Methylation	9

Table 1.1. Macrolide resistance mechanisms found in pathogens and their respective numbers of rRNA operons. Adapted from Vester and Douthwaite paper (Vester and Douthwaite, 2001).

Additional mutations in the apparatus of the ribosome, such as ribosomal proteins L4 and L22, have been linked to reduced susceptibility to macrolides. It is believed that mutations within these proteins can alter protein-RNA interactions thus giving resistance (Gregory and Dahlberg, 1999).

The first mechanistic description of macrolide resistance in *Ureaplasma* was presented by Palu *et al.* (Palu *et al.* 1989). Investigators described that a highly resistant strain also had a dramatically reduced ribosomal binding of radiolabelled erythromycin. With this evidence, as well as the association between rRNA operons and methods of resistance, the most likely mechanism would be via selection of point mutations in the 23S rRNA. A series of macrolide resistant *U. parvum* strains were produced *in vitro* by serial passage of a sensitive *Ureaplasma* strain in increasing sub-inhibitory concentrations of antibiotic. Resistance to various macrolides resulted from point mutation of A2058 with or without mutation of additional adjacent bases, occasionally combined with additional mutations within the ribosomal accessory proteins L4 and L22 (Pereyre *et al.* 2007). Prior to the work presented in this thesis, the mechanism of macrolide resistance had yet to be demonstrated in any clinical isolate.

Data from the genome of *U. parvum* serovar 3 has shown two multi-drug and toxin extrusion (MATE) exporters as well as an array of ATP-binding cassette (ABC) transporters (Glass *et al.* 2000). Inhibition of an ABC transporter in *Mycoplasma hominis* can increase intracellular erythromycin concentrations greater than two-fold, although the organism is intrinsically resistant due to established mutations within its 23S rRNA (Pereyre *et al.* 2002). The efflux systems encoded within the genome of *Ureaplasma* may also play a role in any erythromycin resistant strains.

1.4.3. The tetracyclines

1.4.3.1 Tetracycline structure and mode of action

The tetracylines are a second family of antibiotics which function to inhibit bacterial protein synthesis (Figure 1.8). Many of the tetracyclines in clinical use today, such as doxycycline, are synthetic derivatives of chlorotetracycline which was originally isolated in 1948 from *Streptomyces aureofaciens*. They act bacteriostatically by binding to the 30S ribosomal subunit and prevent the attachment of charged tRNAs to the A site. They have a broad spectrum of activity against most Gram-positive and Gram-negative bacteria and due to their ability to enter eukaryotic cells, tetracyclines are active against intracellular pathogens such as *Chlamydia trachomatis* and *Rickettsia rickettsii*.

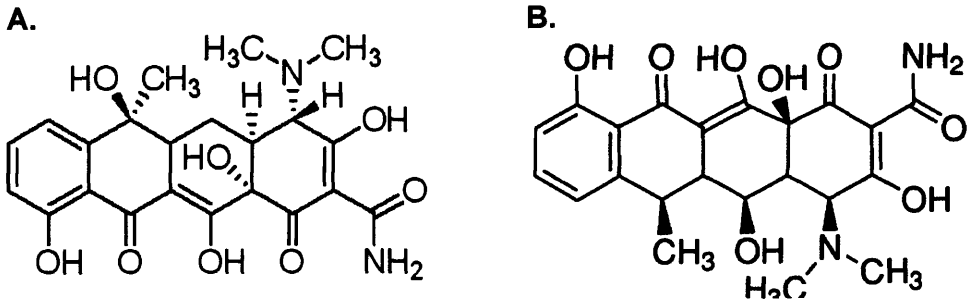


Figure 1.8. Chemical structures of (A) tetracycline and (B) doxycycline.

1.4.3.2 Mechanisms of tetracycline resistance in *Ureaplasma* spp.

One means by which bacteria mediate tetracycline resistance is due to the acquisition of a tetracycline resistance gene such as *tetM* or *tetO*, which have been found in both Gram-positive and Gram-negative bacteria, or *tet*A – *tet*F which are confined to Gram-negative (Blanchard *et al.* 1992). Tetracycline resistance in *Ureaplasma* was first documented in 1974 by Ford and Smith from a patient with NGU (Ford and Smith, 1974). In 1986, Roberts and Kenny determined that resistance to tetracycline was mediated via the presence of *tet*M gene (Roberts and Kenny, 1986a). To date, only *tetM* has been isolated from *Ureaplasma* spp. and has been shown to be present upon the Tn1545-like transposon (de Barbeyrac *et al.* 1996). It has been hypothesized that transposition of the *tetM* gene from a Streptococci to *Ureaplasma* may have been the original source of the tetracycline resistance determinant (Roberts and Kenny, 1986a).

An early report suggested that tetracycline resistance was present in 10% of *Ureaplasma* isolates from men with NGU (Evans and Taylor-Robinson, 1978) which was later confirmed in 1986 when 10% resistance was again found in *Ureaplasma* isolated between 1973 and 1983 (Taylor-Robinson and Furr, 1986). Interestingly, it was noted that 39% of the tetracycline resistant strains also showed resistance to erythromycin, whereas only 9% of the tetracycline sensitive strains were erythromycin resistant. Roberts and Kenny determined that the incidence of tetracycline resistance was 20% in 63 isolates, all of which contained the *tetM* gene (Roberts and Kenny, 1986b).

1.4.4. The fluoroquinolones

1.4.4.1 Fluoroquinolone structure and mode of action

The quinolones are a family of bacteriocidal antibiotics which disrupt DNA replication by binding to enzyme-DNA complexes during replication leading to the death of the cell. The quinolones have under gone numerous structural re-engineering to both broaden their spectrum of activity and increase their potency against resistant isolates. The basic structure of all quinolones is orientated around the 4-quinolone centre with modifications leading to the recognised classification into four groups (Figure 1.9). The first group consisted predominantly of nalidixic acid which had a limited antimicrobial activity against Gram-negative organisms such as *E. coli*. The addition of fluorine at C-6 greatly expanded the spectrum of activity to include *P. aeruginosa* giving the first of the fluoroquinolones, ciprofloxacin. Levofloxacin is another example of a second (and sometimes argued third) generation fluoroquinolone. This drug has an extended spectrum of activity against Gram-positives with excellent activity against *Ureaplasma* both *in vitro* (Bebear *et al.* 2000; Waites *et al.* 2003) as well as *in vivo* (Eilers *et al.* 2007) with only a limit number of resistant strains identified (Duffy *et al.* 2006). Further modifications to the structural core lead to the generation of groups 3 and 4 which had greater Gram-positive properties as well as some anaerobic activity. Moxifloxacin is an example of a group 4 fluoroquinolone in which chemical modification, the addition of a methoxy moiety at C-8, has been used to overcome cross-resistance.

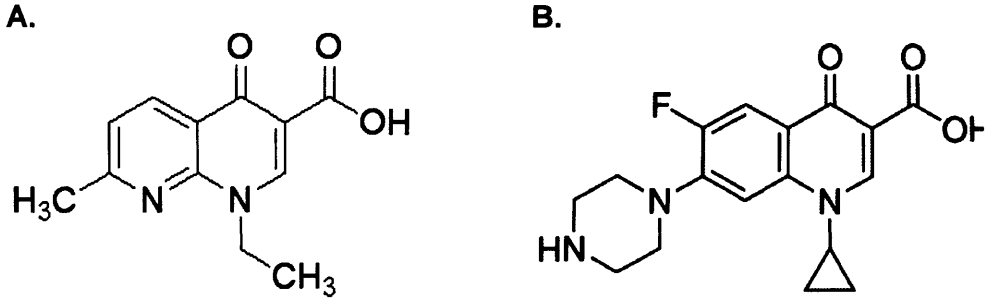


Figure 1.9. Chemical structure of a group 1 quinolone, naladixic acid (A), and a group 2 ciprofloxacin (B). The addition of fluorine at C-6 as seen in ciprofloxacin greatly enhanced the drugs spectrum of activity and improved its pharamacokinetic properties by increasing the half life of nalidixic acid from around 1.5 hours to 3-4 hours in ciprofloxacin.

Quinolones act by binding to the type II topoisomerase enzymes of bacteria, namely DNA gyrase and topoisomerase IV. DNA gyrase is composed of four structural subunits; two encoded by the *gyrA* gene and two from the *gyrB* gene. Topoisomerase IV is structurally homologues to DNA gyrase with two subunits encoded by the *parC* gene and two from the *parE* gene, respectively. It is hypothesised that the negatively charged DNA binds to the enzymes along a positively charged stretch within the alpha helix near the tyrosine active site involved in DNA cleavage. Although the enzymes are structurally similar they have marginal differences in activity. DNA gyrase predominantly functions to introduce positive supercoils in DNA ahead of the progressing replication fork, whereas topoisomerase IV's primary role is to de-catenate the two circular daughter chromosomes following replication. Additional differences are preferential sites of action: the gyrase proteins for Gram-negative bacteria and the topoisomerase IV proteins for Gram-positive bacteria (Blanche *et al.* 1996). This data is supported by quinolone resistant mutants as Gram-negative resistant mutants generally contain mutations within the *gyrA* or *gyrB* genes, whereas resistant Gram-positive organisms contain mutations within the *parC* or *parE* genes (Gellert *et al.* 1977; Fournier and Hooper, 1998).

1.4.4.2 Mechanisms of fluoroquinolone resistance in *Ureaplasma* spp.

Prior to this thesis, a total of 32 clinical isolates of fluoroquinolone-resistant *Ureaplasma* had been described from five individual reports. Substitutions of Ser83Leu in the ParC protein, or a triple substitution of Asp112Glu in GyrA

protein along with Ala125Thr and Ala136Thr in ParC protein, all within the quinolone resistance determining regions (QRDRs), being most commonly noted (Bebear *et al.* 2000; Bebear *et al.* 2003; Geissdorfer *et al.* 2008; Xie and Zhang, 2006; Zhang *et al.* 2002).

1.4.5 Sensitivity testing of Ureaplasma

The inability of the *Ureaplasmas* to grow as a lawn upon solid media prevents the use of conventional disk diffusion techniques by which antibiotic impregnated disks are placed upon confluent lawn and zones of inhibition are recorded. Although *Ureaplasma* do not grow to a visible turbidity in liquid culture, the use of a pH indicator within culture media, gives a visible and qualitative result of *Ureaplasma* growth (Purcell et al, 1966). This property has been adapted for use in the microbroth dilution technique whereby organisms are grown in a series of broth cultures which contain doubling dilutions of antibiotics in the range of around 64 μg/ml through to 0.025 μg/ml.

In 1993 Kenny and Cartwright demonstrated three variables which significantly alter the MIC of erythromycin result of a single *Ureaplasma* isolate (Kenny and Cartwright, 1993). The most dramatic variable was pH. It is common knowledge that pH of test media can dramatically affect the efficacy of erythromycin, which is not limited to studies on *Ureaplasma*. The MIC for ATCC 29213 *S. aureus* was 64-fold different between culture at pH 6 and 7.4 (Waites *et al.* 1992). Kenny and Cartwright described that MICs were 4- to 16-fold higher at the more acidic pH of 6 than at a neutral 7 with

MICs of 8 μg/ml when the media was pH 6 dropping to as low as 0.25μg/ml at pH 7.

When using the microbroth technique for sensitivity testing of other organisms, the McFarland standards have been used as an approximate reference for number of bacteria in an inoculum, which are based on densitometric measurement of culture turbidity. As *Ureaplasma* do not grow to a visible turbidity, and the degree of colour change is not indicative of cell numbers, it is difficult to adjust inoculum size without prior knowledge of cell numbers. Kenny and Cartwright found that as the inoculum size increased so did the MIC. When testing serovar 8, they observed up to an 8-fold increase in MIC from an inoculum size of 45 CCU_{50} to 4500 CCU_{50}. This result was likely due to overwhelming of the antibiotic by numbers of cells. A result of this observation, many groups set up 10-fold serial dilutions to titre the inoculum size and agree that the result is only valid if the input inoculums was between 10^4 to 10^5 CCU (Matlow *et al.* 1998; Waites *et al.* 1992).

The final parameter that was observed was incubation time. MICs increased 4 to 16 fold from 2 to 5 days post inoculation irrespective of media pH and inoculum size. Recommendations that were proposed at the conclusion of the study were, several inoculum sizes should be used in each sensitivity test, the media should be of a standard pH and incubation time should be 48hrs.

1.4.6 Antibiotic resistant Ureaplasma in neonatal samples

A limited number of reports have been made regarding the trends in resistance among *Ureaplasma* isolates from neonates, but due to the lack of a standardised methodology, results are difficult to compare. The methodology described by Waites *et al.* has formed the basis for many later studies (Waites *et al.* 1992). Using this methodology the *in vitro* activity of six antibiotics including erythromycin and doxycycline, were screened against 43 *Ureaplasma* isolates from neonates. *In vitro* resistance was seen in 1 or more isolates to each antibiotic except erythromycin. Although resistance to erythromycin was not seen, a proportion of isolates were designated to have intermediate resistance. A small study of 10 isolates from ETA samples from Malaysian neonates with RDS identified one tetracycline resistant isolate, two ciprofloxacin resistant isolates, with all ten isolates being susceptible to erythromycin. As expected all isolates were resistant to lincomycin and sulfamethozazole-trimethoprim (Tay *et al.* 1997). A third study examined the sensitivity of twenty-one *Ureaplasma* isolates obtained from ETA samples from VLBW neonates. Isolates were screened against ciprofloxacin, gentamicin, chloramphenicol erythromycin, azithromycin and doxycycline. Although the authors did not define if isolates were sensitive or resistant, due to the absence of standardised breakpoints, they did show that chloramphenicol, doxycycline and azithromycin had the greatest activities *in vitro* (Matlow *et al.* 1998).

1.5 General hypothesis

Ureaplasma and complement

- Human serum will kill *Ureaplasma* by activation of all three complement activation pathways
- Serovars vary in susceptibility to complement-mediated killing

Role of infection in the development of CLD

- Neonates with presence of *Ureaplasma* in lung samples are more predisposed to developing CLD
- Neonates with presence of 16S rRNA in lung samples are more predisposed to developing CLD
- *Ureaplasma* are more prevalent with decreasing gestational age

Antibiotic resistance in Ureaplasma spp.

- Individual clinical isolates of *Ureaplasma* with resistance to three separate classes of antibiotics exist in samples taken from neonates.
- Resistance to macrolides will result from mutations in 23S rRNA
- Resistance to tetracycline will result from presence of the *tetM* gene
- Resistance to fluoroquinolone will result from mutations within the QRDRs of the type II topoisomerase proteins

1.6 Aims

Ureaplasma and complement

- To develop an assay for determining the predominant complement activation pathway involved in killing of *U. parvum*
- Determine if any variation in susceptibility occurs between different serovars of *U. parvum*

Role of infection in the development of CLD

- Determine the best way to detect *Ureaplasma* in samples from neonates
- Address the role of *Ureaplasma* in the development of CLD
- Address the role of general bacterial infection in the development of CLD

Antibiotic resistance in Ureaplasma spp.

- To develop a method of detecting antibiotic resistance without prior measurement of input bacterial numbers
- To determine the rate of antibiotic resistance in a retrospective cohort of neonatal patient samples
- Identify the molecular mechanisms behind identified resistant phenotypes to macrolide, tetracycline and quinolone antibiotics

Chapter 2

Materials and Methods

2.1 Culturing *Ureaplasma* spp.

2.1.1 Ureaplasma selective culture medium and culture conditions

Ureaplasma selective medium (USM) was purchased from Mycoplasma Experience Ltd. (Surrey, UK). USM consisted of a simple broth base medium supplemented with yeast extract, 1 g/L urea, and 10% heat-inactivated porcine serum, with phenol red as the pH indicator (starting pH, 6.65) and with 2.5 μg/ml amphotericin B and 0.25 mg/ml ampicillin. Cultures were either performed in flat-bottom or "V"-bottomed 96-well plates, where indicated, covered with adhesive sealing tape (Elkay, Basingstoke) or in 7 ml bijoux containers (Elkay, Basingstoke) and incubated in a humidified tissue culture incubator set at ambient CO_2 concentration at 37 ^{o}C.

2.1.2 Ureaplasma isolates

The prefix UHW represented samples obtained from the University Hospital of Wales whereas the prefix HPA represented those kindly donated by Dr Victoria Chalker from the Health Protection Agency, Colindale. Type strains of known serovar were also obtained from HPA, although SV1 (DKF-1) and SV9 (Vancouver isolate) were originally obtained from the Institute of Medical Microbiology, University of Aarhus, Denmark. The main strains used in this study are outlined in Table 2.1

DKF-1	U.p	1
HPA11	U.p	1
HPA18	U.p	1
UHWO10	U.p	1
UHWP2	U.p	1
UHWQ3	U.p	1
HPA1	U.p	3
HPA5	U.p	3
HPA7	U.p	3
HPA14	U.p	3
HPA29	U.p	3
HPA2	U.p	6
HPA23	U.p	6
HPA10	U.p	14
HPA32	U.p	14
UHWJM	U.p	N.D
Vancouver	U.u	SV9
HPA3	U.u	N.D
HPA6	U.u	N.D
HPA12	U.u	N.D
HPA17	U.u	N.D
HPA20	U.u	N.D

Table 2.1. Overview of the *Ureaplasma* isolates examined in this thesis. U.p indicates *U. parvum* and U.u indicates *U. urealyticum*. *Spp.* indicates the species of *Ureaplasma* and SV the serovar. Serovar of *U. parvum* was determined whereas the serovar of *U. urealyticum* was not. N.D = not determined.

2.1.3 Culturing of Ureaplasma from clinical samples

Initially 25µl of either gastric fluid (GF), nasopharnaneal aspirate (NPA), endotracheal aspirate (ETA) or bronchial alveolar lavage (BAL) was added to 2 ml of USM within a 7 ml bijoux. Cultures were incubated at 37 °C and checked for colour change every 24 hours for 1 week. 200 µ of pure positive cultures were pelleted by centrifugation at 16 000 xg for 10 min followed by removal of all USM supernatant. Pellets were resuspended in 50 µl sterile water, heat lysed by incubation at 95 °C for 10 min. 1 µl, as well as 1 µl of a 1:100 and 1:1000 dilution, were used to confirm the presence of *Ureaplasma* by PCR as described in section 2.4.3.3. Mixed cultures were prepared in the same way as pure *Ureaplasma* cultures but were resuspended in 100 µl sterile water. A "false-negative control" was included where samples were spiked with 1:1 *Ureaplasma* DNA to sample DNA to rule out the presence of PCR inhibitors.

2.1.4 Quantification

When quoting numbers of *Ureaplasma* the standard unit stated is colour changing units (CCU). To determine the number the number of CCU within a sample or culture a series of 1:10 dilutions are made as follows. 180 µl of USM was added to a single column (8 wells) of a 96 well plate. 20 µl of sample was inoculated into the first well to give an initial 1:10 dilution. From this well the process is repeated down the plate to the 7^{th} well give a dilution gradient to 10^{-7}. The final well remained uninoculated to serve as a negative

control, to rule out false positives. Plates were then incubated for 48 hours at which time colour change had ceased. The final well in which colour change occurred was denoted as 1 CCU. By knowing the dilution of the well giving 1 CCU, the number of CCU in the initial sample could be calculated (Figure 2.1).

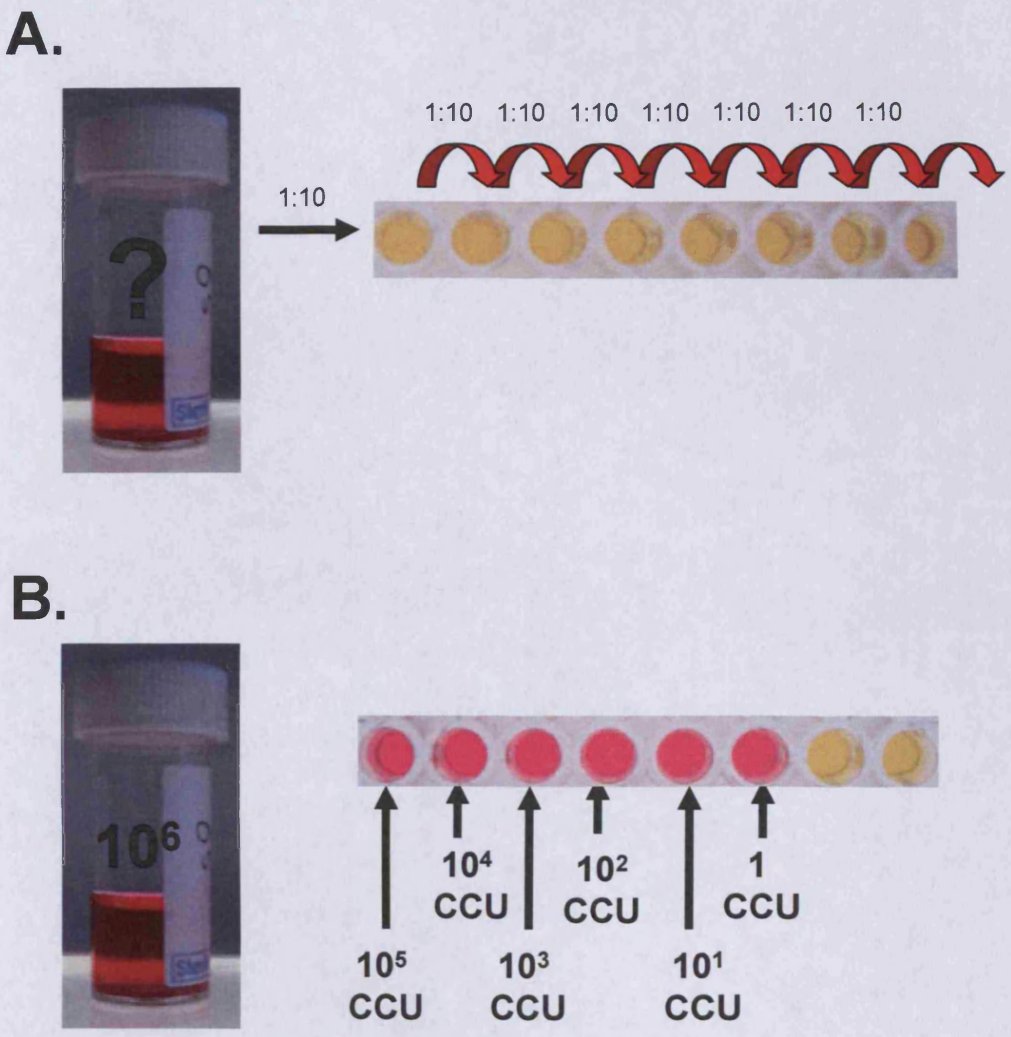


Figure 2.1. Quantification of *Ureaplasma*. The number of colour changing units (CCU) is calculated by titrating out culture of unknown CCU 1:10 in a microtitre plate (A). Cultures are then incubated at 37 oC for 48 hours until colour change stops. The final well in which colour change occurs is designated as 1 CCU. By working back through the dilution gradient it is possible to determine that in the original culture there was 10^6 CCU.

2.1.5 Long term storage and thawing of Ureaplasma spp. isolates

Ureaplasma positive cultures were sub-cultured 1:100 and 1:1000 into 2 ml cultures and incubated overnight. 250 µl aliquots were made into 2 ml cryovials (Starlab, Milton Keynes) which were then frozen directly at -80 °C. When frozen isolates were required, cryovials were defrosted on ice then sub-cultured 1:10 into 180 µl USM followed by serially diluted out to 10^{-5} and incubated overnight. The next day, inoculum was prepared by diluting the final well which was starting to show colour change by 1:10 into USM to provide enough culture of a uniform number of CCU for multiple samples. If greater than 180 µl of O/N culture was needed, then the final two wells showing colour change were pooled and expanded to an appropriate final volume. This method provided an inoculum of about 10^4-10^5 CCU

2.2. Complement studies

2.2.1 Preparation of human serum

Volunteer blood was collected from 9 healthy females (VxF) and 3 healthy males (VxM) of unknown *Ureaplasma* colonisation status into 20 ml glass universal bottles. Blood was allowed to clot at room temperature for 1 hour. Following incubation, the clot was dislodged from the wall of the bottle and incubated for an addition hour on ice to allow the clot to contract further. Samples were centrifuged at 1000 xg for 10 min. Supernatant (serum) was then dispensed into 1.5 ml tubes and centrifuged again at 16 000 xg for 1 min to remove uncoagulated red blood cells. 250 µl aliquots of normal human serum (NHS) were made into 0.6 ml tubes which were then stored at -80 °C until required. To obtain heat-inactivated serum (HI-NHS), aliquots were incubated at 56 °C for 30 min. This process inactivates C2 (required for the classical and lectin pathways) and factor B (required for the alternative pathway). 50% NHS and 50% HI-HNS were prepared by mixing 1:1 v/v with veronal buffered saline (Complement Fixation Buffer; Oxoid, Baisingstoke) unless stated otherwise.

2.2.2 Complement killing assay

Ureaplasma cultures were prepared as stated in section 2.1.5. 200 µl of culture was added, in duplicate, to the well of a point bottomed 96 well plate (Figure 2.2). Plates were sealed and centrifuged at 3600 xg for 10 min followed by careful aspiration of the supernatant by angling the plate towards

the operator and drawing the liquid from the top of the conical ridge so to avoid disrupting the pellet. Pellets were resuspended in 200 µl 50% NHS diluted in VBS. A 50% serum matched heat inactivated control (50% HI-NHS) was run in parallel as a direct comparison for later calculation of killing. Plates were incubated at 37 °C for 1 hour then centrifuged as described above. Pellets were resuspended in 200 µl USM and transferred to a fresh 96 well flat bottom plate where both NHS and HI-NHS exposed *Ureaplasma* were titrated 1:10 for 3 dilutions then 1:2 for 8 dilutions giving a dilution gradient to 2.56 x 10^{-5}. Plates were sealed and incubated for 48 hours until colour change had ceased. Degree of killing was determined by calculating the fold decrease in CCU from the HI-NHS relative to the NHS. For experiments in which the classical and lectin pathways were knocked-out leaving the alternative pathway intact, VBS was substituted with alternative pathway buffer (APB) (VBS supplemented with 10 mM EGTA & 10 mM Mg^{2+}). EGTA was used in the alternative pathway buffer as it specifically chelates Ca^{2+} from serum which the classical pathway (CP) and lectin pathway (LP) are dependent on for activation. Only Mg^{2+} is required for the assembly of the C3bBb complex of the alternative pathway (AP), and alternative pathway buffer contains an additional 10 mM Mg^{2+} even though EGTA has a very low affinity for this cation.

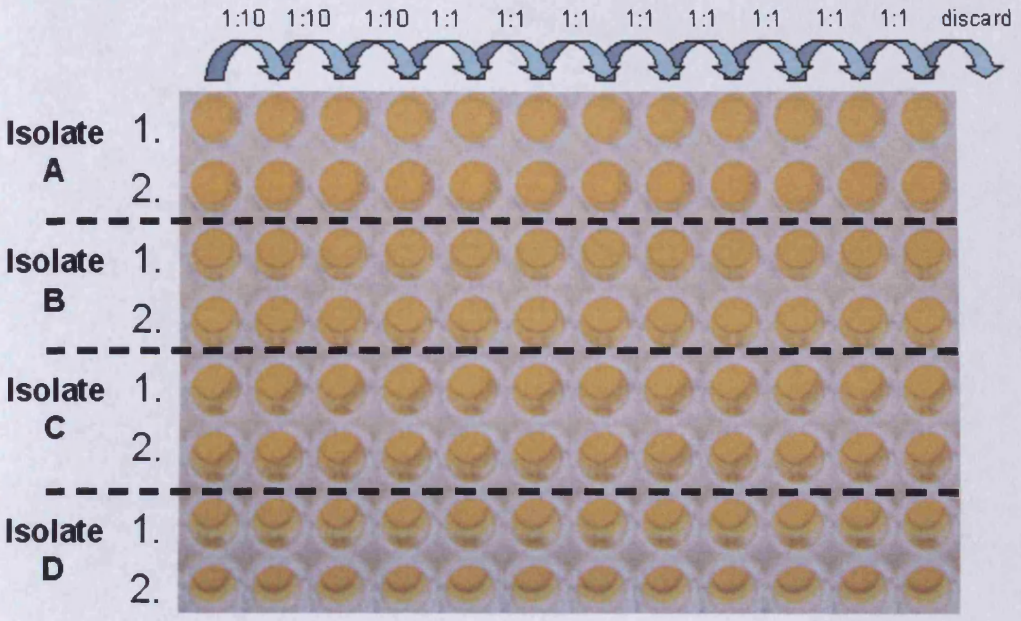


Figure 2.2. Schematic diagram of the 96-well plate set up of complement killing assay for four *Ureaplasma* isolates (A - D) under two conditions. (1) 50% heat inactivated normal human serum (HI-NHS) and (2) 50% normal human serum (NHS). Following complement attack *Ureaplasma* were titrated out as set out in the plate above, initially 1:10 dilutions followed by 1:1 dilutions, to determine the fold reduction of colour changing units between HI-NHS and NHS.

2.2.3 IgG depletion of serum with protein A and protein G-sepharose columns

A HiTrap™ Protein A-sepharose column (GE HEALTHCARE, Slough) was used to deplete IgG (sub-classes 1, 2 and 4) from three volunteers with low killing (including two sero-negative sera) and three with high killing to the four representatives of the serovars. Columns were regenerated by passing 10 volumes of VBS through at a flow rate of 1ml/min using a 10 ml syringe (ELKAY, Basingstoke). One ml of serum was loaded onto the column and pushed through with 1 ml VBS. Eluted serum was passed through a second time to ensure full IgG depletion and was stored at a 50 % concentration in VBS. Bound antibody was eluted with 200 mM glycine pH2.5. Columns were then stored in 20 % ethanol. For complete depletion of all IgG subclasses (IgG1, 2, 3 and 4) a 5ml HiTrap™ Protein G-sepharose column (GE HEALTHCARE, Slough) was used. IgG depletion was identical to that described above for the Protein A column, but with the modification of adding 2 ml of human serum, instead of only 1ml, and only passing the contents through the column once (this was due to the larger column size of the Protein G column (5 ml) verses the Protein A (1 ml)).

2.2.4 MBL depletion and ELISA

Levels of MBL in each of the samples were assayed using the MBL Oligomer ELISA Kit (BIOPORTO Diagnositics, Denmark) as per manufacturer's instructions. MBL was depleted from 1 ml NHS by circulating ice cold serum through a mannan-agarose column on ice (carried out by Dr Brad Spiller). To

confirm successful MBL depletion, MBL levels in each serum sample were measured using the commercial MBL ELISA kit post-depletion. No samples were found to have more than 49 ng/ml MBL (which represents non-functional MBL as it would not bind mannan-sepharose).

2.2.5 Adapting Ureaplasma into a pig-serum free system

HPA5 (SV3) was adapted to growing in USM without 10 % pig serum, but supplemented with 1 % HI-NHS from either MBL depleted VxF1 or VxF9. *Ureaplasma* cultures grown in USM with pig serum were titrated out to 10^{-6} in USM supplemented with 1% HI-NHS from either MBL depleted VxF1 or VxF9 and incubated at 37 °C for 24 hours. The final well in which colour change occurred was subsequently titrated out in modified USM with HI-MBL depleted serum. This process was repeated a total of three times to ensure that no residual pig MBL-decorated bacteria would be present.

2.2.6 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

5 ml *Ureaplasma* cultures were centrifuged at 16000 xg for 10 min and the resultant pellets washed three times in sterile PBS. Pellets were then resuspended in 25 µl PBS and 25 µl LDS buffer (Invitrogen, Paisley). Samples were boiled for 2 min and 7 µl sample loaded on to SDS-PAGE gel which was set up as follows: Gel casting apparatus was set up as directed by manufacturer (BIORAD, Hertfordshire, UK) and resolving gels were made up to using the volumes indicated in Table 2.2 and poured leaving 1.5 – 2 cm

space. 200 µl of butanol was immediately overlaid to level out the top of the resolving gel which was washed off with de-ionised water after the gel had polymerised. The stacking gel was prepared as shown in Table 2.2 and poured on top of the set resolving gel with the addition of a gel comb which was removed after the gel had polymerised. Plates containing set gels were set up in the running tank with the central reservoir filled with 1x running buffer (25 mM tris, 192 mM glycine and 0.1% SDS at pH 8.3). 11.5 µl of total ladder containing a mixture of 7.5 µl of SeeBlue® Plus 2 Prestained standard (Invitrogen, Paisley) ladder combined with 4 µl of MagicMark™ XP Western Protein Standard (Invitrogen, Paisley) and loaded along side samples. Gels were electrophoresised 150 V at approximately 120 mA for about 1 hour (until the dye front reached the bottom of the gel).

Reagent	4 %
40% bis/acrylamide 37.5:1	1.012 ml
dH_2O	6.4 ml
Upper buffer buffer pH 6.8	2.4 ml
10% APS w/v	100 µl
TEMED	40 µl

Reagent	7.5%
40% bis/acrylamide 37.5:1	1.93 ml
dH_2O	5.47 ml
Lower buffer buffer pH 8.8	2.5 ml
10% APS w/v	100 µl
TEMED	10 µl

Table 2.2. Recipes for pouring a 7.5 % resolving SDS-PAGE gel with a 4 % stacking gel for the resolution of proteins between 50 - 120 kDa.

2.2.7 Western blotting

Proteins were transferred from gels to 0.22 μm nitrocellulose membranes using BioRad Mini-Protean 3 system as indicated by manufacturer instructions. Transfer was carried out in a transfer tank with the transfer cassette, 1x transfer buffer (14.4 g/L, 3.03g Tris 20 % methanol), an ice pack to keep the transfer buffer cool and a magnetic stir bar to circulate buffer. Transfers were run at 100 V for 1 hour. Nitrocellulose membranes containing transferred proteins were placed within a 50 ml falcon tube (Elkay, Basingstoke) and blocked for 1 hour with 10 ml blocking buffer (5% w/v non-fat milk and 2% w/v BSA) on a roller. Membranes were probed with 1:100 HI-NHS, diluted in blocking buffer, for 30 min followed by three washes in PBST. To detect bound human IgG, a goat anti-human IgG (Fc specific) peroxidise-conjugated secondary antibody (Sigma, UK), diluted 1:1000 in blocking buffer, was incubated for 30 min. Membranes were washed three times in PBST and subsequently in PBS before developing with Pierce® ECL Western Blotting Substrate (Thermo scientific, Loughborough). For blots which were IgG sero-negative a goat anti-human IgG, A, M polyvalent peroxidise-conjugated antibody, diluted 1:1000 in blocking buffer, (Sigma, UK) was used.

2.2.8 Isolation of Ureaplasma lipoproteins using Triton X-114 (TX-114)

A 5 ml overnight culture of *Ureaplasma* was grown as described in section 2.1.5. The culture was centrifuged at 13, 000 xg and pellets were washed three times in ice cold tris buffered saline (TBE). The pellet was then

resuspended in 200 µl ice cold TBS with 0.5 % TX-114 and vortexed several times, but while keeping the sample chilled. This suspension was then layered on top of 300 µl of frozen TBS with 6 % sucrose and 0.06 % TX-114 and incubated in a water bath at 37 °C for 3 min in which time cloud point will be reached. The sample was then centrifuged at 300 xg at room temperature for 3 min followed by removing the upper aqueous phase. To the lipid phase was added 40 µl 10 % SDS and 40 µl of LDS and frozen at -20 °C until analysis.

2.2.9 CH50 assay

The CH50 assay was used to asses the optimal concentration of α-C1q globular head antibody so to identify the role of direct C1q binding on SV3. Two lots of 1.5 ml sheep erythrocytes were aseptically decanted into 1.5 ml micro-centrifuge tubes. These were centrifuged at 5000 rpm for 3 min. Supernatant was removed and pellets were washed in 1.5 ml PBS three times. 5 ml of PBS was added to two lots of universal containers, one of which received 25 µl of amboceptor antibody (rabbit α-sheep erythrocyte antibody) while the other received 200 µl of washed sheep erythrocyte pellet. These two solutions were pooled together giving a 2x solution of erythrocytes. This was incubated at 37 °C for 30 min with vortex every 10 min. Following sensitisation the 2% mix was centrifuged at 1000 xg for 2 min, resuspended with 10 ml PBS and washed in PBS twice. Two washes in VBS followed and the sensitised erythrocytes were diluted to 1% in VBS. The α-C1q globular head antibody (clone 2204) was kindly donated by Professor Moh Daha

(Leiden University, Netherlands) and was incubated with serum samples by adding 7 µl and 14 µl of antibody (1 mg/ml anti-C1q, final concentrations 70 µg/ml and 140 µg/ml, respectively) and then incubated for 30 min to compete out any C1q. As the antibody was obtained in a PBS buffer, an equal volume of PBS was additionally tested in parallel to determine the inhibition of classical pathway by calcium chelation mediated by the PBS carrier. Samples were then diluted 1:20 in VBS, in preparation for the assay. In each row of a flat bottom 96-well plate, 50 µl of water was added to row A. 50 µl of the 1:20 serum was added to row B. Wells C-H received 50 µl VBS. Well C received an additional 100 µl of the 1:20 serum dilution. 2:3 dilutions were made down the plate from well C to well G where 100 µl was discarded. Well H remained as a serum-free VBS negative lysis control. The 2% erythrocyte solution was diluted 1:1 to give a working 1% solution of which 50 µl was added to every well. The plate was then incubated at 37 °C for 30 min, with absorbance readings being taken every 3 min in a plate reader at wavelength 690nm.

2.2.10 Concentration of α-C1q globular head antibody

The results from the CH50 assay showed some effect from the PBS in the αC1q buffer therefore to reduce the non-specific effects of PBS in the killing assay the stock was concentrated to 5 mg/ml as follows. 600 µl of 1 mg/ml stock was added to a Vivaspin centrifugal protein concentrator column (molecular mass cut-off 50 kDa) (Fisher, Loughborough). The column was spun at 5 000 rpm in a bench top centrifuge for 20 min. This was sufficient

time for 480 µl of solution to pass through leaving 120 µl of concentrated antibody at 5 mg/ml.

2.2.11 Preparation of serum for C1q-blocked killing assay

100 µl of serum was incubated with 7 µl of 5 mg/ml α-C1q antibody (350 µg/ml final concentration) for 30 min prior to the killing assay. Run conditions were then as described in section 2.2.2. An isotype matched control antibody (anti-HHV8 KCP antibody) was run along side to control for any non-specific differences as a result of protein concentration (Mark *et al.* 2007).

2.3 Clinical samples

2.3.1 Ethics

All samples were collected after ethical approval from patients located on the Neonatal intensive care or high dependency units at the University Hospital of Wales, Cardiff, U.K. by the following; Dr Nicola Maxwell or Dr Phillip Davies (BAL samples), Sister Diane Nutall (GF, ET and NPA) and Sisters Jenny Webb or Louise Bridge (GF).

2.3.2 Gastric fluid (GF)

Infants admitted to the neonatal unit frequently have a nasogastric or orogastric tube inserted as part of their routine neonatal care. The tubes are aspirated at the time of insertion to obtain gastric fluid which is pH tested in order to assess the correct situation of the tube in the stomach. Aspirated gastric fluid was saved in an occluded syringe and refrigerated until appropriate, signed informed consent could be obtained from the infant's parents. Gastric fluid was taken once only from infants who had a naso- or oro-gastric tube inserted within 2 hours of birth and who had not previously had a milk feed.

2.3.3 Endotracheal aspirate (ETA)

Infants requiring mechanical ventilation regularly have endotracheal suction performed as part of their routine care. Following signed, informed parental consent, endotracheal secretions were collected in a suction trap during

routine endotracheal suctioning on a twice weekly basis until the infant was 28 days old or until extubated, whichever occurred first. Briefly, a size 6 French Gauge (FG) suction catheter was introduced into the endotracheal tube and advanced to a predetermined length so that the tip of the catheter would reach but not extend beyond the tip of the endotracheal tube and a suction pressure of 8-12kPa was applied as the catheter was withdrawn, in order to obtain any secretions remaining in the catheter following the suction procedure, 2 ml of sterile saline was suctioned through the catheter into the suction trap before it was disconnected from the suction tubing. Samples were immediately refrigerated and then transferred to the laboratory within 12 hours. If an infant was having bronchoalveolar lavage performed (see section 2.3.5 below), the BAL procedure replaced routine endotracheal suctioning and thus no ET sample was obtained at the same time.

2.3.4 Nasopharyngeal aspirate (NPA)

Non-intubated infants receiving continuous positive airway pressure (CPAP) and infants who had been extubated to CPAP following a period of ventilation intermittently require nasopharyngal suction to keep their upper airway patent and free of a build up of secretions. Again following signed, informed parental consent, the secretions obtained at such routine suctioning procedures were collected in a suction trap on a twice weekly basis until the infant was 28 days old or no longer required nasopharyngeal suction. The samples were refrigerated until transfer to the laboratory (always <12 h post-collection).

2.3.5 Bronchioalveolar lavage (BAL)

BAL is a well established clinical and research technique that is safe and well tolerated in mechanically ventilated neonates, provides reproducible results and no long term adverse effects.

A large number of the infants included in this study were involved in another study of neonatal BAL. For the purpose of the study, BAL was performed daily for the first week of life and then twice weekly until the infant was 28 days old or until the infant was extubated, whichever occurred first. Preterm infants in the neonatal unit in Cardiff routinely receive exogenous surfactant therapy at birth and again at 12 hours of age. In order to minimise any wash out of surfactant, the first BAL was performed at 12 hours of age, immediately prior to the administration of the second surfactant dose. Timing of subsequent lavages was co-ordinated in order to replace the routine endotracheal suction. If an infant was judged too unwell by the attending clinician to tolerate a BAL, the procedure was withheld.

BAL was performed using the guidelines set out by the European Respiratory Society task force on BAL in children (de Blic 2000) and as previously described, Briefly, with the infant lying supine and the head turned to the left, a size 6FG catheter was gently introduced down the endotracheal tube until resistance was felt. Then 1ml/kg of 0.9% saline (up to a maximum of 2ml) was instilled via the catheter. The catheter was then connected to 8-12 kPa of suction pressure and the lavage fluid was suctioned back and collected in a suction trap as the catheter was withdrawn. The ventilator was reconnected

and the infant's heart rate and saturations, which may have dipped during suction, were allowed to return to normal before the procedure was repeated a second time. The BAL fluid was pooled from the two lavages, placed on ice and immediately transported to the laboratory for analysis.

2.3.6 Measurement of IL-6 and IL-8 concentrations in BAL samples by ELISA

IL-6 and IL-8 ELISA's were run by Dr Eamon McGreal and Dr Mallinath Chakraborty from the Department of Child Health. IL-6 in BAL samples were measured in 11 term, 21 RDS, 24 CLD and 7 babies who died by ELISA (IL-6 DuoSet ELISA kit, R&D Systems, UK) according to manufacturer's instructions. IL-8 was measured in all the above samples less 1 infant who died (OptiEIA ELISA kit, BD, UK).

2.4 Molecular genetic methods

2.4.1 Preparation of clinical samples prior to DNA/RNA extraction

Samples were thawed on ice for 20 min then centrifuged at 2400 xg to collect the sample in the bottom of the tube. Samples were then transferred to 1.5ml microcentrfuge tube (Elkay, Basingstoke) and centrifuged in a bench top centrifuge at 16,000 x*g* for 10 min followed by the removal of 25μl aliquots of supernatant which were frozen at -20 °C for future analysis.

2.4.2 DNA/RNA extraction of GA, NPA, ET and BAL samples

DNA/RNA extractions were carried out using the commercially available QIAGEN® RNA/DNA Mini kit (25) (Qiagen, Crawley). 100μl of cell pellet and supernatant was transferred to a 1.5ml tube. Cells were lysed with 500μl lysis Buffer QRL1 and vortexed for 10 seconds then passed 5 times through an 18- or 20- gauge needle. 500μl of Buffer QRV1 was added followed by thorough vortexing. Lysed samples were then centrifuged at 16,000 x*g* for 20 mins at 4 °C to pellet cellular debris. Supernatant containing released nucleic acid was transferred to a fresh 2ml collection tube. Nucleic acids were precipitated with 0.8 volumes (0.8 ml) of ice cold isopropanol on ice for 5 min. Samples were then centrifuged at 16,000 x*g* for 30 min at 4 °C. During this time QIAGEN-tips were equilibrated with 1ml of Buffer QRE. Following centrifugation supernatants from samples were discarded with the nucleic acid pellet resuspended and dissolved in 150 μl of Buffer QRL1 by heating tubes for 6

min at 60 °C. To increase binding of RNA to the QIAGEN-tip 1.35 ml of Buffer QRV2 was added to the nucleic acid suspension. To prevent any non-dissolved nucleic acid from blocking the QIAGEN-tip the sample was centrifuged at 5,000 x*g* for 5 min at 4 °C. Supernatant was added to the QIAGEN-tip and allowed to pass by gravity. Flow-through was collected and stored at room temperature for later isolation of DNA. 2 ml of Buffer QRW was applied to the column and allowed to pass through removing any contaminating proteins or polysaccharides while retaining nucleic acid binding. 2 ml of Buffer QRU^{R}, which had been preheated to 45 °C, was used to elute off bound RNA from the column. Collected RNA was precipitated using 1 volume of ice-cold isopropanol and placed on ice while the DNA from the earlier steps was rebound to the QIAGEN-tip and washed with 3 ml of Buffer QC. DNA was eluted off the column with 2 ml of preheated (45 °C) Buffer QC. DNA was precipitated with 0.7 volumes of RT isopropanol for 10 min. Both DNA and RNA were centrifuged at 16,000 x*g* for 30 min at 4 °C. Supernatant was carefully removed and pellets washed with 500 µl of ice-cold 70 % ethanol, vortexed and centrifuged at 16,000 x*g* for 20 mins at 4 °C. This step was then repeated. DNA and RNA pellets were left to air dry for 10 min at RT by inverting tubes on paper towel. DNA and RNA were resuspended in 100 µl and 50 µl of RNAse free water, respectively. To dissolve nucleic acids samples were heated to 60 °C for 3 min, vorterxed for 5 seconds and pulsed-down to collect the dissolved nucleic acid in the bottom of the tube. The heating and vortexing process was repeated twice more to ensure full solubilisation of nucleic acid. Both DNA and RNA were then stored at – 20 °C until later analysis.

2.4.3 Polymerase chain reaction (PCR)

All polymerase chain reactions (PCR) were carried out in 200µl PCR tubes (Starlab, Milton Keynes) with all batches containing a known positive control, specific for the PCR in question, and a negative control (sample mix without DNA) to rule out false negatives and false positives, respectively. Mixtures for PCR can be found in Table 2.3 with a full list of primers in Table 2.4 with all reactions following the cycling parameters, unless stated otherwise, of denaturation of 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at the temperature stated in Table 2.4 for 1 min followed by an extension of 72 °C for 1 min/Kb of product. A final extension of 10 min followed and products were cooled to 4 °C until needed. All PCR reactions were run in QB96 Server Gradient Thermal Cyclers (Quanta, Surrey).

dH_2O	11.15 µl
5x Taq buffer	4.0 µl
$MgCl_2$ (25 mM)	1.2 µl
dNTP (10 mM)	0.4 µl
Forward primer	1 µl (5 pmol)
Reverse primer	1 µl (5 pmol)
GoTaq	0.25 µl
DNA template	1 µl
Final volume	20 µl

Table 2.3. Reagents and volumes used for a 20 µl polymerase chain reaction (PCR).

Table 2.4. Primers used in this thesis

Name	Sequence	Tm	Size	Ref
Detection of human DNA				
Human mitochondrial cytochrome oxidase (HMCO)				
H6A	5' ATGACCCACCAATCACATGCCTATCA 3'	59	823	(Fry *et al.* 2004)
H6B	5' ACTAGTTAATTGGAAGTTAACGGTACTA 3'			
Detection of bacterial DNA				
16s rRNA gene				
27f	5' AGAGTTTGATC(AC)TGGCTCAG 3'	See	1465	(Davies *et al.* 2009)
1492r	5' TACGG(CT)TACCTTGTTACGACT T 3'	methods		
***Ureaplasma* spp. detection**				
Urease gene				
U4	5' ACGACGTCCATAAGCAACT 3'	54	430	(Blanchard *et al.* 1993)
U5	5' CAATCTGCTCGTGAAGTATTAC 3'			
Multiple banded antigen (5' region)				
UM-1				
UMS-125	5' GTATTTGCAATCTTTATATGTTTTCG 3'	52	403(*U.p*)	(Teng *et al.* 1994)
UMA226	5' CAGCTGATGTAAGTGCAGCATTAAATTC 3'		448(*U.u*)	
Macrolide resistance primers				
Domain V of the 23S rRNA – Operon I				
MH23S-11	5' TAACTATAACGGTCCTAAGG 3'	56	1,339	(Pereyre *et al.* 2007)
UP23S-OP1	5' ACCACCATTCAATGTTTGAC 3'			
Domain V of the 23S rRNA – Operon II				
MH23S-11	5' TAACTATAACGGTCCTAAGG 3'	56	1,427	(Pereyre *et al.* 2007)
UP23S-OP2R2	5' CGTATACTTTGCCATAGTGTTGCC 3'			*
L4				
UPL4-U	5' TCTATTGATGGTAACTTCGC 3'	60	392	(Pereyre *et al.* 2007)
UPL4-R	5' GTTGAAGGTGTTTCTAAATCGC 3'			
L22				
UPL22-U	5' TTCGCACCGTAAAGCTTCTC 3'	60	458	(Pereyre *et al.* 2007)
UPL22-R	5' GTTCTGGATCAACGTTTTCG 3'			
Tetracycline resistance primers				
tetM primers for screening				
TetMF	5' TTATCAACGGTTTATCAGG 3'	48	397	(Blanchard *et al.* 1992)
TetMR	5' CGTATATATGCAAGACG 3'			
Sequencing remaining tetM gene				
TetMF-78	5' GTATACCTATGGTTATGC 3'	48	901	*
TetMR	5' CGTATATATGCAAGA CG 3'		(Blanchard *et al.* 1992)	
TetMF	5' TTATCAACGGTTTATCAGG 3'	54	1,715	(Blanchard *et al.* 1992)
TetMR 2123	5' GCATTTCGGACAATAGAGGGGG 3'			*
Ciprofloxacin resistance primers				
GyrA QRDR				
gyrA-1	5' TTGCTGCTTTCGAAAACGG 3'	50	336	(Bebear *et al.* 2000)
gyrA-2	5' CTGATGGTAAAACACTTGG 3'			

Sequencing remaining gyrA gene				
gyrA-1	5' TTGCTGCTTTCGAAAACGG 3'	50	1456 (Bebear *et al.* 2000)	
gyrA 1655R	5' CCTTGTACACCTACACCAC 3'			*
gyrA 1504F	5' GGTGATGAGCGTCGTACAG 3'	50	1258 (*U.p*)	*
gyrA 2743R	5' CCTGCAACTTCACCAATTG 3'		1270 (*U.u*)	*
GyrB QRDR				
gyrB-3	5' CCTGGTAAATTAGCTGACTG 3'	55	310 (Bebear *et al.* 2000)	
gyrB-4	5' TTCGAATATGACTGCCATC 3'			
Sequencing remaining gyrB gene				
gyrB -98F	5' GAAAATAGACGTGGTCG 3'	50	1668	*
gyrB-4	5' TTCGAATATGACTGCCATC 3'		(Bebear *et al.* 2000)	
gyrB-3	5' CCTGGTAAATTAGCTGACTG 3'	50	1249 (Bebear *et al.* 2000)	
gyrA-2	5' CTGATGGTAAAACACTTGG 3'			
ParC QRDR				
parC-5	5' ACGCAATGAGTGAATTAGG 3'	55	309 (Bebear *et al.* 2000)	
parC-6	5' CACTATCATCAAAGTTTGGAC 3'			
Sequencing remaining parC gene				
parC-5	5' ACGCAATGAGTGAATTAGG 3'	50	1397	
par 1545R	5' GTATCCATCACGACTTATTAC 3'			*
parC 1367F	5' GCTAAAACGCTACAAGAACG 3'	50	1318 (*U.p*)	*
parC 2553R	5' CAACGTTGGCATAAATTGG 3'		1324 (*U.u*)	
ParE QRDR				
parE-7	5' ATGGGCGGAAAATTAACGC 3'	55	313 (Bebear *et al.* 2000)	
parE-8	5' CTTGGATGTGACTACCATCG 3'			
Sequencing remaining parE gene				
parE -54	5' CAGCAACCATTTTATGCCGATCC 3'	50	1576	*
parE-8	5' CTTGGATGTGACTACCATCG 3'			
parE-7	5' ATGGGCGGAAAATTAACGC 3'	50	1182 (Bebear *et al.* 2000)	
parC-6	5' CACTATCATCAAAGTTTGGAC 3'			

Table 2.3 continued. All primers used in this thesis. * indicates primers designed in this study. U.p = *U. parvum*. U.u = *U. urealyticum*

2.4.3.1 PCR for the Human mitochondrial cytochrome oxidase (HMCO) gene

To assess the success and integrity of DNA extractions from clinical samples a PCR for the HMCO gene was carried out (Table 2.4) (Fry *et al.* 2004). Presence of an 823 bp product indicated that DNA was successfully isolated from the sample.

2.4.3.2 PCR for detection of the bacterial 16S rRNA gene

Detection of bacterial DNA was carried out by the amplification of the bacterial 16s rRNA gene; a highly conserved region across the bacterial kingdom (Lane 1991). Cycling parameters differed to those stated in section 2.4.3 as follows. Initial denaturation was performed at 94 °C for 10 min. This was followed by a touch-down protocol where samples were denatured at 94 °C for 1 min with annealing at 67 °C for 40 seconds and extension at 72 °C for 1 min. This was repeated for a total of 20 cycles in which the annealing temperature decrease by 1 °C per cycle. An immediate second round of cycling consisted of denaturation at 94 °C for 1 min followed by 47 °C for 1 min and extension at 72 °C for 1.5 min. This was repeated for a total of 20 cycles in which the extension time increase by 1 second per cycle. A final extension followed at 72 °C for 8 min. Samples were then cooled and stored at 4 °C.

2.4.3.3 PCR for the urease gene of *Ureaplasma* Spp. and use in confirming positive clinical samples

To detect or confirm the presence of *Ureaplasma* DNA within samples an established PCR method was used which amplified a 430 bp region of the urease operon (Table 2.4) (Blanchard *et al.* 1993). This region is unique to *Ureaplasma* species and universal across both *U. parvum* and *U. urealyticum*.

2.4.3.4 PCR for speciation of *Ureaplasma* isolates and serovar determination of *U. parvum*

Ureaplasma isolates were divided into either *U. parvum* or *U. urealyticum* using a previously documented PCR protocol (Teng *et al.* 1994). Primer set UM-1 amplified both *U. parvum* and *U. urealyticum* but gave a 403 bp and 448 bp product, respectively (Table 2.4). *U. parvum* isolates were classified to a serovar level by PCR sequencing of the UM-1 primer set (See section 2.4.5 for sequencing protocol). Returned sequence data was then aligned with the known MBA sequences of *U. parvum* serovars 1, 3, 6 and 14 (Kong *et al.* 2000). Two regions between -54 to -56 and -81 to -83 of the MBA gene were used to differentiate between the four *U. parvum* serovars as seen in Table 2.5.

Serovar	Nucleotide position within MBA gene					
	-84	-83	-82	-56	-55	-54
SV1	G	C	T	A	C	A
SV3	A	C	C	A	T	A
SV6	A	A	T	T	A	G
SV14	A	A	T	A	T	A

Table 2.5. Serovar specific nucleotide differences which can be used to differentiate between *U. parvum* serovars.

2.4.3.5 PCR for macrolide resistant *Ureaplasma*

To selectively amplify each of the two 23S rRNA operons independently, the following primer combinations were used (Pereyre *et al.* 2007). For operon 1, the cross-23S rRNA operon primer MH23s-1 was used in conjunction with the operon 1 selective primer UP23S-OP1 (which annealed within the 5' region of the adjacent *folA* gene). For operon 2, MH23s-1 was used with the operon 2 selective primer UP23S-OP2R2, which annealed to the 5' region within the adjacent *greA* gene. Genes were then sequenced as described in section 2.4.5.

Amplification of the L4 and L22 genes, previously associated with macrolide resistance in *Ureaplasma,* were carried out using PCR and sequencing protocols.

2.4.3.6 PCR for the *tetM* transferable element associated with tetracycline resistance

The presence of the *tetM* transferable element in *Ureaplasma* isolates was determined by PCR using the primers described by Blanchard *et al.* (Table 2.4), which amplified the 398 bp product (Blanchard *et al.* 1992). The whole *tetM* coding sequence from positive isolates was determined by using the primer combinations set out in Table 2.4 and sequences as described 2.4.5.

2.4.3.7 PCR for identification of mutations within the *Ureaplasma gyrA*, *gyrB*, *parC* and *parE* genes associated with ciprofloxacin resistance and additional whole gene sequencing

Primers *gyrA*-1 and *gyrA*-2 were used to amplify the quinolone resistance determining region (QRDR) of the *gyrA* gene, *gyrB*-3 and *gyrB*-4 were used to amplify the QRDR of the *gyrB* gene, *parC*-5 and *parC*-6 were used to amplify the QRDR of the *parC* gene and the *parE*-7 and *parE*-8 primer pair were used to amplify the QRDR of the *parE* gene all of which were described by Bebear *et al.* (Bebear *et al.* 2000). To determine the full sequence for each of the respective amplicons from primer combinations in Table 2.4, standard PCR protocols were used, with exception of primer combination *parE* -54 and *parE*-8 which required 40 cycles instead of 35. Products were sequenced as stated in section 2.4.5.

2.4.4 Agarose gel electrophoresis

To determine the presence and size of amplified DNA following a PCR reaction, DNA fragments were separated using agarose gels. Gels were prepared by dissolving molecular grade agarose (Invitrogen, Paisley) in 1x tris/borate/EDTA buffer (MP biomedicals, France) at 0.7 – 1.5% w/v (dependent upon size of product expected). The mixture was heated in a microwave until all agarose granules were dissolved then cooled to around 55 °C. When cooled and immediately prior to gel casting, ethidium bromide (MP biomedicals, France) was added to a final concentration of 1 μg/ml. Gels were poured into a sealed casting tray and allowed to set at room temperature. When the gel was set, casting trays were placed in to a running tank in the orientation of well to be loaded at the cathode. 5μl of DNA ladder, either Hyperladder I or IV (Bioline, London), was added to the first well as a size marker. Gels were then run at 130 V for one hour. Ethidium bromide stained gels were visualized in a BioDoc-IT™ UV Tansilluminator (Ultra-Violet products, Cambridge).

2.4.5 Sequencing of PCR products

If a PCR product was to be sequenced, five replicate PCR reactions (identical to the successful PCR) were performed, the reactions pooled and the amplicons purified (below) prior to sequencing to yield a sufficient quantity of DNA. For each sample 5 μl of the reaction was ran on an agarose gel to confirm that reaction was successful.

2.4.6 Purification of DNA PCR products for sequencing

Novagen SpinPrep™ PCR Clean-up Kits (Novagen, Nottingham) were used to purify PCR products in preparation for sequencing. The five PCR replicates were pooled in a 1.5 ml tube with the addition of 4 volumes of SpinPrep Bind Buffer (A) (400µl per 100µl PCR reaction). The PCR/Bind Buffer mixture was transferred to a SpinPrep PCR Filter which was inserted into a 2-ml Receiver Tube. The unit was then spun at ≥10,000 x*g* for 1 min. 400µl of SpinPrep Bind Buffer (A) was added to the filter followed by a second spin at ≥10,000 x*g* for 1 min. Flow-through from the spin steps were discarded. The filter was then placed back into the receiver tube and 500µl of reconstituted SpinPrep Wash Buffer (B) was added and passed through the filter by centrifugation at ≥10,000 x*g* for 1 min. Flow through was discarded and the unit was spun for an additional 2 min at ≥10,000 x*g* so to remove any residual SpinPrep Wash Buffer (B). The filter was then transferred to a 1.5 ml Eluate Reciever Tube. 15µl of prewarmed (70 °C) SpinPrep Elute Buffer (C) was added directly to the filter membrane and incubated at RT for 3 min. The unit was then centrifuged at ≥10,000 x*g* for 1 min. To increase the yield of DNA, a second elution with 15µl of prewarmed SpinPrep Eluate Buffer (C), followed by a 3 min RT incubation and centrifugation at ≥10,000 x*g* was performed. Assessment of quantity and purity of purified DNA was made by measuring DNA and protein concentrations at wavelengths A260/A280, respectively, in a BIO-SPEC-1601 dual beam spectrophotometer (Shimadzu, Milton Keynes) by diluting 2µl of purified product into 98µl of de-ionized water (1:50).

2.4.7 BigDye Terminator v3.1 sequencing

Sequencing reactions were set up in 200 µl PCR tubes containing the reagents listed in Table 2.6a and performed using the conditions listed in Table 2.6b using QB96 Server Gradient Thermal Cyclers (Quanta, Surrey) . Following the sequencing reaction, products were transferred to a 1.5 ml tube with the addition of 50µl ice cold 100% ethanol, 2 µl 125 mM EDTA and 2 µl 3M sodium acetate. Tubes were vortexed and incubated at room temperature for 15 min. Tubes were then spun at 16, 000 x*g* in a bench top centrifuge for 30 seconds. Supernatants were removed and DNA pellets were washed with 70 µl 70% ice cold ethanol, spun again for 15 min and washed and pelleted for a final time. Supernatants were removed and DNA pellet was air dried. The sequencing reaction was run in an ABI Prism Genetic Analyzer 3100 (Applied Biosystems Warrington, UK) by central biotechnology services at Cardiff University.

A. Reagent	Volume
Better buffer	5 µl
ABI big-dye	1.5 µl
Primer (5 pmol)	1 µl
DNA template (200 – 500 ng)	7.5 µl
Total	15 µl

B. 25 cycles		
Denaturation	50 °C	30 sec
Annealing	50 °C	15 sec
Extension	60 °C	4 min
Store	4 °C	∞

Table 2.6. Reaction mix (A) and cycling parameters (B) for BigDye® Terminator v3.1Cycle Sequencing protocol.

2.4.8 Sensitivity of primers

The sensitivity of the urease, UM-1 and 16S rRNA primers against CCU of *Ureaplasma* was determined as follows. A 1 ml logarithmic culture of SV8 was titrated out to 10^{-8} in a microtitre plate and incubated at 37 °C for 48 hours. The 1ml culture was immediately centrifuged at 16,000 x*g* for 20 min. All supernatant was removed and the pellet was resuspended in 50 µl sterile H_2O. The sample was boiled (lysed) at 95 °C for 10 min with the sample then diluted to 10^{-8} with 1 µl of each dilution used in each PCR reaction as described in 2.4.3. Degree of colour change was then compared with that of PCR positivity at each dilution.

2.5. Antibiotic susceptibility testing

2.5.1 Stocks of antibiotics

All antibiotics were purchased from Sigma-Aldrich (Dorset, UK) and reconstituted to concentrations indicated in Table 2.7. Erythromycin was bought as a 1 mg/ml stock solution in water. Azithromycin was prepared to a concentration of 10 mg/ml in ethanol. Clarithromycin was prepared to a concentration of 1 mg/ml in methanol. Tetracycline and doxycycline were both prepared to a concentration of 1 mg/ml in sterile water. Ciprofloxacin was prepared to a concentration of 5 mg/ml in DMSO. Working stocks were diluted immediately prior to use, in USM, to the indicated concentrations. The sensitivity of isolates to ethanol, methanol and DMSO (used to dilute antibiotics) were tested against USM only to ensure no non-specific antibacterial activity was mediated by these substances.

Antibiotic	Solvent	Stock concentration
Erythromycin	Water	1 mg/ml
Azithromycin	Ethanol	10 mg/ml
Clarithromycin	Methanol	1 mg/ml
Tetracylcine hydrochloride	Water	1 mg/ml
Doxycycline hyclate	Water	1 mg/ml
Ciprofloxacin	DMSO	5 mg/ml

Table 2.7. Antibiotics used to examine the sensitivity of *Ureaplasma* isolates. The solvents used to prepare each drug and the stock concentrations are indicated.

2.5.2 Break point screening of isolates

A break-point titration was used to identify resistant *Ureaplasma* from a cohort of 61 isolates from UHW and HPA. As reference ranges were not available for levels of resistance in *Ureaplasma*, values were determined from previous studies looking at the MIC as well as data on within the literature (Waites *et al.* 2001) as well as personal communication with C. M. Bebear. In a flat-bottom 96 well plate row A1-H1 contained 180 µl of 4 µg/ml erythromycin (all given as final concentrations), row A2-H2 contained 180 µl 4 µg/ml ciprofloxacin, row A3-H3 contained 180 µl 2 µg/ml tetracycline, and row A4-H4 contained 180 µl antibiotic free USM (Figure 2.3). Twenty microlitres of *ureaplasma* culture was then added to each well of A1-A4, and serial 10-fold dilutions performed

as above (diluting *Ureaplasma* but not antibiotic), but restricted to the 4 test rows. In this manner, the 96-well plate was organised to investigate 3 different isolates per plate by repeating the layout for A5-H8 and A9-H12 as above, but using different test isolates of *ureaplasma*. SV9 (Vancouver strain) was used as a positive control for tetracycline resistance, however, no known characterised resistant strains were available to use as controls for erythromycin and ciprofloxacin resistance. Any strains that showed growth in wells which corresponded to 10^3-10^4 CCU in the antibiotic free positive control row was subsequently fully investigated using the full plate method.

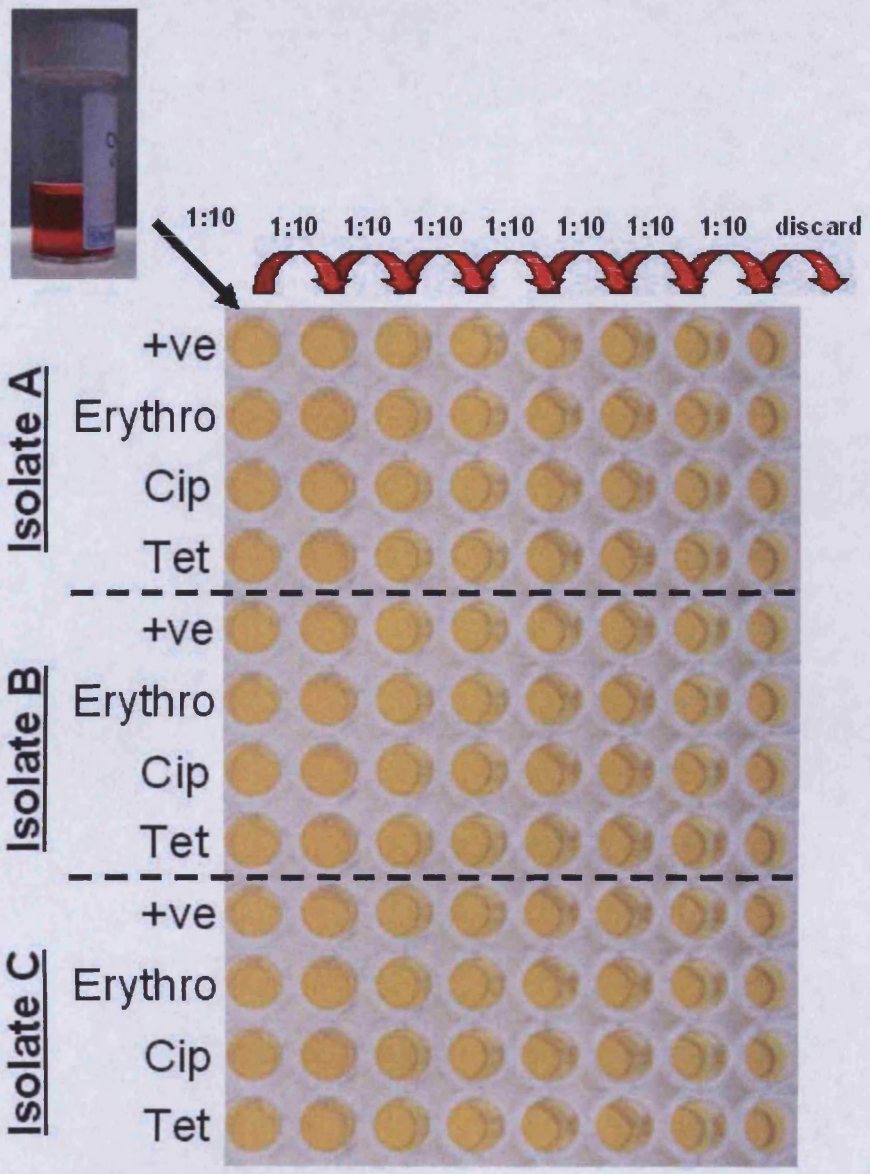


Figure 2.3. Break point analysis used to screen three different isolates (A, B and C) per 96-well plate. A culture of unknown CCU was titrated across the x-axis of the plate with set break point concentrations of each antibiotic and a growth control for each isolate. Erythro = erythromycin 4 μg/ml, Cip = ciprofloxacin 4 μg/ml and Tet = Tetracycline at 2 μg/ml.

2.5.3 96-well plate set up for determination of MIC for a single isolate with varying inoculum sizes

In a 96-well plate, wells A1-H1 received 360 µl of 64 µg/ml antibiotic in USM and 180 µl sterile USM was added to the remaining wells of the plate. Using a multi-channel pipette, rows of doubling dilutions were made by transferring 180 µl from wells in row 1 through to wells in row 11 with excess 180 µl discarded as seen in Figure 2.4. Thus an antibiotic gradient was created from 64 µl/ml to 0.0625 µl/ml (by rows). Row 12 remained free of antibiotic for unrestricted growth comparison. *Ureaplasma* isolates to be tested were prepared as stated in section 2.1.5. Twenty microlitres of *Ureaplasma* from the prepared culture was added to each well in the column A (1:10 dilution). A ten-fold dilution of bacteria was then titrated at 90 degrees across the antibiotic gradient. The plate was sealed and incubated at 37 °C in a humidified cell culture incubator and observed for colour change at 24h intervals for 4 days. The MIC was defined as the lowest concentration of antibiotic that prevented a colour change after 48h when read at 10^3 - 10^4 colour change unit (CCU) (relative to the growth in the antibiotic free medium). USM with and without antibiotics were also incubated in the absence of added U*reaplasma* to serve as a negative colour change control.

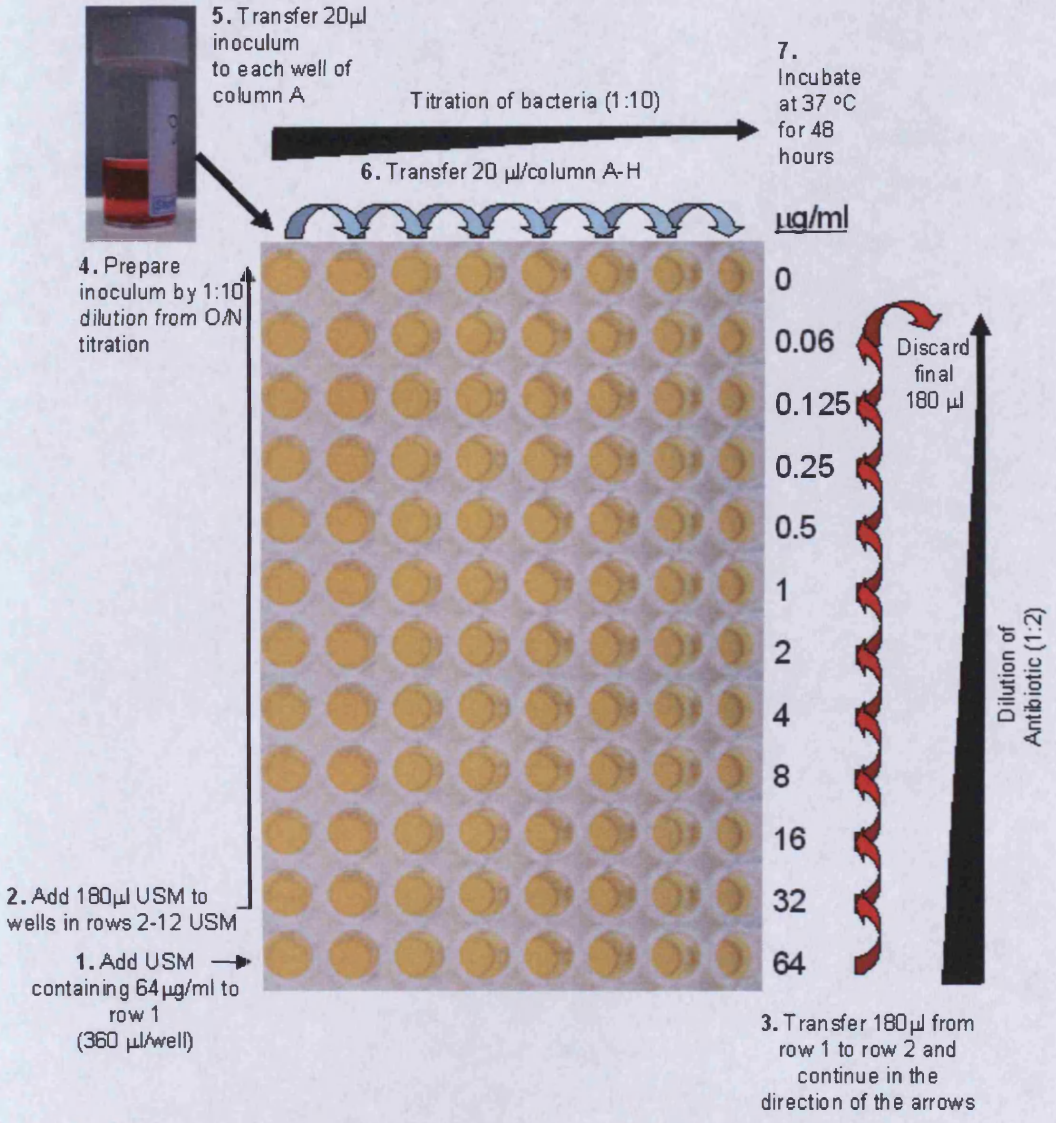


Figure 2.4. A schematic diagram of the 96-well plate was set up to determine the MIC of an *Ureaplasma* isolate to a single antibiotic

2.5.4 Control isolates

To ensure that the conditions for susceptibility testing were correct the MIC values were determined for standardised control isolates, as recommended by the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2001) in Mueller-Hinton broth using the methodology described in section 2.5.3. Control isolates were kindly donated by Dr Vicki Chalker from the HPA and were as follows *S. aureus* ATCC 29213, *E. coli* 25922 and *E. faecalis* 29212.

2.6. Bioinformatics

2.6.1 Sequence analysis

Analysis and manipulation of DNA sequence data was carried out using DNASTAR SeqMan™ II expert sequence analysis software Version 5.07. DNA and protein alignments were prepared using DNASTAR MegAlign™ expert sequence analysis software Version 5.07.

2.6.2 Phylogenetic trees

Phylogenetic trees were initially produced in DNASTAR MegAlign™ expert sequence analysis software Version 5.07 with final versions produced by Dr Vicki Chalker (HPA) using CLUSTALW with distances calculated using maximum likelihood. The evolutionary history was inferred using the Neighbour-Joining method and the optimal trees after 500 bootstraps. Repeat testing using Jukes Cantor and Tamura Nei distance calculations gave identical tree topology. Trees were rooted using respective gyrase or topoisomerase genes from *Mycoplasma pneumoniae*.

2.7 Statistics

All statistics in this thesis were calculated using GraphPad Prism Version 5.01.

Chapter 3

The effect of human complement upon killing of *U. parvum*

3.1 Introduction

Invasive *Ureaplasma* infections primarily occur in preterm neonates with an immature immune system or in immune compromised patients such as those who are hypogammaglobulinaemic or receiving immunosuppressive therapy. This suggests an essential role for a healthy immune system to fight off *Ureaplasma* infection.

Little is know about the interactions between *Ureaplasma* and the complement system. The predisposition of hypogammaglobulinaemic patients to *Ureaplasma* infections as well as reports of an antibody response to *Ureaplasma* suggests an essential role for the classical pathway (Watson *et al.* 1990; Furr *et al.* 1994). A single report has commented on the ability of MBL to bind to *Ureaplasma*, but no function was determined (Benstein *et al.* 2004). No reports have been made regarding the impact of the alternative pathway upon *Ureaplasma*, but due to being recognised as "foreign" by the immune system would be predicted as having an effect.

In this first results chapter I focus on the ability of the complement system to naturally fight off infection in an *in vitro* system using serum from 12 healthy volunteers and determine which complement activation pathways are responsible for the killing of representative serovars of *U. parvum*.

3.2 Results

3.2.1 Titration of serum for optimal killing

To determine the optimal concentration of serum for the complement killing assays which gave results similar to that of 100 % serum, but scaled down the volume of serum required, a series of doubling dilutions of 100 % serum in VBS were set up. As can be seen from Figure 3.1 the killing of *Ureaplasma* was dependent upon the concentration of serum used with levels of killing noticeably declining from 25 % serum. Killing of SV3 decreased by a log at every dilution from 25 % to 3 %, whereas SV1 killing began to decrease by a log at every dilution from 12 % to 3 %. As the use of 50 % serum gave comparable results to that of 100 % serum it was therefore determined to be suitable for use in the complement killing assays.

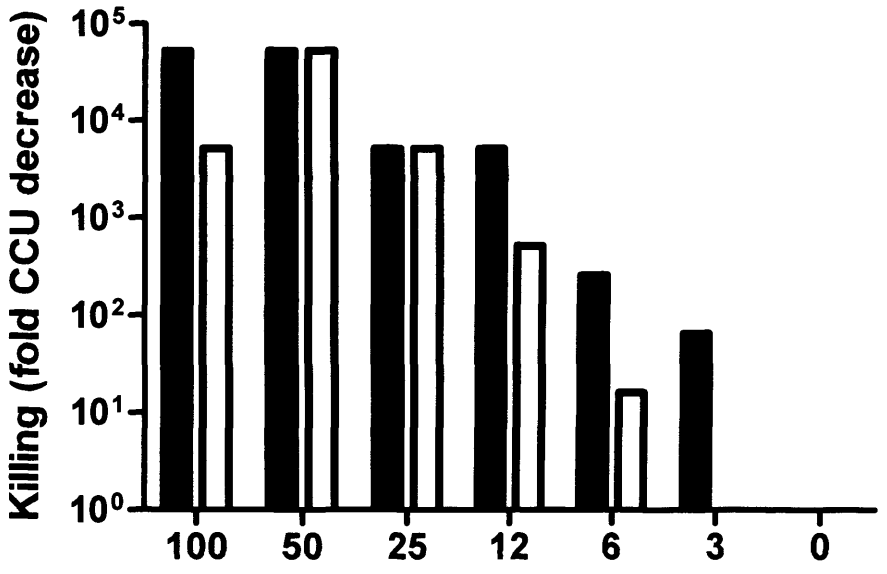


Figure 3.1. The ability of varying concentrations of human serum to kill *Ureaplasma*. Black bars represent killing of SV1 and white bars represent the killing of SV3. Numbering of the x-axis represents concentration of human serum.

3.2.2 Analysis of the complement killing assay

The killing assay described in the materials and methods chapter 2.2.2. allowed the determination of degree of bactericidal killing of serum from twelve human volunteers. As degree of killing was calculated via the decrease in CCU from a HI-NHS matched control, there was no need to know the CCU of the initial inoculum. Occasionally wells were observed in which colour change was absent although subsequent wells further down the titration were positive (Figure 3.2). To keep consistency in determining 1 CCU (the final well in which colour change occurs), negative wells were counted as positive if a single negative well was subsequently followed by two or more positive wells. To additionally rule out any bias in spontaneous negative wells within the titration all killing assay experiments were repeated in triplicate on independent days, and the mean and standard error of the mean determined prior to comparison. As degree of killing was dependent upon the reduction in NHS wells relative to a matched HI control, the poor growth in the HI control would result in an artificially low kill. Using the preparation methodology described, all serovars grew to at least 10^3 CCU in all experiments therefore ruling out any possible methodological bias in degree of killing.

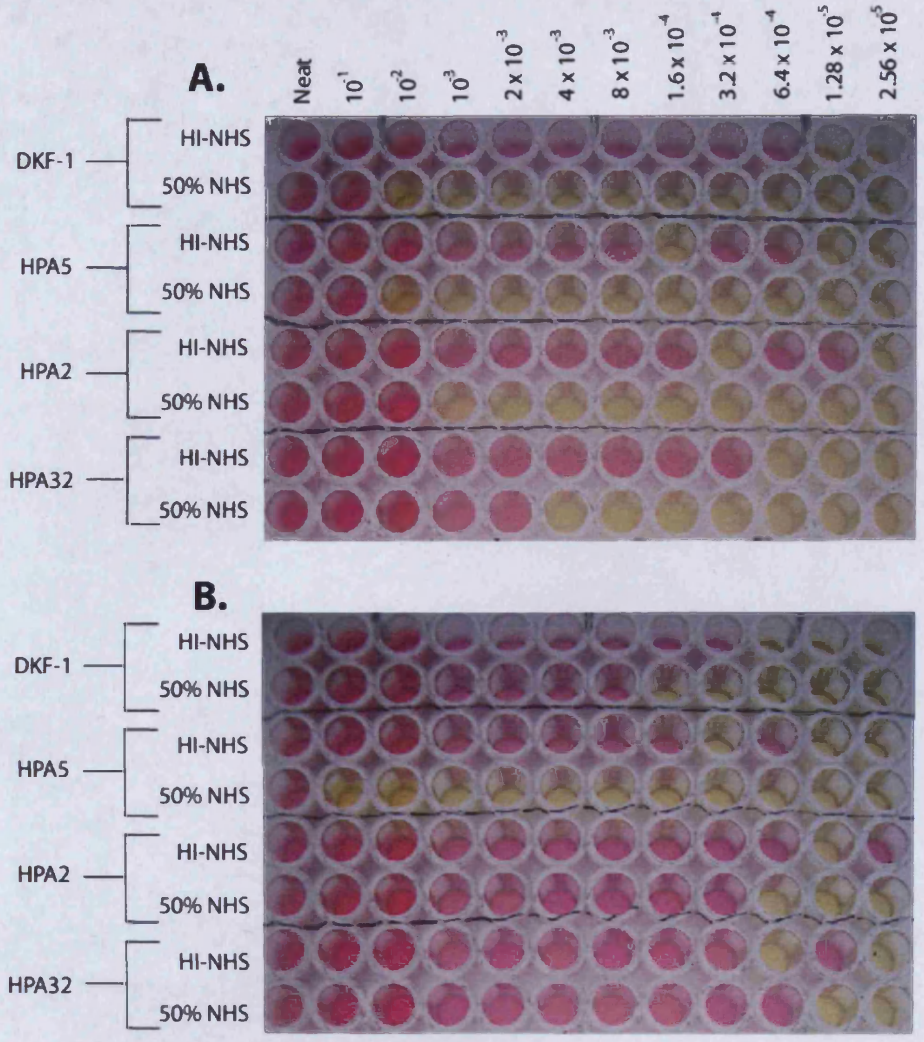


Figure 3.2. Complement killing assay to determine the bactericidal activity of human serum. The four representative serovars of *U. parvum* were DKF-1 (SV1), HPA5 (SV3), HPA2 (SV6) and HPA32 (SV14). The killing capacity of 50 % normal human serum (NHS) from the pan-ureaplasmacidal VxF3 serum (A) verses that of the low ureaplasmacidal VxF1 (excluding SV3 killing) (B). Pink wells represent growth of *Ureaplasma* whereas yellow wells represent absence of growth. Killing was determined by calculating the fold-decrease in CCU from the NHS relative to the heat-inactivated normal human serum (HI-NHS) control.

3.2.3 Variations in SV1, 3, 6 and 14 killing by all complement pathways compared to alternative pathway only

The ability of 50% human serum to kill representative strains of each *U. parvum* serovar is shown in Figure 3.3a-d. With all three activation pathways intact, the sera from 5/12 volunteers reduced the CCU of DFK-1 (SV1) by greater than 1000-fold, 2/12 reduced CCU by 100 to 1000-fold, 3/12 volunteers reduced CCU by 10 - 100-fold and the remaining 2 volunteers reduced CCU by less than 10-fold (Figure 3.3a). All volunteers serum reduced HPA5 (SV3) CCU by greater than 1000-fold (Figure 3.3b). One third (4/12) of sera reduced HPA2 (SV6) CCU by 100 to 1000-fold, one third (4/12) of sera reduced CCU between 10 to 100-fold and the final third (4/12) reduced CCU by less than 10-fold (Figure 3.3c). A single serum decreased HPA32 (SV14) CCU by greater than 1000-fold, two sera decreased the CCU by 100 to 1000-fold and the remaining nine sera decreased numbers by less than 10-fold (Figure 3.3d). The alternative pathway from all sera had a negligible effect upon killing of all serovars by reducing the CCU by less than 10-fold after one hour (Figure 3.3a-d).

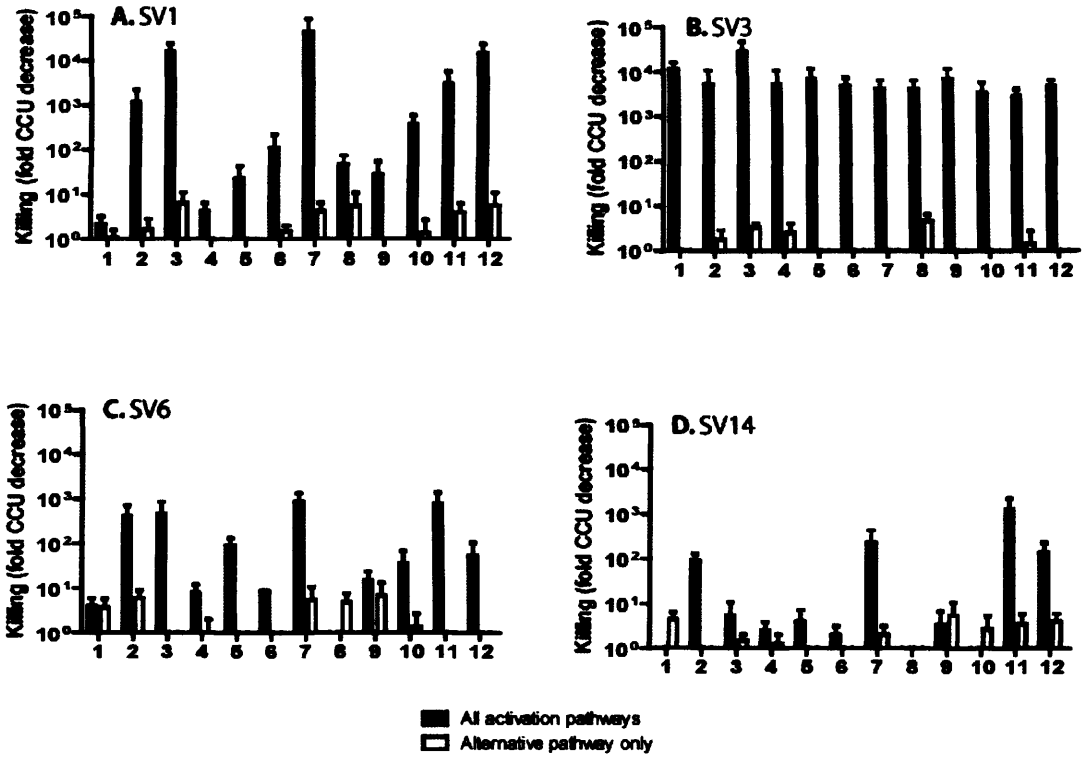


Figure 3.3. The killing capacity of 12 human sera against the four serovars of *U. parvum*. (A) SV1 isolate DKF-1, (B) SV3 isolate HPA5, (C) SV6 isolate HPA2 and (D) SV14 isolate HPA32. Dark bars indicated killing in the presence of three activation pathways. White bars indicate killing in the presence of the alternative pathway only. Numbering on the x-axis refers to volunteer serum used.

3.2.4 Detection of anti-Ureaplasma IgG response with volunteer serum by western blot

Figure 3.4 shows immunoblots from the 12 volunteers against the four serovar representatives of *U. parvum*. The blot from VxF1 shows the first of two seronegative sera identified in this study. This blot was subsequently probed with seropositive serum to rule out the possibility of a false negative result. Blots from VxF2 and VxF3 had similar banding patterns for each respective serovar. DKF-1 lanes produced a dark band of just over 60 kDa with a second, less intense, band of just greater than 70 kDa. This band was conserved across all serovars blotted with these sera, but with greater intensities. Faint bands were also seen for all serovars of about 85 – 90 kDa. In addition, the blot using VxF3 serum produced intense, low molecular weight bands which ran with the dye front. These were noticed in blots from VxF5 and VxF7. Serum from VxF4 revealed a conserved band from all serovars of less than 50 kDa with an additional, lower molecular weight band present for HPA5. In addition to the low molecular weight band already described for VxF5, faint bands at approximately 60 kDa were seen. A serovar specific band around 70 kDa was present in for DKF-1 with a 85 – 90 kDa band present for the remaining isolates. HPA2 had a unique band with a slightly higher molecular weight than the 85 – 90 kDa band just described. Only faint bands were present for VxF6 in the lane containing DKF-1 (85 kDa), HPA5 (less than 50 kDa) and HPA2 (70 – 75 kDa). No bands were present for HPA5 or HPA32. VxF7 produced a number of bands to each isolates with varying intensities. As described earlier, an intense low molecular weight

band was seen in all lanes. Further intense bands were present for DKF-1 one at 65 kDa and a second at 90 kDa. A final intense band of high molecular weight (less than 220 kDa) was seen in the lane containing HPA32. Although a gel loading control was not present, the comparable intensities of additional bands suggested that loading was equal therefore not a reason for variation in some band intensities. Weaker conserved bands across all isolates were additionally noticed. Blots probed with VxF8 serum resembled those probed with VxF2: with three bands present for DKF-1 and a single 70 kDa band for the remaining isolates. VxF9 represented the second seronegative blot. Blots from using this serum consistently had a high background, but not so bad as to obscure any potential bands. As with VxF1, these were then serially probed with seropositive serum to rule out false-negatives. VxM10 blots were similar to that of VxF2 and VxF8. VxM11 blots bound to a number of unique bands (not observed for the other sera) of high molecular weight. A faint 70 kDa band was present in the lane containing DKF-1, as well as a < 50 kDa band in the HPA5 lane. VxM12 serum produced 70 kDa and 50 kDa bands for DKF-1 and HPA5, respectively, similar to that of VxM11, but with a greater intensity. A high molecular weight band of 220 kDa was noticed along with a number of doublet bands of around 90 kDa.

Due to using whole-cell lysates the bands present on the western blot represented both internal (non-complement activating) and external (complement activating) antigens. As only the external antigens would be able to activate complement attempts were made to isolate these antigens using a tritonX 114 methodology. Unfortunately the methodology described in

section 2.2.8 failed to yield a large enough quantity of protein therefore cloud point was never reached and these experiments were abandoned.

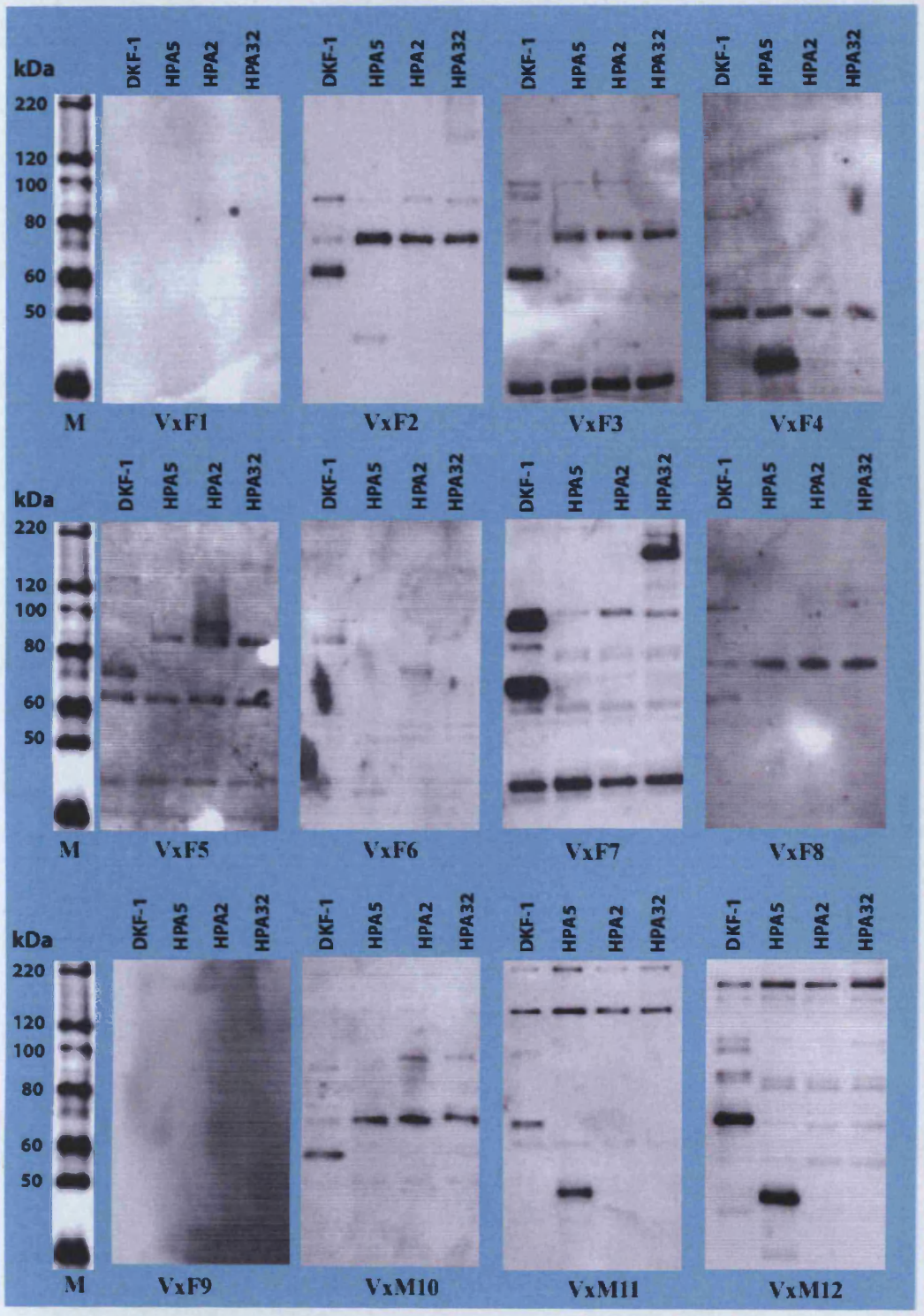


Figure 3.4. Immunoblots of SV1 isolate DKF-1, SV3 isolate HPA5, SV6 isolate HPA2 and SV14 isolate HPA32 probed with volunteer serum (Vx1-12).

3.2.5 Killing of U. parvum with human serum following depletion of IgG using a protein A column

A protein A-sepharose column was used to deplete IgG from the serum of six volunteers and assayed as in section 3.2.2 against the respective serovar isolates (Figure 3.5-d). These sera were selected on the basis of three having a low killing capacity (VxF1, VxF6, VxF9) as well as three having a high killing capacity (VxF3, VxF7, VxM12) in addition to these sera having the greatest amount of aliquots remaining following previous experiments. In those sera which killed SV1, IgG depletion reduced the killing by 53-fold for VxF6, 9057-fold for VxF3, 16261-fold for VxF7 and 10787–fold for VxM12 (Figure 3.5a). Similar patterns were observed for killing against SV6 and SV14 (Figure 3.5c-d). SV6 killing in the absence of IgG was reduced by 663-fold for VxF3, 231-fold for VxF7 and 296–fold for VxM12. SV14 killing in the absence of IgG was reduced by 108-fold for VxF3, 163-fold for VxF7 and 2827-fold for VxM12. Little difference in killing was seen when SV3 was challenged with IgG depleted sera with depleted sera still killing by greater than 1000-fold with exception to VxF1 and VxF9 which was reduced by 4901 and 3829-fold, respectively (Figure 3.5).

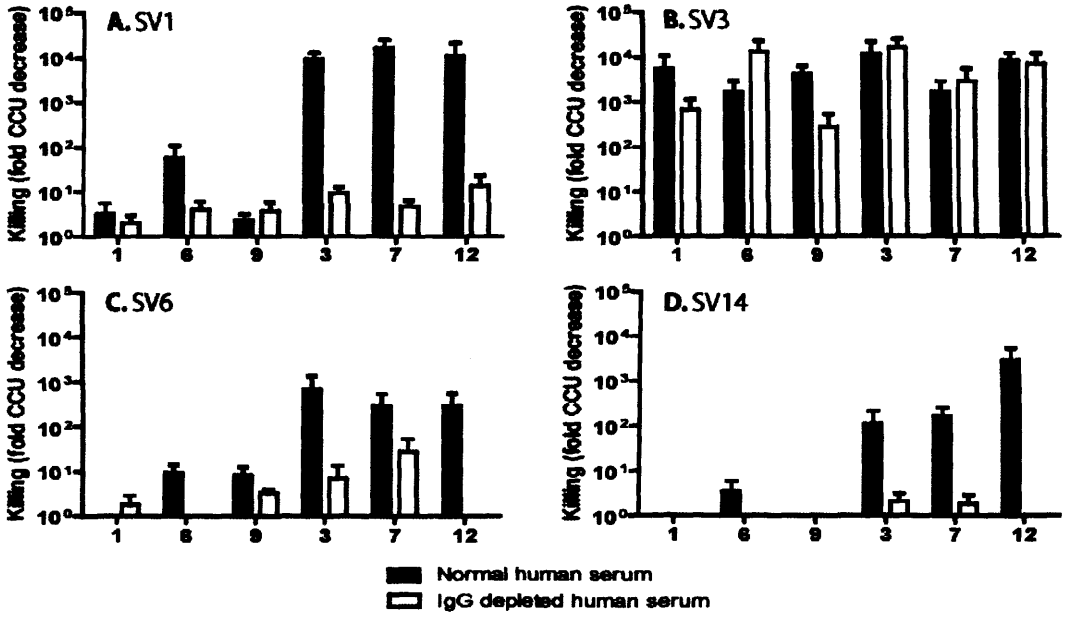


Figure 3.5. Effects of various sera depleted of IgG using a protein A sepharose column, on the killing capacity of the four serovars of *U. parvum*. (A) SV1 isolate DKF-1, (B) SV3 isolate HPA5, (C) SV6 isolate HPA2 and (D) SV14 isolate HPA32. Dark bars indicated killing in the presence IgG sufficient normal human serum. White bars indicate killing from IgG depleted human serum. Numbering on the x-axis refers to volunteer serum used.

3.2.6 MBL ELISA data and killing assay with MBL depleted serum

All sera were found to be above the cut-off for normal MBL levels (< 190 μg/L) (Crosdale *et al.* 2000) as defined by the commercial ELISA kit as seen in Table 3.1. The highest MBL concentration was seen in VxF1 (2673 ng/ml) and the lowest concentration VxF9 (348 ng/ml). MBL was depleted from both seronegative sera (VxF1 and VxF9) and used in an attack assay against HPA5 following confirmation of depletion via ELISA (Figure 3.6). Due to the possibility of complement activation occurring as a result of pre-decoration of *Ureaplasma* by pig lectins present in the growth media, HPA5 (SV3) was adapted to grow in USM absent of pig serum, but was supplemented with 1% HI-MBL depleted serum from either VxF1 or VxF9. By passaging HPA5 three times in the modified USM, the isolate was completely free of all pig lectins and human MBL. When the "adapted" HPA5 isolate was attacked with the respective MBL depleted serum from either VxF1 or VxF9 no decrease in killing was seen in neither the MBL depleted sera or that of HPA5 adapted to growth in modified USM without pig serum and supplemented with 1% HI-MBL depleted sera.

Serum	μg/L
VxF1	2673
VxF2	441
VxF3	2530
VxF4	1979
VxF5	1386
VxF6	862
VxF7	850

Serum	μg/L
VxF8	1260
VxF9	348
VxM10	585
VxM11	826
VxM12	634
VxF1 dep	48
VxF9 dep	49

Table 3.1. Serum MBL levels from the 12 volunteer sera. The data also includes MBL depleted seronegative sera from VxF1 & VxF9 as determined by ELISA. "dep" refers to MBL depleted serum.

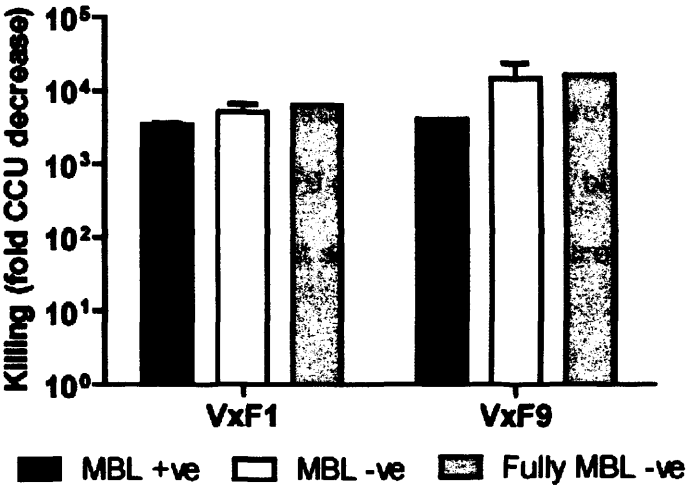


Figure 3.6. Effects of MBL depleted sera upon killing of *U. parvum* SV3 isolate HPA5. Two seronegative sera (VxF1 & VxF9) were depleted of MBL and assayed as normal. Black bars represent normal human serum (MBL sufficient). White bars represent MBL depleted human serum. Grey bars represent HPA5 grown in modified MBL without pig serum, but supplemented with 1% HI-MBL depleted sera and subsequently attacked.

3.2.7 Confirmation of complement mediated killing of SV3 using C6 deficient serum

To fully confirm the killing of SV3 was complement mediated, the serum from a naturally C6 deficient patient was used. Figure 3.7 shows the killing capacity of C6 deficient serum against that of the seronegative VxF9. As consistent with previous results VxF9 did not kill DKF-1, but reduced HPA5 by 10000-fold whereas the C6 deficient serum did not reduce the CCU of neither DKF-1 nor HPA5.

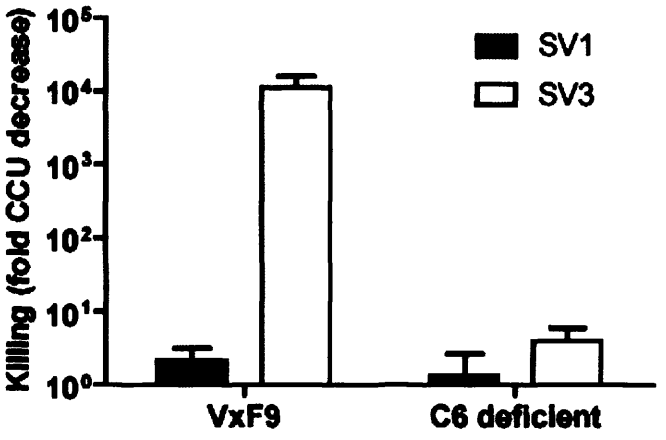


Figure 3.7. Killing capacity of C6 deficient serum against SV1 isolate DKF-1 (black bars) and SV3 isolate HPA5 (white bars) versus the C6 sufficient seronegative VxF9 serum.

3.2.8 CH_{50} assay to determine concentration of anti-C1q function blocking antibody.

The data from Figure 3.8 shows the CH50 assay for the determination of optimal concentration of the function blocking anti-C1q antibody required to block the classical pathway (clone 2204, (Roos *et al.* 2001). Figure 3.8a shows that normal human serum could lyse sensitised sheep red blood cells at a dilution of 1/126 (the dilution at which the red control line intersects the 50 % lysis line) where as in the presence of 140 µg/ml of blocking antibody a greater amount of serum was required to achieve 50 % lysis (less than 1/67 serum), suggesting the successful blocking of the classical pathway. As the antibody was received in a PBS buffer, which is known to chelate cations and therefore inhibit the classical pathway, equal volumes of PBS were run to see if any non-specific inhibition occurred. There was a small degree of inhibition of the classical pathway by the PBS which inhibited activation to a similar extent as the 70 µg/ml concentration of blocking antibody. To reduce volume of antibody used, and therefore reduce any potential side affects from the PBS the antibody was concentrated to a 5 mg/ml stock. The assay was then repeated with the same volumes of sample added (7 µl and 14 µl) along with control PBS, but this time the PBS was obtained from the flow through of the column (Figure 3.8b). These results showed much greater inhibition with 700 µg/ml of blocking antibody completely inhibiting activation even at a 1/20 dilution of serum. Using a volume of 7 µl (giving a final concentration of 350 µg/ml) gave inhibition at a dilution of (1/56), which was significantly different to that from the PBS flow through examined (1/100) as well as the control serum (1/152).

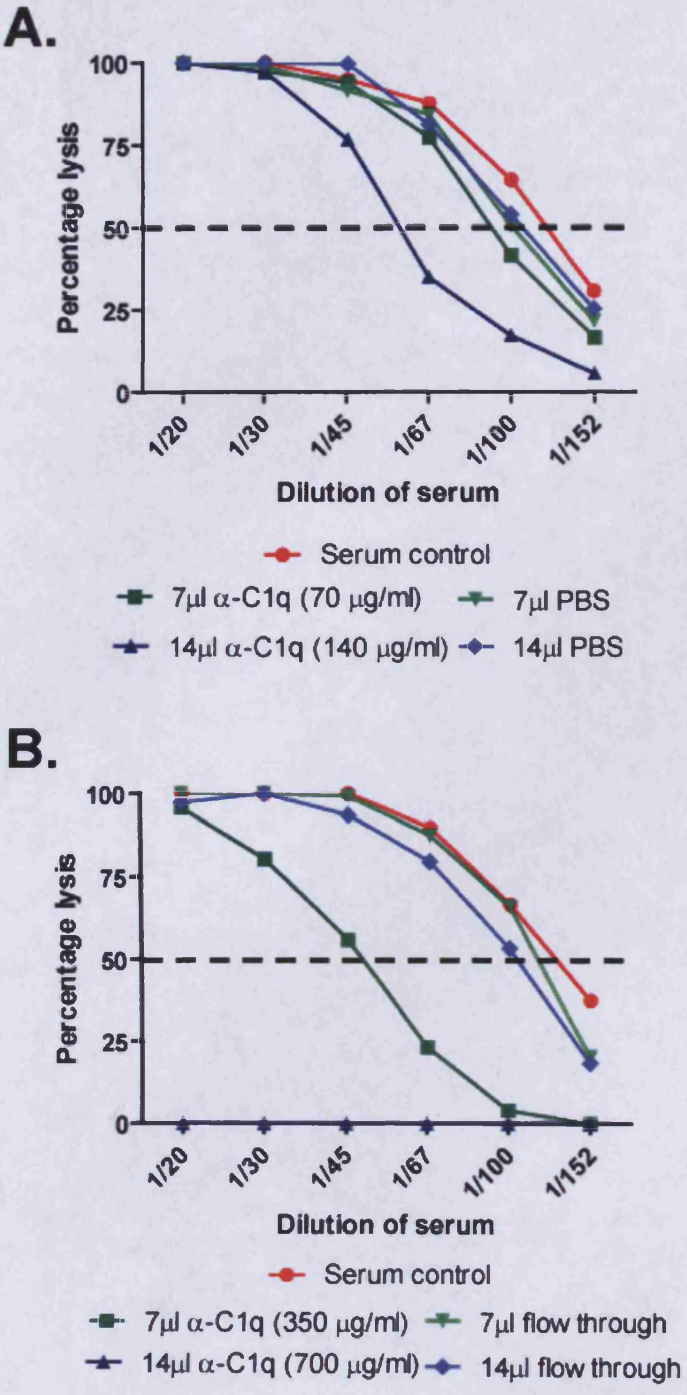


Figure 3.8. CH_{50} data showing the blocking of the classical pathway using an anti-C1q antibody. (A) Data representing the neat 1 mg/ml antibody received and (B) representing data from the concentrated 5 mg/ml antibody with results read at 15 min after the start of the assay. The dashed line represents 50% lysis (CH_{50}). Note the 700 µg/ml data line directly on the x-axis in graph B.

3.2.9 Complete blocking of the classical pathway using a function blocking anti-C1q globular head antibody

Data presented in Figure 3.9 shows the effect of the function blocking anti-C1q antibody upon the killing of SV3 by the seronegative sera from VxF1 and VxF9. Further proof of classical pathway blocking, backing up the CH50 data, was demonstrated by blocking the antibody dependent killing of SV1 from VxF7 serum. NHS from VxF1 and VxF9 SV3 killed SV3 by 10^3 – 10^4-fold, whereas in the presence of C1q blocking antibody this killing was completely removed. An relevant isotype-matched control antibody of the same concentration showed little effect upon the killing of *Ureaplasma* demonstrating this effect was not due to any differences in protein concentration.

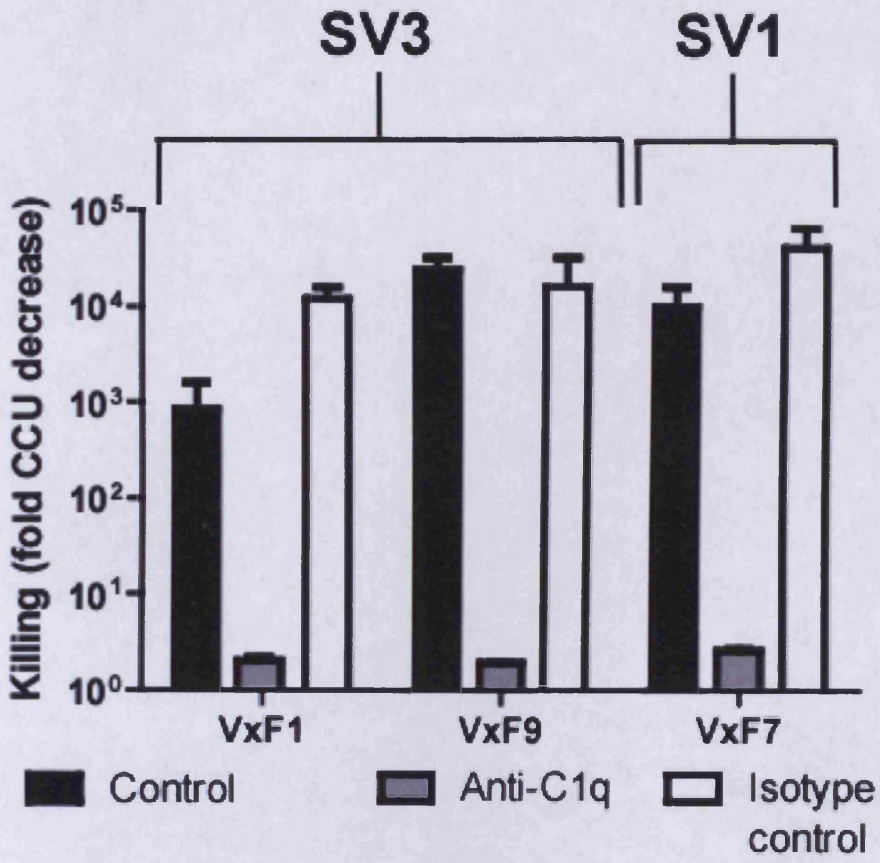


Figure 3.9. Effects of anti-C1q antibody upon killing of SV3 by seronegative sera (VxF1 and VxF9) and SV1 by seropositive serum (VxF7). Black bars represent the killing in normal conditions whereas grey bars, which are absent due to no killing at all, represent serum pre-treated with the anti-C1q antibody. White bars represent the killing in the presence of an isotype control antibody to show any non-specific protein effect.

3.2.10 Killing of U. parvum with human serum following depletion of IgG using a protein G column

Following the protein A depletion experiments it was noted that the protein A column previously used to deplete IgG would only deplete subclasses 1, 2 and 4 whereas a protein G column was able to deplete all four IgG subclasses. The results from these data (Figure 3.10) showed that again the killing for SV1 was removed from those sera with previously high killing capacity following the removal of IgG, but when depleted sera was examined against SV3 the results were variable. Killing was removed for 3 out of the 8 sera with 3 out of the 8 sera retaining full killing and 2 out of the 8 losing 2 logs worth of killing. Interestingly the killing was retained in one of the previous seronegative sera (VxF9), but removed in the second seronegative sera (VxF1).

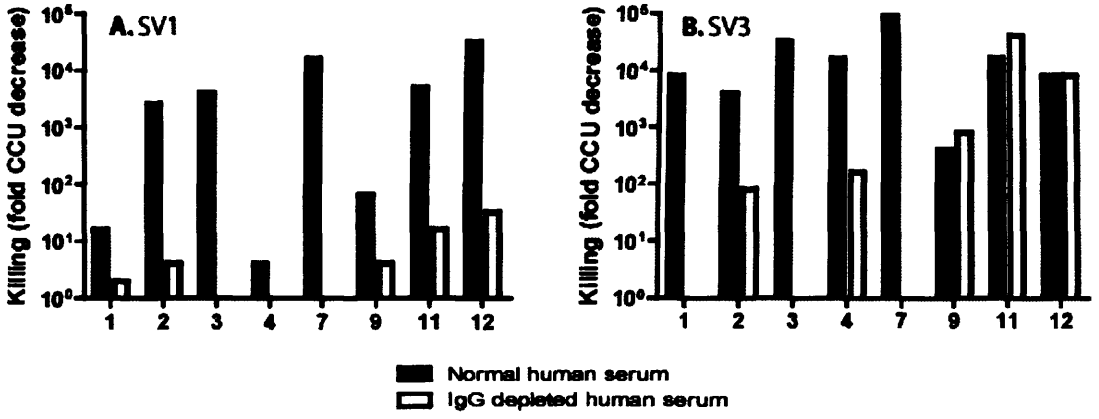


Figure 3.10. Effects of various sera depleted of IgG, using a protein G sepharose column, on the killing capacity of serovars 1 and 3 of *U. parvum*. (A) SV1 isolate DKF-1, (B) SV3 isolate HPA5. Dark bars indicated killing in the presence IgG sufficient normal human serum. White bars indicate killing from IgG depleted human serum. Numbering on the x-axis refers to volunteer serum used.

3.2.11 Intra-serovar variation

To rule out the possibility of any bias in results due to using a single prototype serovar strain, a selection of clinical isolates from each serovar were examined (Figure 3.11a-d). Serum from the seronegative VxF1 reduced the CCU of all five SV1 isolates by greater than 10-fold (Figure 3.11a). VxF4 serum also had poor killing activity for isolates DKF1, UWHQ3 and UHWO10 but greater killing of 300 and 30-fold was seen in isolates HPA11 and HPA18, respectively. The highly SV1-cidal VxF3 and VxM12 reduced the CCU of all isolates by at least 1000-fold with exception to VxF3 which reduced the CCU of UHWQ3 by 333-fold and VxM12 which reduced the CCU of UHWO10 by 110-fold. With only two exceptions, all sera reduced SV3 growth by 100-fold CCU and most reduced the CCU of SV3 isolates by > 1000-fold (Figure 3.11b). Exceptions to this rule was VxF1 serum which reduced HPA1, HPA6 and HPA29 by between 100 to 1000-fold also VxF6 serum which reduced HPA29 by 550-fold. HPA1 and HPA29 were reduced by VxF7 serum by only 48 and 80 fold, respectively. Fewer SV6 and SV14 isolates were available for intra-serovar variation analysis (Figure 3.11c-d). VxF1 and VxF4 consistently failed to reduce the CCU of both SV6 isolates by anymore than 10-fold. Reduction in CCU between the SV14 representative HPA2 and HPA23 with VxF3 serum was similar with a reduction of over 430-fold. HPA23 was less susceptible than HPA2 with a reduction of 45-fold relative to 880-fold, respectively. No killing was seen for either SV14 isolates with VxF1 serum. Similarly VxF6 killed HPA10 and HPA32 by equal or less than 10-fold. The highly SV14-cidal sera reduced the CCU of both isolates by between 100 and 1000-fold.

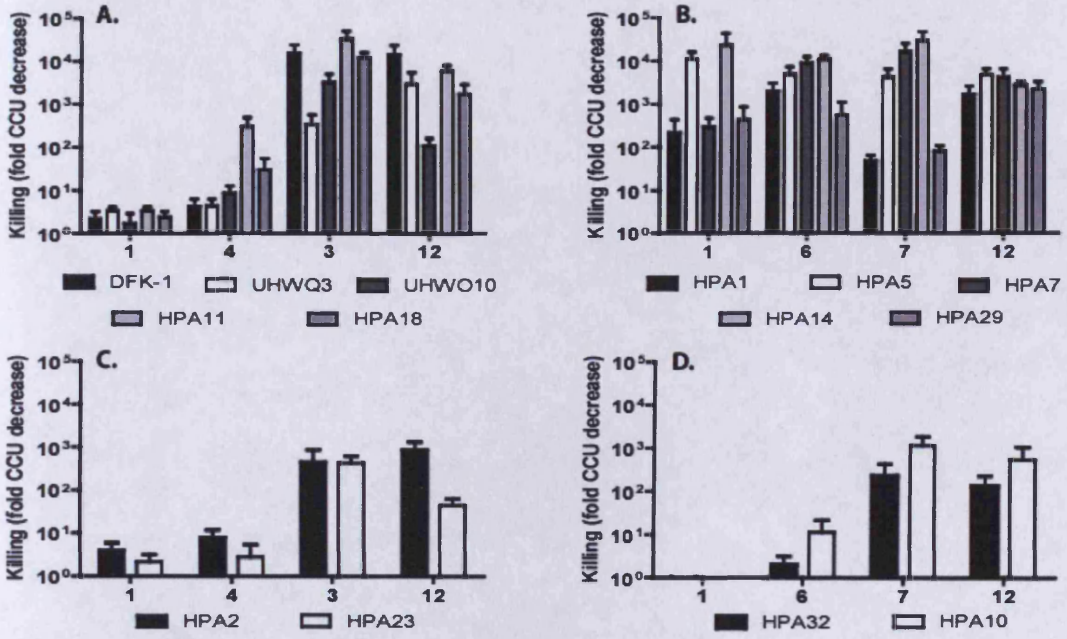


Figure 3.11. Differences in killing capacity of human serum against *U. parvum* isolates of the same serovar. (A) Five SV1 isolates, (B) Five SV3 isolates, (C) two SV6 isolates and (D) two SV14 isolate. Numbering on the x-axis refers to volunteer serum used.

3.2.12 Western blots of intra-serovar variation

For those isolates examined for intra-serovar variation killing were subsequently examined for intra-serovar variation differences in banding pattern. Figure 3.12a,b shows immunoblots of five SV1, five SV3, two SV6 and two SV14 isolates probed with seronegative VxF1 serum as well as seropositive VxF12 serum, respectively. The seronegative serum was consistent in producing negative blots as seen in the first series of immunoblots (Figure 3.12a), whereas conserved large bands of just less than 220 kDa as well as one of around 120 kDa were present in all isolates from all serovars examined using VxM12 serum. A band of approximately 85 kDa was also present in all isolates with exception to the SV1 isolate, UHWO10. Band conservation and intensities were less apparent in lower molecular weight proteins. A number of bands displayed greater intensities between isolates, albeit probed with same serum. A 60 kDa band of the SV1 isolate, DKF-1, as well as two SV3 isolates, HPA1 and HPA14, were noticed. A number of unique bands were seen both at a serovar, but additionally at the intra-serovar level. For example a 90-95 kDa band was seen in the SV3 isolate HPA1 which was absent in all remaining SV3 isolates. HPA14 displayed a lower molecular weight band of less than 60 kDa which was absent from all remaining SV3 isolates. Figure 3.12c shows the banding variation when probed with VxF7 serum. There were a number of bands conserved across the isolates examined, but a few bands showed varying degrees of intensity. The SV1 isolates HPA1, HPA7, HPA14 and SV14 isolate HPA10 produced intense bands of just greater than 60 kDa, but were not present in remaining wells. Additionally the high molecular weight band

seen previously in the SV14 isolate (HPA32) (Figure 3.4) was absent in comparison SV14 isolate HPA10. Immunoblots probed using VxF6 serum again produced a limited number of bands. The low molecular weight band for HPA5 was present again, but absent from all other SV3 isolates. Each isolate contained a single band of unique size of approximately 80 kDa, 50 kDa and 70 kDa for isolates HPA1, HPA14 and HPA29, respectively. No clear band was seen for HPA7. A weak intensity band of greater than 120 kDa band was present for HPA10 whereas no band was present for HPA32. Blots probed with serum from VxF4 produced faint bands of around 50 kDa as previously seen in Figure 3.4. Banding pattern for blots probed with VxF3 serum was similar to those of VxF7 albeit against SV3 and SV14 isolates.

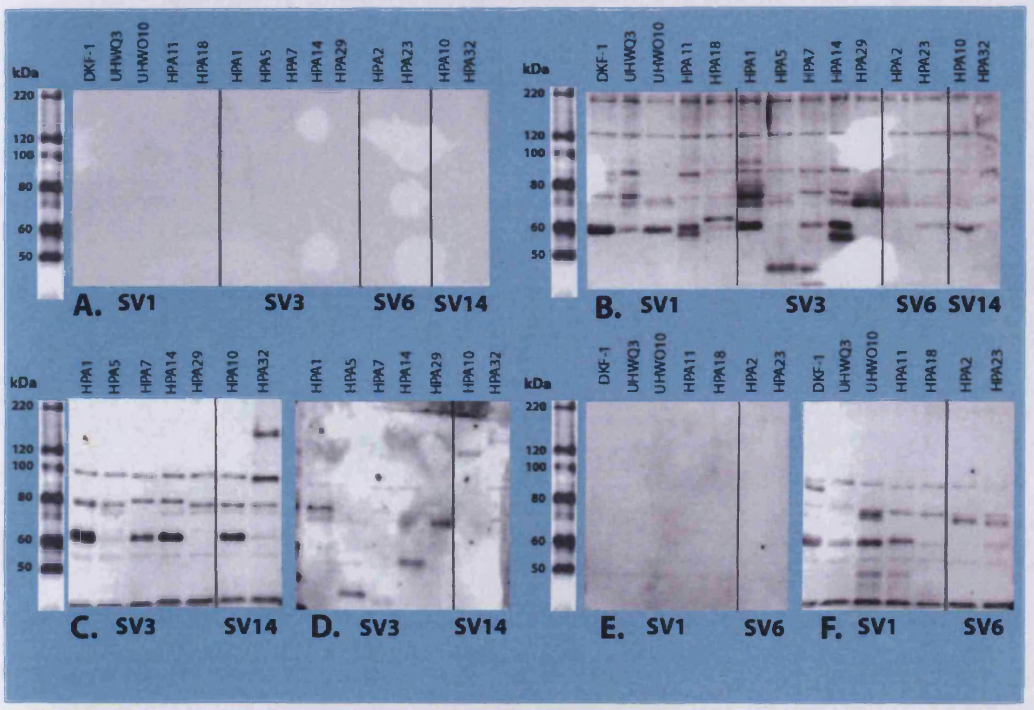


Figure 3.12. Immuno blots showing intra-serovar variations of banding patterns when probed with varying human serum. Blot A. contains 5 SV1, 5 SV3, 2 SV6 and 2 SV14 isolates probed with VxF1 serum. Blot B contains 5 SV1, 5 SV3, 2 SV6 and 2 SV14 isolates probed with VxM12 serum. Blot C contains 5 SV1 and 2 SV14 isolates probed with VxF7. Blot D contains 5 SV1 and 2 SV14 isolates probed with VxF6 serum. Blot E contains 5 SV3 and 2 SV6 isolates probed with VxF4 serum. Blot F contains 5 SV3 and 2 SV6 isolates probed with VxF3 serum.

3.3 Discussion

3.3.1 Analysis of the complement killing assay

The killing assay described in this study was successfully used to firstly, identify serum from a group of volunteers with bacteriocidal activity against representative serovars of *U. parvum* and secondly to elucidate which complement activation pathways were essential for killing of *U. parvum* and finally to determine the serovar specific variations.

Two previous studies examined antibody-mediated killing of Ureaplasma, using similar methods to those described here; however, the focus of these studies was to determine serological status of patient sera, rather than determining the mechanism or sensitivity to complement (Purcell *et al.* 1966; Lin *et al.* 1972). The metabolic inhibition assay described by Purcell *et al.*, in 1966 used guinea pig serum (GPS) as a source of complement to determine the titre of antibody from HI-NHS or hyper-immune serum. *Ureaplasma* cultures were incubated with HI-NHS and GPS in a urea-containing media at 34 °C until colour change was observed in positive control cultures which contained no antiserum. This methodology was successful in categorising *Ureaplasma* into to their respective serovars, but did not take into account the potential effects of the alternative or lectin pathways of GPS in addition to having low levels of sensitivity (Lin *et al.* 1972). Lin *et al.*'s complement-dependent mycoplasmacidal test was a similar assay as that developed by Purcell *et al.*, but differed by the omission of urea containing media during the complement attack stage (Lin *et al.* 1972). The omission of urea was due to

the *Ureaplasma* growth source acting as a dominant nucleophile, abrogating the efficient attachment of C3b and C4b to bacteria (Lin and Kass, 1970). A difference between Lin's assay and the one described here involved the removal of all serum before resuspending in USM to maximise post-attack *Ureaplasma* growth whereas in contrast to Lin *et al.*, who stopped the complement attack by addition of urea containing *Ureaplasma* media. Additionally Lin *et al.,* used low number of *Ureaplasma* for their assay, 10 CCU, whereas my complement attack assay used greater than 10^3 CCU so that those sera with high killing capacity could be distinguished from those with low killing. The method described here additionally differed from these other studies by using modifications to the preparation of the serum (specific calcium chelation or MBL-depletion prior to attack) to determine the contribution of the alternative and lectin pathways upon killing.

3.3.2 Variations in SV1, 3, 6 and 14 killing by all complement pathways and alternative pathway only

Prototype strains for each *U. parvum* serovar were thoroughly characterised for their susceptibility to human serum in the presence of all three complement activation pathways (Figure 3.3). All sera killed SV3, but only a proportion of sera killed the other SVs, although those that killed SV14 seemed to be bacteriocidal for all SV. Serum from VxF2, VxF3, VxF7, VxM11 and VxM12 consistently killed all SV to a greater degree than the remaining seven sera, with exception to VxF3 which had a decreased killing capacity for HPA32 (SV14). To address the role of the alternative pathway in bacteriocidal

activity, a well established method of calcium, but not magnesium, chelation was utilised leaving only the alternative pathway intact. Surprisingly the alternative pathway had little effect upon killing of any *U. parvum* serovar. The alternative pathway is primarily regarded as an important bacteriocidal pathway, due to the failure of bacteria to express sialic acid on their glycoproteins and therefore fail to recruit soluble serum regulator factor H. This is the mechanism that protects the host's cells from significant damage when in proximity to high levels of complement activation. In fact, recruitment of factor H by particular bacterial cell wall proteins, is the often attributed as the mechanism of increased virulence for particularly pathogenic bacterial strains. Examples include *Streptococcus pneumoniae* recruits fH from its surroundings as an evasion mechanism to protect against the bactericidal nature of human serum (Lambris *et al.* 2008; Rooijakkers and van Strijp, 2007). It can be speculated that fH recruitment may be a mechanism by which *U. parvum* is resistant to alternative pathway killing.

3.3.3 Serological status of the 12 volunteers

To determine the serological status of the twelve sera, western blots containing whole-cell lystaes of *Ureaplasma* antigen were probed with volunteer serum (Figure 3.4). From the twelve sera examined ten showed some degree of reactivity, whereas two were fully seronegative for IgG. For these seronegative blots they were subsequently probed with an anti-IgG/M/A antibody to look for any immunoglobulin binding (as IgM is a potent complement activator that is not bound by protein A or protein G), but remained negative. To ensure results were not false negative results, the

blots were subsequently probed with seropositive serum where expected banding was confirmed, therefore ruling out poor transfer. These seronegative sera failed to kill DKF-1, HPA2 and HPA32, but not HPA5 initially suggesting antibody dependent killing for serovars 1, 6 and 14, but antibody independent killing for SV3. For those which were seropositive, a variety of different and conserved banding patterns of varying intensities were seen within blots probed with different sera. High levels of killing were associated with the presence of multiple bands of higher binding intensity. For example, the high level of DKF-1 killing seen with VxF2, VxF3, VxF7 VxM11 an VxM12 correlated with intense binding of bands approximately 62-65 kDa. Further support for the presence of specific bands and killing was seen for SV14 isolate HPA32. Killing was associated with high molecular weight bands or greater than 120 kDa as seen in blots probed with serum form VxF7, VxM11 and VxM12. An outlier to this rule was the serum from VxF2 which showed killing of around a 100-fold, but no large molecular weight band was present. An interesting observation was the banding profiles of blots probed with the sera from VxF2, VxF3, VxF8 and VxM10 as the banding patterns produced by these sera against the SV1 isolate DKF-1 was similar to that of the monoclonal antibody 8B5.2. described by Watson *et al.* which detected bands of 85, 74 and 58 kDa. This suggested multiple bands may represent alternative forms of the same protein or closely related proteins (Watson *et al.* 1990). In a number of cases, such as with VxF4, bands were present, but the degree of killing was minimal. This may represent an antibody response which was raised against internal antigens which would be hidden by intact viable cells. Of course activation of complement can only

occur following binding of the antibody to antigens present upon the surface of bacteria, and as western blot does not distinguish between surface and internal antigens this could account for this finding. Attempts were made to isolate external antigens by using a Triton X114 methodology but failed to produce a large enough quantity of assessable protein. The most likely explanation for this would be due to the low volume of culture used (5 ml). Previous studies have used TX-114 to isolate *Ureaplasma* lipoproteins, but neither of these publications comment on the size of initial culture used (Shimizu *et al.* 2008; Peltier *et al.* 2007).

It can be hypothesised that the primary external candidate protein recognised by the antibody response would be the multiple banded antigen (MBA) based on previous existing work (Watson *et al.* 1990; Zheng *et al.* 1995; Kong *et al.* 2000; Monecke *et al.* 2003; Zimmerman *et al.* 2009). Watson *et al.*, proposed that the MBA of *Ureaplasma* was the predominant antigen recognised by the immune system using sera from *Ureaplasma* positive patients who were attending an infertility clinic (Watson *et al.* 1990). In sera from these patients, the investigators noticed, as observed in this study, there were serovar specific as well as cross-reactive epitopes. In addition, a monoclonal antibody (3B1.5) raised against SV3 produced a laddering effect upon blotting. This was the basis for the naming of the MBA. The size variation seen in the MBA antigen was a result of altering the number of repeat units in the 3' end of the protein and proposed as an antibody evasion mechanism (Zheng *et al.* 1995). The expected size of the MBA are around 70 kDa and consistent with a number of bands present within this study.

Recent work has suggested a more radical method of altering the antigenicity of the surface by full removal of the MBA protein. Monecke *et al.* first described MBA negative variants following selection of strains which were unable to adhere to HeLa cells (Monecke *et al.* 2003), but Zimmerman *et al.* were first to propose a mechanism for this removal of antigen. These authors suggested that the *mba* gene and the adjacent UU376 gene (encoding a 26.9 kDa protein named *Ureaplasma* phase-variable membrane protein 376 (Upvmp376)) are separated by an inverted promoter, such that only one gene can be expressed for each bacterium. They suggested that within a mixed population of *Ureaplasma* a proportion of variants would express MBA whereas another proportion would express Upvmp376. By selection pressures imposed by antibodies to either of these proteins, selection would select for the survival of the bacterial sub-group expressing the less dominant immunoepitope. Blots for VxF3 identified a low molecular weight protein, but the potential identity in relation to the low molecular weight Upvmp376 protein is unknown. Only N-terminal sequencing, or post-decay analysis of mass-spectroscopy of purified bands would identify if the immunoreactive proteins on my blots were MBA (higher) or Upvmp376 (lower bands). Future studies would focus on protein sequencing methods, such as n-terminal sequencing, to determine the identity of the dominant immunoreactive epitopes or scaling up of *Ureaplasma* cultures for use in triton X114 lipoprotein isolations.

3.3.4 The role of the classical pathway as assessed by IgG depleted serum (using a protein A column) and western blot

As the alternative pathway had little effect upon killing, three sera with low killing and three sera with high killing were depleted of IgG using a protein A sepharose column with depleted sera then used in the standard assay (Figure 3.5). The depletion of IgG from the sera resulted in a loss of the majority of killing of DKF-1 (SV1), HPA2 (SV6) and HPA32 (SV14) relative to non-depleted bacteriocidal serum. HPA5 (SV3), which was killed by all sera tested, retained most of the bacteriocidal activity following depletion. By using the same batch of protein A depleted serum for all serovars, the presence of reduced killing of the non-SV3 isolates served as an internal control to rule out the result being due to either dilution or activity loss caused by the depletion process.

3.3.5 The role of the lectin pathway

As mentioned from the twelve sera studied, two of volunteers were seronegative for IgG by western blot (VxF1 and VxF9). Although via IgG depletion experiments antibody was shown to be essential for the killing of DKF-1, HPA2 and HPA32 these seronegative sera were still able to kill HPA5 to the same degree as those which were seropositive. One possibility included complement activation occurring via the lectin pathway. Depleting the MBL from the two seronegative sera and applying this serum to the killing assay has shown that killing of HPA5 still occurred (Figure 3.6), but this was not to suggest that MBL opsonisation does not occur. Benstein *et al.*,

demonstrated the ability of rabbit and human MBL to bind *Ureaplasma* (Benstein *et al.* 2004), although they did not demonstrate functional killing (or complement activation) as a result, nor provide evidence for non-specific guinea pig anti-rabbit MBL binding. An additional finding from this group was the ability of horse MBL from serum components of the growth media to bind *Ureaplasma*. This was not a surprising finding as *Ureaplasma* had previously been shown to contain mannose within the outer-membranes (Whitescarver *et al.* 1975; Smith, 1985). To rule out the possibility of pig MBL from within the USM binding and eliciting killing in the MBL depleted sera experiments, HPA5 cultures were grown in USM without pig serum and supplemented with 1% heat-inactivated MBL depleted serum from either VxF1 or VxF9 prior to complement attack. This ensured no decoration of *Ureaplasma* with MBL during the growth of target bacteria, which would remain bound prior to serum exposure. This did not result in any reduction of killing further supporting the inability of MBL binding to result in terminal complex formation.

Although classical, alternative and MBL in the lectin pathway were ruled out as the mechanisms of HPA5 killing, the inability of C6 deficient serum to mediate killing proved that terminal complex formation was essential (Figure 3.7).

3.3.6 The role of the classical pathway as assessed by blocking of C1q function and IgG depletion using a protein G column

To this point the mechanism of SV3 killing was unknown with all three activation pathways being ruled out. To fully rule out the classical pathway a function blocking anti-C1q antibody was used to fully block the classical pathway with proof of principle demonstrated by inhibition in a CH50 assay (Figure 3.8).

The findings from the C1q function blocking experiments proved the essential role for the classical pathway in SV3 killing and therefore contradicted the results from IgG depletion data, which suggested killing of SV3 seemed to be IgG independent, as well as questioning the data from the western blots, which suggested that VxF1 and VxF9 were seronegative for both IgG and IgM.

The most likely explanation for the contradiction of the IgG depletion data would be due to a methodological error. By looking back at the literature regarding protein A columns, it is now clear that a protein A only depletes IgG subclasses 1, 2 and 4, therefore leaving the potent complement activator IgG3 within the serum, whereas a protein G column would deplete all subclasses (IgG1, 2, 3 and 4). For this reason a selection of sera were depleted of total IgG by this methodology. The results from these data were mixed, with half of the sera examined retaining killing post depletion, including one of the

previous seronegatives (VxF9), whereas the killing was removed in the remaining half, including the second seronegative sera (VxF1) (Figure 3.10).

These results from the protein G data (Figure 3.10) additionally contradict the results from the western blot data (Figure 2.4) by suggesting that the previously designated seronegative serum from VxF9 did contain anti-*Ureaplasma* IgG. A possible reason for the absence of reactive bands upon western blot probed with serum from VxF9 include the possibility that the IgG3 antibodies recognise an conformational dependent epitope, therefore when the protein is denatured and separated on a western blot, the binding is lost. A second possibility may be due to the low sensitivity of the western blot technique. IgG3 makes up only 7% of total IgG and is found at a circulating concentration of 0.8 mg/ml whereas the most abundant IgG, IgG1, makes up around 65 % of circulating IgG and exists at serum concentrations of ten times that of IgG3 (8 mg/ml).

As mentioned the results from the protein G depletion experiment accounted for the removal of killing of sera from VxF1, 3, 7 and partial removal of killing for VxF2 and 4, but could not explain the retention of killing for sera from VxF9, VxM11 and VxM12. This killing in the previously designated seronegative sera of VxF9 could be attributed to an undetectable IgM response by western blot. Unfortunately a IgM specific anti-μ antibody was not used to look for this antibody in IgG positive VxM11 and VxM12 sera as the polyvalant anti-IgM/G/A antibody used to confirm the absence of IgM response in the previously designated seronegative sera detected IgM as well

IgG and IgA. For this reason future work should use a IgM specific anti-μ antibody to rule out its role.

Although this methodological error initially suggested an IgG independent killing mechanism for SV3, it presented interesting data on what subclass of IgG is raised to this serovar. From the combination of the protein A and protein G depletion data we can speculate an IgG response raised to SV1, 6 and 14 would be predominantly skewed towards the IgG1 subclass as IgG4 does not activate complement and IgG2 has relatively poor activating properties. In contrast the removal of all IgG results in the removal of SV3 killing in a number of sera suggesting a bias towards an IgG3 response.

The data from the anti-C1q function blocking experiment also confirmed previous results regarding the lack of killing from the alternative and lectin pathways. If these pathways were functional, killing would be present following the blocking of C1q and therefore the classical pathway.

This evidence for the essential role of IgG in complement mediated killing of *Ureaplasma* supports the association between *Ureaplasma* infection and hypogammaglobulinemic patients who have an inherent inability to produce antibodies and therefore receive intravenous IgG on a regular basis. Furr *et al.*, described their observations of the occurrence of *Mycoplasma* and *Ureaplasma* infections in hypogammaglobulinaemic patients over a period of 20 years (Furr *et al.* 1994). They found that from 91 hypogammaglobulinaemic patients 21 (23%) had septic arthritis in the

absence of either aerobic or anaerobic bacteria, with 8 of these (38 %) positive for *Mollicutes*, 4 of which were *Ureaplasma*. In one case *Ureaplasma* were isolated from four independent joints (shoulder, elbow, wrist, knee).

3.3.7 Variations in killing at the intraserovar level

Results from the four *U. parvum* serovar representatives suggested that SV3 was the most serum sensitive isolate identified in this study. This finding was counter-intuitive to what would be expected, due to the most sensitive serovar being the most commonly isolated as shown by my results in chapter 5 as well as other investigators (Nelson *et al.* 1998; Glass *et al.* 2000). To rule out that this was not a strain specific finding of HPA5, the variation of killing at the intra-serovar level was examined for all serovars against a selection of high and low bacteriocidal sera (as judged against the prototype strains; Figure 3.11). Data from VxF1 consistently showed an inability to kill five different SV1, two SV6 and two SV14 strains, whereas killing was present against all five strains of SV3, albeit to varying amounts. Western blot analysis again failed to show any banding for any of the 14 different strains examined (Figure 3.12). A similar picture was seen for the low killing VxF4 which had reduced killing to three out of five SV1 and all SV6 isolates. VxF6 had low killing against both SV14 strains, but killed all SV3 strains. All the high killers, VxF3, VxF7 and VxM12 killed all strains in a similar manor to that of the prototype strains used. An interesting exception to this rule was the 1624-fold reduced ability, relative to other SV1 isolates, of VxM12 serum to kill UHWO10. This correlated with the absence of an approx 85 kDa band which was present in all remaining SV1 isolates (asterix in Figure 3.12) and of the same size as the

SV1 specific 85 kDa band described by Watson *et al.* from *Ureaplasma* infected patients (Watson *et al.* 1990). As discussed later in Chapter 5 this isolate was additionally resistant to macrolide antibiotics, but it is unlikely that there is any link between the mechanism of macrolide resistance and this finding. Further discrepancies were seen in the reduced killing capacity of serum form VxF7 against SV3 strains HPA1 and HPA14.

3.3.8 Previous findings on complement activation and Ureaplasma

The results described in this study contradict those found previously by Webster *et al.*, who concluded that complement alone had little effect upon the killing of *Ureaplasma* (Webster *et al.* 1988). Two possible reasons can account for these discrepancies. Firstly Webster *et al.*, added human serum directly into actively growing cultures in *Ureaplasma* media. As described in section X Urea is a nucleophile and would abrogate the efficient attachment of C3b and C4b to bacteria, hence the reason for this assay in which *U. parvum* were transiently exposed to human serum in the absence of urea. Secondly evidence present here has shown that the presence of antibody is essential for the killing of *U. parvum* serovars, but the presence of antibodies alone may not be enough to elicit killing if the dominant antigenic targets are internal. Webster *et al.*, demonstrated low concentration of antibodies to the SV5 *U. urealyticum* isolate used in the experiments, but these, as discussed, may have been against internal antigens and be ineffective.

3.3.9 Relevance to the clinical picture

The work presented in this chapter only takes into consideration a very small component of the complement system and its role in modulating the host immune system. This work has shown that SV3 is particularly susceptible to MAC-mediated killing, but MAC killing only represents a small fraction of the role of complement in the clearance of infection and potentially of serovars 1, 6 and 14. The complement system bridges the gap between the innate and cellular immune response so that even in the absence of MAC-mediated killing, complement activation can result in the release of anaphlatoxins (C3a and C5a) which will recruit phagocytes to the site of infection and aid in clearing infection. Clearance of *Ureaplasma* can also occur as a result of opsonisation with either antibodies, C3b or MBL. As previously mentioned Benstien *et al.*, demonstrated MBL can bind *Ureaplasma*, but, as my data suggests, does not lead to MAC mediated killing (Benstein *et al.* 2004). For this reason we cannot rule out the possibility that MBL may opsonise *Ureaplasma* therefore allowing clearance by macrophages by recognition through collectin receptors such as calrecticulin in complex with CD91.

As *Ureaplasma* are primarily found on mucosal surfaces, such as the lung, an understanding of the complement system at this area is needed. Complement components C1r, C1s, C4, C3, C5, C6, C7, C8, C9, fB, fH and fI have all been shown to be produced within the lung by either alveolar type II epithelial cells or alveolar macrophages (Rothman *et al.* 1989; Strunk *et al.* 1988). Later work by Rothman *et al.*, showed that expression of complement components could be regulated by cytokines in a dose dependent manner with IL-1β

producing a two-fold increase in C3, but a 50 % decrease in C5 (Rothman *et al.* 1990). Using clinical BALF samples Watford *et al.*, confirmed the earlier *in vitro* cell line data along with functional studies (Watford *et al.* 2000). These data present evidence for a local source of complement within the lung which *Ureaplasma* would have to overcome. The complement system in the context of CLD may be important as clearance by MAC mediated lysis could potentially reduce the number of neutrophils' entering the lung and releasing tissue damaging proteases.

Although classical complement components are found locally within the lung the predominant activators, IgG and IgM, are in significantly lower abundance relative to the imunoglobulins of the IgA class. Unlike IgG and IgM, IgA does not have the capacity to activate complement, but is thought to inhibit the adherence of invading pathogens and therefore prevent further invasion (Underdown and Schiff, 1986). IgA must pose a significant hurdle for *Ureaplasma* to overcome as all serovars have been found to contain human-specific IgA proteases (Robertson *et al.* 1984), but has little role in complement mediated killing. Therefore an IgG and IgM response may play more of a role in the prevention of further systemic infection and confining the bacteria to mucosal surfaces. Clinical evidence for this was discussed earlier with hypogammaglobulinaemic patients being prone to invasive disease caused by *Ureaplasma* (Watson *et al.* 1990; Furr *et al.* 1994).

3.4 Summary

This chapter has firstly described an assay to quantify the bactericidal effects of human serum upon *Ureaplasma*. This methodology was manipulated in a way to pin-point the key complement activation pathways involved in *U. parvum* killing. The key findings identified that the classical pathway as the predominant pathway involved in killing all serovars. Killing of SV1, 6 and 14 was dependent upon the presence of IgG subclasses 1, 2 and 4 whereas killing of SV3 was dependent, in half of the cases, on IgG3. It can be speculated that the remaining sera which killed SV3 an anti-SV3 IgM was present. The alternative and lectin pathways were ruled out due to negligible killing in the absence of Ca^{2+} and MBL, respectively, as well as lack of killing in the absence of the classical pathway as demonstrated by the anti-C1q function blocking antibody.

Chapter 4

The role of antenatal infection in neonatal patients at risk of developing CLD

4.1 Introduction

Chronic lung disease of prematurity (CLD) remains a cause of significant morbidity and mortality in preterm infants. Although it's risk factors have been defined for many years, its exact aetiology remains unknown. A strong link between microbial infections, with particular reference to bacteria within the genus *Ureaplasma*, has been strongly debated

In the first results chapter I examined the body's innate ability to prevent infections caused by *Ureaplasma* via complement mediated killing. This results chapter will therefore focus on the consequences if the balance between immunity and infection tips in favour of the infecting organism. I aim to address the question of the role of bacterial infection, with particular focus on *Ureaplasma* spp., on the development of CLD in a cohort of 192 babies. By sequencing 16S rRNA products, the identity of infecting organism can be matched to clinical outcome. Data from co-workers on the inflammatory response in these samples using the markers IL-6 and IL-8 will be used to compare the presence of bacteria with an immune response.

4.2 Results

4.2.1 Differences in DNA integrity between clinical sample type relative to sampling method and site

DNA was extracted from 392 bronchioalveolar lavage (BAL), 134 gastric fluids (GF), 66 endotracheal aspirates (ETA) and 248 nasopharyngeal aspirate (NPA) samples from 192 patients. Successful isolation of intact human DNA was determined by positive PCR reaction amplifying the human mitochondrial cytochrome oxidase gene (HMCO). Although there is no clear relationship between host DNA integrity and bacterial DNA integrity, following extraction, failure to amplify host DNA would suggest that a failed 16S PCR could be due to poor DNA integrity rather than lack of bacteria. The return in HMCO DNA is shown in Figure 4.1 and expressed as percentage of the total number of samples. NPA samples had the greatest return in DNA (80%) while ETA and BAL were similar (77 and 74%, respectively) and GF were lowest (63%). BAL samples analysed came from two independent studies, both of which used different numbers of eukaryotic cells in initial experiments which therefore could potentially influence the amount of residual material available for extraction. When analysed separately 82 % of BAL samples from the protease study were positive (greater residual for processing) compared with 66 % of samples from the flow cytometry and apoptosis study (sometimes very little residual sample).

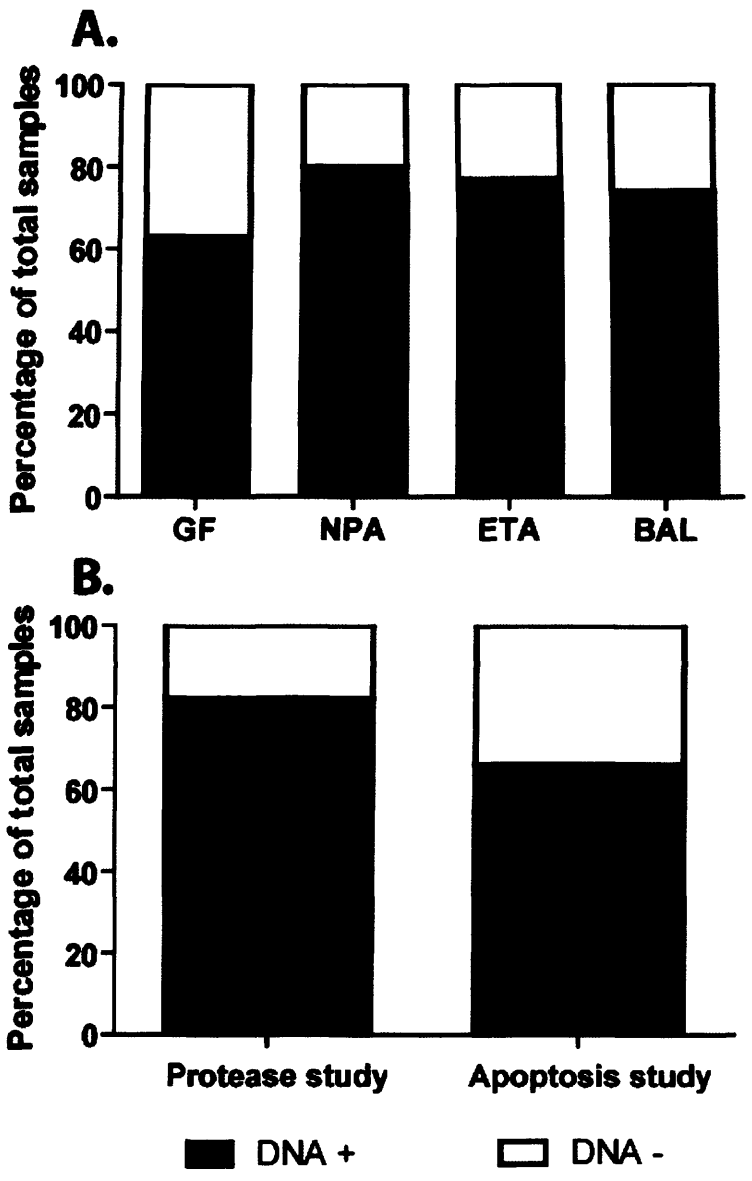


Figure 4.1. DNA recovery from various sample types following extraction with the QIAGEN® RNA/DNA Mini kit (25). (A) DNA integrity from all sample types; GF - Gastric fluid, NPA - nasopharyngeal aspirate, ETA - endotracheal aspirate, BAL - Bronchioalveolar lavage (all BAL samples). (B) Difference in DNA integrity between BAL samples from a study examining neutrophil proteases and one studying neutrophil apoptosis

4.2.2 Culture versus PCR on DNA extracts for the detection of *Ureaplasma*

Table 4.1 examines the longitudinal relationship between DNA integrity, as measured by presence of HMCO, with ability to detect *Ureaplasma* by PCR in a selection of BAL samples. These results are compared to results from direct culture of samples and subsequent PCR confirmation. From the 12 BAL samples taken from Baby O only four were positive for HMCO. This intermittent positivity was additionally seen in *Ureaplasma* positivity as assessed by PCR on the extracted DNA. Two of the HMCO positive samples correlated with *Ureaplasma* positive samples (O2 and O6). The third *Ureaplasma* positive sample, O3, correlated with a negative HMCO sample. In comparison, the results by culture were consistently positive. All 12 samples were *Ureaplasma* culture positive (colour change) as were the urease PCR reactions performed on the cultures (6 out of the 12 were cloudy indicating additional bacterial presence). Four out of six 16S rRNA positive samples for Baby O were positive despite an HMCO negative result. A similar trend in relationship between HMCO, *Ureaplasma* PCR on DNA extract, 16S rRNA PCR and *Ureaplasma* culture were seen for babies Q, W and Y (Table 4.1).

4.2.3 Sensitivity of primers

To determine the detection limits of the urease (pan-*Ureaplasma*), UM-1 (*Ureaplasma* speciation) and 16S rRNA primers 10-fold dilutions of DNA of a known 10^6 CCU culture were tested. From Figure 4.2 it is clear that the

urease primers were most sensitive by detecting DNA as low as 1 CCU. Primer set UM-1, used for identifying *U. parvum from U. urealyticum* isolates, could weakly detect 10^2 CCU, but intense bands were only seen at 10^4 CCU. Finally the 16S rRNA primers required 10^5 CCU for a successful PCR reaction.

Baby O	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12
Ureaplasma culture	+	+	+	+/-	+/-	+/-	+/-	+	+	+	+/-	+/-
PCR confirmation[1]	+	+	+	+	+	+	+	+	+	+	+	+
Ureaplasma PCR[2]	-	+	+	-	-	+	-	-	-	-	-	-
16s rRNA PCR[2]	+	+	+	-	+	+	-	-	+	-	-	-
HMCO PCR[2]	-	+	-	-	-	+	-	+	-	-	+	-

Baby Q	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10
Ureaplasma culture	-	+	+	+	+	+/-	+	+	+	-
PCR confirmation[1]	-	+	+	+	+	+	+	+	+	-
Ureaplasma PCR[2]	-	-	+	+	+	-	-	+	+	-
16s rRNA PCR[2]	-	-	-	-	-	-	-	-	-	-
HMCO PCR[2]	+	-	+	+	+	-	+	+	+	-

Baby W	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10
Ureaplasma culture	+	+	+	+	+	+/-	-	-	+/-	+/-
PCR confirmation[1]	+	+	+	+	+	-	-	-	+	+
Ureaplasma PCR[2]	+	+	-	+	-	-	-	-	-	-
16s rRNA PCR[2]	-	-	-	-	-	-	-	-	-	-
HMCO PCR[2]	+	+	-	+	-	-	-	+	-	-

Baby Y	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	Y10	Y11	Y12
Ureaplasma culture	-	-	-	-	+/-	+/-	+/-	+/-	+	+/-	+/-	+
PCR confirmation[1]	-	-	-	-	+	+	+	+	+	+	+	+
Ureaplasma PCR[2]	-	-	-	-	-	-	-	-	-	-	+	-
16s rRNA PCR[2]	-	-	-	-	-	+	-	-	-	-	-	-
HMCO PCR[2]	+	+	+	+	-	+	-	-	+	+	+	-

Table 4.1. A longitudinal examination of *Ureaplasma* colonisation status in four neonates comparing PCR results for HMCO, *Ureaplasma*, and 16S rRNA versus specific *Ureaplasma* culture. +/- denotes a mixed culture. [1]*Ureaplasma* positive cultures were confirmed via PCR amplification of the urease gene on DNA extracted from cultures. [2]Template DNA for these PCR reactions was obtained from direct DNA extraction of clinical samples

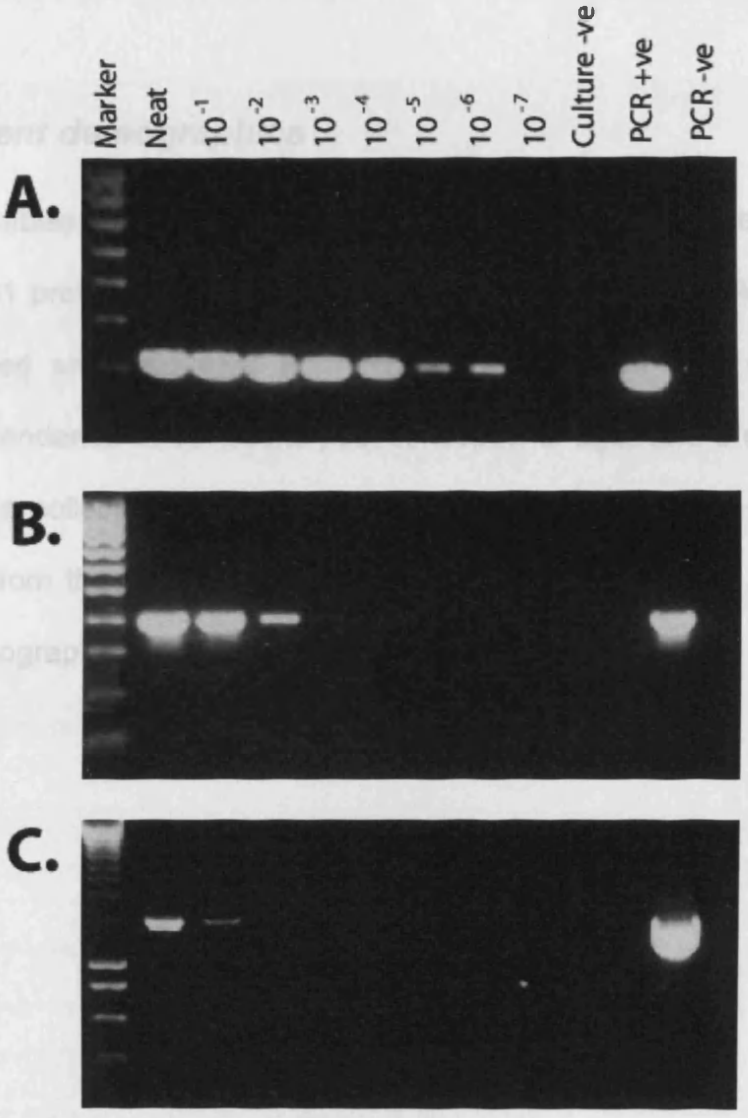


Figure 4.2. Agarose gels showing the sensitivity of (A) urease primers, (B) UM-1 speciation primers and (C) 16S rRNA primers against *Ureaplasma*. The final dilution to show colour change (i.e. 1 CCU) was to the dilution of 10^{-6} (10^{6} CCU).

4.2.4 Patient demographics

From 192 babies enrolled to the study 61 were born at term (gestation ≥37 weeks). 131 preterm infants of ≤34 weeks gestation were enrolled of which 88 developed and recovered from RDS, 35 developed CLD (defined as oxygen dependence at 36 weeks post-conceptional age) and 8 died. Of the 840 samples collected 88 were from the term group, 408 from infants with RDS, 309 from the CLD group and 35 from babies who died. A full list of patient demographics are shown in Table 4.2.

	CLD	RDS	Term	Death
Number of patients	35	88	61	8
Number of samples	309	408	88	35
GF:BAL:NPA:ET	10:260:9:30	69:73:236:30	51:37:0:0	4:22:3:6
Gestational age (weeks)	$26^{+5} \pm 2^{+2}$	$30^{+5} \pm 2^{+2}$	$38^{+5} \pm 1^{+5}$	$26^{+6} \pm 1^{+2}$
	$(23^{+4} - 31^{+6})$	(26 – 34)	$(37 - 42^{+1})$	$(25^{+6} - 30^{+3})$
Birth weight Kg	1.03 ± 0.65	1.50 ± 1.43	2.92 ± 0.64	0.87 ± 1.59
(Range)	(0.53 – 2.73)	(0.61 - 3.41)	(0.91 – 4.35)	(0.55 - 1.49)
Male:Female	18:17	41:47	41:20	6:2
Vaginal:Caesarean Delivery	19:16	24:64	33: 28	3:5
Antenatal steroids >24 hours	22/35 (63%)	71/88 (81%)	2/61 (3.3%)	7/8 (88%)
Surfactant	35/35 (100%)	43/88 (49%)	1/61 (1.6%)	8/8 (100%)
pPROM	6/35 (17%)	15/88 (17%)	7/61 (11%)	1/8 (13%)
Ventilation (IPPV:CPAP:SV)	35:0:0	47:28:13	19:13:29	8:0:0
***Ureaplasma* spp positive**	13/35 (37%)	9/88 (10%)	9/61 (15%)	3/8 (38%)
16S rRNA positive	27/35 (77%)	35/73* (48%)	14/34** (41%)	2/8 (25%)

Table 4.2. Patient characteristics for those who developed CLD, RDS and those who were born at full term gestation. *Only 73 patients within the RDS group were examined for presence of 16S rRNA. Values are mean or proportion of total patients within each group. ** Only 41 patients within the term group were examined for presence of 16S rRNA. IPPV = intermittent positive-pressure ventilation. CPAP = continuous positive airway pressure. SV = self-ventilation.

4.2.5 Association between infection and development of CLD

An association was noted between the presence of *Ureaplasma* and development of CLD as shown in Table 4.3a. Of the 35 babies in the CLD group, 37% were positive for *Ureaplasma* relative to 10% of RDS babies and 15% of terms. When the preterm groups who developed either RDS or CLD were compared, the association with development of CLD and presence of *Ureaplasma* was highly statistically significant by the Mann-Whitney unpaired t-test (p=0.0004). 16S rRNA PCR was performed on samples from 164 infants. When the association of presence of microbial genes and development of CLD samples were examined, 77% of the infants in the CLD were positive compared with 48% of the RDS babies and 41% of terms (p=0.004) (Table 4.3b).

In order to determine if the infection occurred antenatally or postnatally, I divided the samples into those which were positive on or before three days (likely ante- or perinatally acquired) of age and those who only became 16S PCR positive after the first three days of life (postnatal acquired). The association between day of detection of colonisation with *Ureaplasma* and other microbes relative to development of CLD is shown in Table 4.4a and 4.4b, respectively. There was a significant association between *Ureaplasma* and 16S rRNA genes within the first three days of life (p=0.005 and p=0.003, respectively) whereas the association was significant post three days, but to a lesser extent. (p=0.01 and 0.04, respectively).

A.

	Ureaplasma detected	*Ureaplasma* not detected	TOTAL
Term	9 (15%)	52 (85%)	61
RDS	9 (10%)	79 (90%)	88
CLD	13 (37%)	22 (63%)	35
Died	3 (38%)	5 (62%)	8
Total	34	158	**192**

B.

	16S rRNA detected	16S rRNA not detected	TOTAL
Term	14 (41%)	20 (59%)	34
RDS	35 (48%)	38 (52%)	73
CLD	27 (77%)	8 (23%)	35
Died	2 (25%)	6 (75%)	8
Total	78	86	**164**

Table 4.3. Contingency tables demonstrating the relationship between (A) the presence of *Ureaplasma* spp. and (B) 16S rRNA with clinical outcome. The relationship between *Ureaplasma* and 16S rRNA was statistically significant between RDS and CLD groups (p=0.0004 and p=0.004, respectively)

A.

	Ureaplasma < 3 days		Ureaplasma + 4 days		Total
	+	-	+	-	
RDS	6 (7%)	82 (93%)	3 (3%)	85 (97%)	88
CLD	8 (23%)	27 (77%)	5 (14%)	30 (86%)	35
Total	**14**	**109**	**8**	**115**	**123**

B.

	16S < 3 days		16S + 4 days		Total
	+	-	+	-	
RDS	14 (19%)	59 (81%)	21 (29%)	52 (71%)	73
CLD	14 (40%)	21 (60%)	13 (37%)	22 (63%)	35
Total	**28**	**80**	**34**	**74**	**108**

Table 4.4. Contingency tables demonstrating the relationship between the initial detection of (A) *Ureaplasma* and (B) 16S rRNA before and post three days of birth. There was statistical significance between presence of *Ureaplasma* and development of CLD within three days ($p = 0.005$) and additionally to a lesser extent for post three days ($p= 0.01$). Similarly there was statistical significance between presence of 16S rRNA and the development of CLD within three days ($p = 0.003$), but again to a lesser extent post three days ($p= 0.04$).

4.2.6 Relationship between IL-6 and IL-8 with infection and clinical outcome

Concentrations of the pro-inflammatory cytokines IL-6 and IL-8 were examined by ELISA (Dr Eamon McGreal and Dr Mallinath Chakraborty) in BAL samples from 63 and 62 infants, respectively. The peak results for each individual infant were correlated with the presence or absence of either *Ureaplasma* or 16S rRNA genes as shown in Figures 4.3a and 4.3b. Samples positive for either *Ureaplasma* or microbial genes had median BAL fluid IL-6 and IL-8 values of 12,389 pg/ml and 9,690 pg/ml, respectively, which were 6-fold and 4-fold greater than the respective median values in the microbial negative samples (both $p<0.0001$). Only when samples within the first three days were examined a significant relationship was also noted between IL-6 ($p=<0.05$) and IL-8 concentrations ($p=<0.05$) and presence of microbes (Figure 4.3c and 4.3d, respectively).

The peak values for the IL-6 and IL-8 for each diagnostic group is shown in Figure 4.4 with presence of microbes shown as filled (present) and unfilled (absent) markers. BAL fluid samples from term babies had both the lowest IL-6 and IL-8 median values of 980 and 12,107 pg/ml, respectively. RDS babies had median IL-6 and IL-8 levels of 5,170 and 17,502 pg/ml, respectively, whereas CLD babies had median levels of 15,594 and 122,550 pg/ml, respectively. Using a Mann Whitney U-test the differences between RDS and CLD IL-6 and IL-8 were statistically significant ($p=<0.005$ and $p=<0.0001$, respectively).

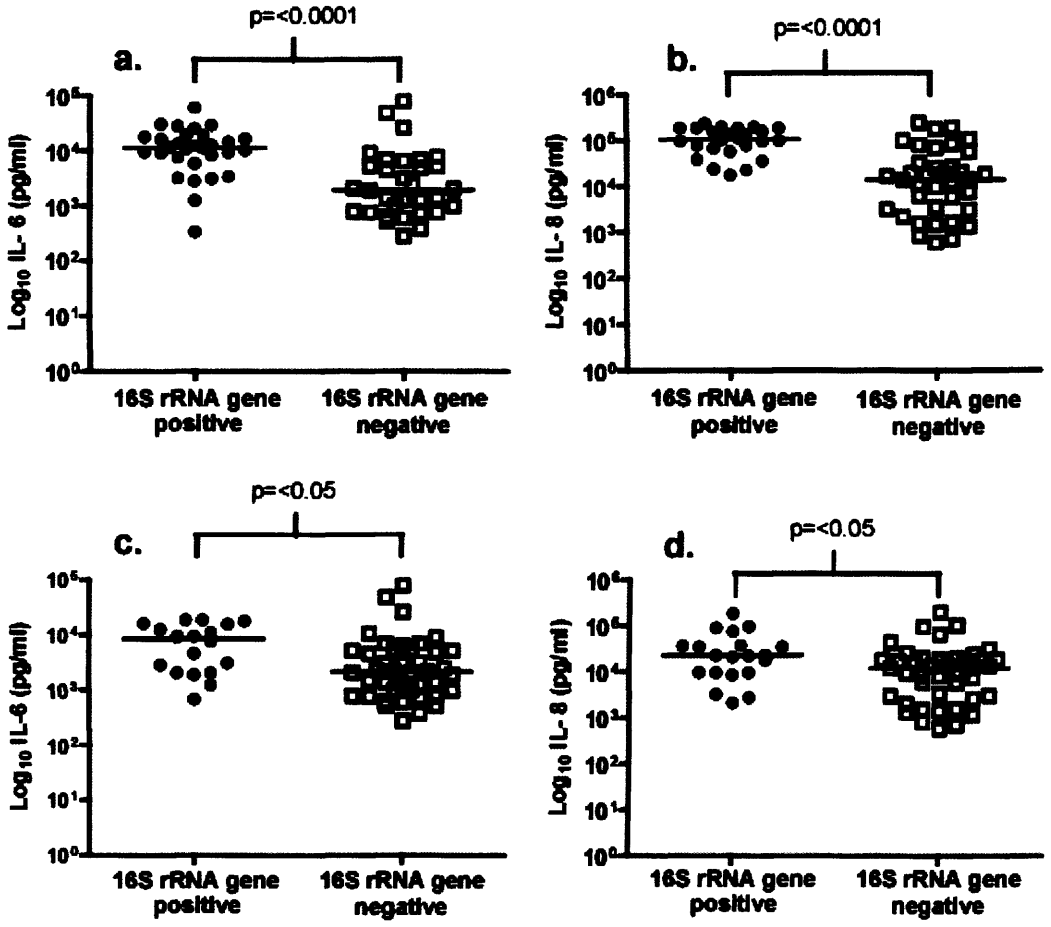


Figure 4.3. Correlation between peak IL-6 and IL-8 levels and presence of bacteria within BAL samples. (A) Peak IL-6 levels across sampling period, (B) peak IL-8 levels across sampling period, (C) peak IL-6 within three days and (D) peak IL-8 within three days.

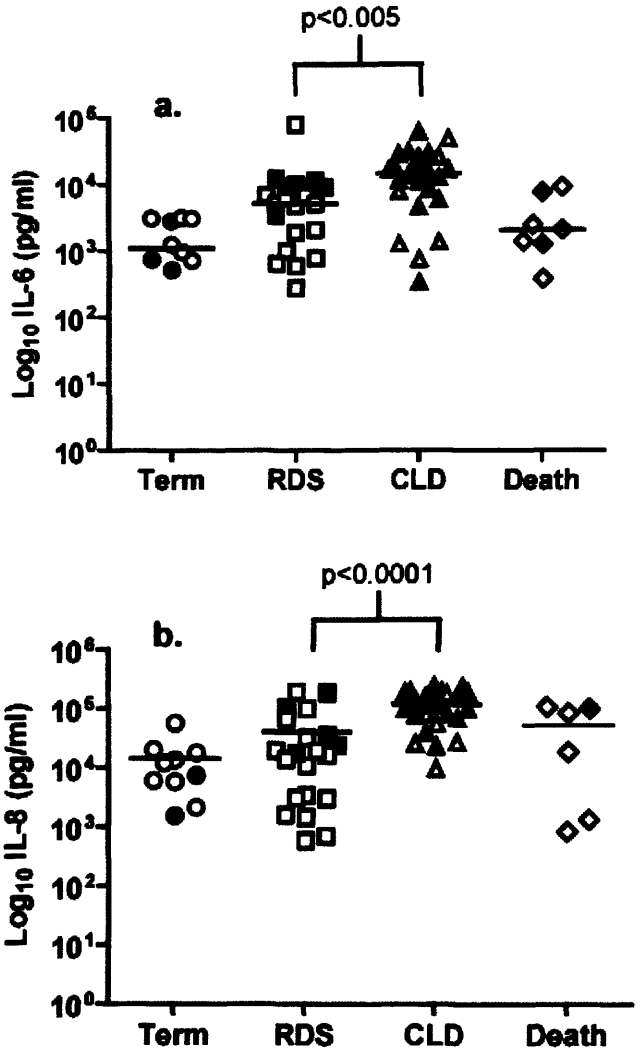


Figure 4.4. Peak levels of IL-6 (A) and IL-8 (B) from BAL samples for term, RDS, CLD and infants which died. Open shapes represent bacteria negative patients and closed shapes represent bacteria positive patients.

4.2.7 Bacteria associated with peaks in IL-6 and IL-8

Amplicons from 16S rRNA positive samples which coincided with peaks in IL-6 and IL-8 were sequenced to determine the species of infecting organism (Table 4.5). *Staphylococcus epidermidis* was the most prevalent organism which was isolated from infants who went on to develop RDS with one peak being associated with *Fusobacterium nucleatum*. Gram-negative organisms were more frequently associated with peaks in CLD babies and included *Escherichia coli*, *Haemophilus influenzae*, *Enterobacter* spp. and *P. aeruginosa*, although Gram-positives including *S. epidermidis* and *Staphylococcus haemolyticus* were additionally identified. Twice as many *U. parvum* were isolated relative to *U. urealyticum* in both RDS and CLD babies.

4.2.8 Sequence homology between 16S rRNA primers and bacteria detected.

To rule out the possibility of any bias in the binding of either the 27f or 1492r 16S rRNA primers, the binding sequence for each organism detected in this study was examined using genome sequence data (Figure 4.5). As can be seen the degeneracy in each of the primers allows specific and unbias binding to all of the bacteria identified in this study.

4.2.8 Day of initial 16S rRNA positive sample and clinical outcome

The relationship between 16S rRNA positivity and day of first acquiring an infection is presented in Figure 4.6. From the 27 CLD babies which were 16S rRNA gene positive, 56% (15/27) were positive within the first 3 days, which decreased to 26% (7/27) at days 4 to 6 and then 7% (2/27) at days 7-9 and 10-12. In the RDS group, 37% (13/35) of babies were colonised within the first 3 days of with subsequent colonisation occurring in 17% (6/35) of babies on days 4-6, 14 % (5/35) on days 7-9.

RDS		CLD	
Gram-positive	Incidence	Gram-positive	Incidence
Staphylococcus epidermidis	3	*Staphylococcus epidermidis*	4
		Staphylococcus haemolyticus	1
Gram-negative		Gram-negative	
Fusobacterium nucleatum	1	*Escherichia coli*	2
		Haemophilus influenzae	1
		Enterobacter spp.	1
		Pseudomonas aeruginosa	1
Other		Other	
Ureaplasma parvum	2	*Ureaplasma parvum*	4
Ureaplasma urealyticum	1	*Ureaplasma urealyticum*	2
		Mixed	2

Table 4.5. Bacteria associated with peaks in IL-6 and IL-8 from BAL samples. Peak levels of IL-6 and IL-8 from BAL fluids from preterm infants were correlated with the presence of infection. 16S rRNA positive samples were then sequenced to identify the infecting organism. The number of samples containing each organism is indicated.

16S rRNA primers
(with degeneracy in green)

	27f	1492r
	AGAGTTTGATCATGGCTCAG	TACGGCTACCTTGTTACGACTT
	AGAGTTTGATCCTGGCTCAG	TACGGTTACCTTGTTACGACTT

Bacteria	Primer binding sites	
S. epidermidis	TCTCAAACTAGGACCGAGTC	ATGCCGATGGAACAATGCTGAA
S. haemolyticus	TCTCAAACTAGGACCGAGTC	ATGCCGATGGAACAATGCTGAA
F. nucleatum	TCTCAAACTAGGACCGAGTC	ATGCCAATGGAACAATGCTGAA
U. parvum	TCTCAAACTAGGACCGAGTC	ATGCCAATGGAACAATGCTGAA
U. urealyticum	TCTCAAACTAGGACCGAGTC	ATGCCAATGGAACAATGCTGAA
H. influenzae	TCTCAAACTAGTACCGAGTC	ATGCCAATGGAACAATGCTGAA
Enterobacter spp	TCTCAAACTAGTACCGAGTC	ATGCCAATGGAACAATGCTGAA
E. coli	TCTCAAACTAGTACCGAGTC	ATGCCAATGGAACAATGCTGAA
P. aeruginosa	TCTCAAACTAGTACCGAGTC	ATGCCGATGGAACAATGCTGAA

Figure 4.5. 16S rRNA primer homology to sequences from bacteria detected in BAL supernatants. Green highlighted areas show the area of degeneracy in the primers (A/C at position 19 in primer 27f and T/C at position 1497 in primer 1492r). Yellow highlighted areas show region of divergence in primer binding sequence which coincide with primer degeneracy. 16S rRNA sequences were from the following organisms; *S. epidermidis* (ATCC 12228), *S. haemolyticus* (JCSC 1435), *F. nucleatum* (ATCC 25586), *U. parvum* (ATCC 700970), *U. urealyticum* (ATCC 33699), *H. influenzae* (Rd KW20) *Enterobacter* spp (638), *E. coli* (K12 MG1655) and *P. aeruginosa* (PAO1).

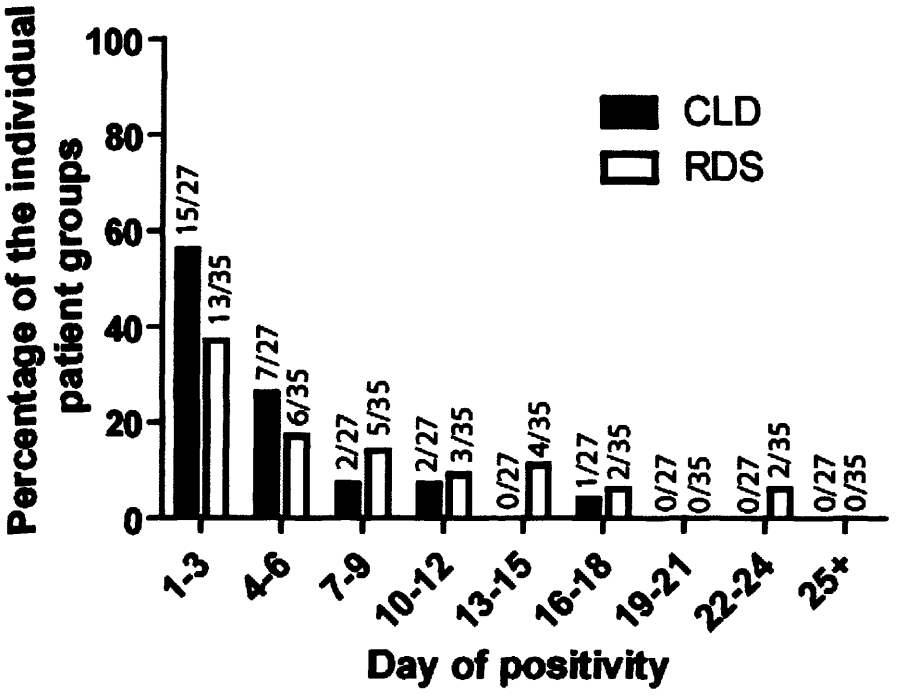


Figure 4.6. Rate at which samples first became 16S rRNA by day. Closed and open bars represent the number of babies which were initially 16S rRNA positive within the designated day and went on to develop CLD or RDS, respectively. The number of individual patients per the total of each group are represented above the respective bar.

4.2.9 Relationship between Ureaplasma infection and gestational age

Figure 4.7 shows the relationship between gestational age and acquisition of *Ureaplasma* broken down into clinical outcome. Percentage of colonisation between 23 and 26 weeks ranged between 41 and 67 %. All infants colonised with *Ureaplasma* between 23 and 25 weeks developed CLD whereas of the 7/17 colonised, born at 26 weeks: 3 developed CLD (18 %), 2 developed RDS (12 %) and 2 died (12 %). The percentage of colonisation dropped to 20 % in infants born at 27 weeks and continued to decline as gestation increased.

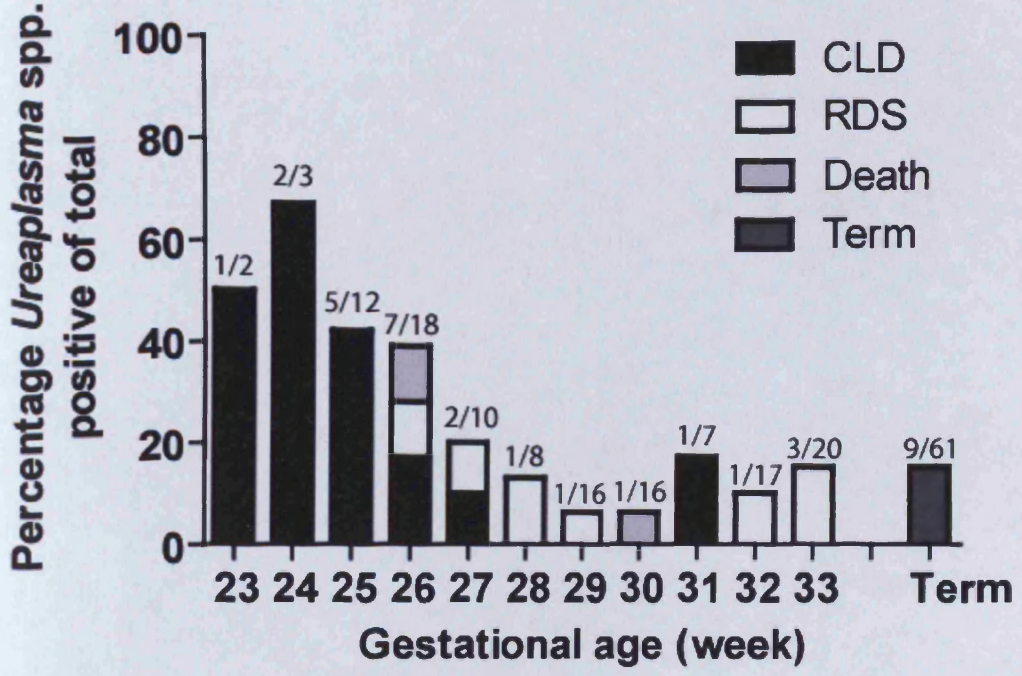


Figure 4.7. The association between *Ureaplasma* positivity and gestational age. Closed, open, light grey and dark grey bars represent percentage of babies born at respective gestational age who were colonised with *Ureaplasma* and developed CLD, RDS, died or were born at term, respectively. The number of *Ureaplasma* positive patients per the total born for each week are shown above the respective bar.

4.3 Discussion

The primary aim of this chapter was to examine the role of infection, with particular focus to *Ureaplasma*, in development of CLD. From 192 neonates a total of 840 samples (392 BAL, 134 GF, 66 ETS and 248 NPA) were examined for the presence of infection and correlated with both clinical outcome as well as presence of inflammatory markers IL-6 and IL-8.

4.3.1 Quality of DNA and from extraction

Detection of the HMCO gene was used as a method of confirming successful DNA isolation from a variety of clinical samples. Of the four sample types NPA gave the greatest return in HMCO followed by ETA, BAL and finally GF. Factors which may potentially influence the presence of HMCO following extraction include the amount of start material, DNA degradation during the extraction process or false-negatives on PCR due to the presence of PCR inhibitors. Nelson *et al..*, examined the presence of PCR inhibitors in a selection of 115 DNA extracts from ETA samples which were negative for *Ureaplasma* DNA (Nelson *et al..* 1998). By adding the equivalent of 10 – 20 CCU of *Ureaplasma* DNA to each sample they identified 7% of samples contained inhibitors. This may account for a number of the negative samples identified in my studies. Additionally they stated that samples which were mucoid in consistency were more likely to contain PCR inhibitors as demonstrated by the presence of samples which were PCR negative following extraction, but positive by culture. This could account for the lowest level in

DNA return from GF samples as a number of these were mucoid in consistency.

The BAL samples that I investigated were obtained following prior use in two independent studies. Those BAL samples which came from the protease study had greater rate of DNA positivity (82%) than those from the apoptosis study (62 %). The different rate of positivity likely reflects the amount of cells remaining for DNA extraction following use in the primary projects. A greater number of cells were most likely remaining following preparation of cytospins for the protease study whereas in some cases almost all of the cells were required for the apoptosis study which utilised flow cytometry as well as cytospins. If the BAL samples from the apoptosis study were removed, then BAL samples from the protease study would be the most reliable sample to obtain HMCO DNA (82% *vs.* 80% from NPA).

4.3.2 Culture versus PCR for the detection of Ureaplasma

Table 4.1 shows a selection of four babies (Baby O, Q, W and Y) comparing the ability of PCR upon DNA extracts and culture based methods to detect *Ureaplasma*. Additionally it shows the presence of 16S rRNA and the HMCO DNA quality control marker. HMCO results were intermittently positive for all babies suggesting, as discussed in section 4.3.1, either no human DNA was isolated or the presence of PCR inhibitors. Although inhibitors are a possibility, a number of samples, for example O1, O3, O5 and O9, were positive for 16S rRNA, but HMCO negative. One possible explanation for this would be the lack of human cells in the sample, yet presence of bacteria. In

some samples, the amount of human start material may have been low, whereas bacteria may have been present in the saline supernatant which was extracted along with the pellet. External contamination may be ruled out as sequencing of the 16S rRNA product for Baby O revealed the presence of *E. coli*, which correlated with the clinical findings of the NHS diagnostic laboratories. Due to the HMCO being an indicator for successful isolation of human DNA, and not prokaryote DNA, and the presence of 16S rRNA and *Ureaplasma* PCR positive in HMCO negative samples, little emphasis was placed on those samples which were HMCO negative. In addition, multiple samples were taken from each baby; therefore intermittent positivity for HMCO was not of significant concern.

An unexpected finding was the poor ability of the 16S rRNA primers to amplify *Ureaplasma* DNA. For example extracted DNA for samples Q3, Q4, Q5, Q8 and Q9 were positive by urease PCR primers, suggesting presence of *Ureaplasma* DNA, but were negative by 16S rRNA. By looking at the data on the sensitivity of primers in Figure 4.2, it was seen that the lower level of detection for the 16S rRNA primers against *Ureaplasma* was 10^5 CCU; therefore, any samples containing less than this threshold limit would not be detected. One explanation for this is may be the due to the low copy number of ribosomal operons present in *Ureaplasma* (two) relative to other organisms, such as the most commonly identified *S. epidermidis* (five). Poor homology between the 16S rRNA primers and the respective binding sequence in each of the *Ureaplasma* spp was ruled out as seen in Figure 4.5. The degenerate primers used take into account the small degree of divergence, a single base

in each region, seen within this highly conserved region from all bacteria. Although the ability to amplify *Ureaplasma* 16S rRNA was poor, 16S rRNA sequences for samples 34.4 and 41.8 came back as *U. urealyticum* and *U. parvum* respectively.

The general consensus, although not as expected, suggested that culture was more reliable than PCR on DNA extractions for the detection of *Ureaplasma*. As seen in Table 4.1 no samples were positive by PCR if negative by culture. Nelson *et al.*. reported that PCR was a more sensitive method relative to culture (Nelson *et al.*. 1998). Figure 4.2 shows that the sensitivity of the urease primers within my studies were similar to that described by Nelson which could detect to 1 CCU. As the sensitivity for detection of both culture and PCR were the same, the reason for the poor correlation between PCR on DNA extracts and culture, could be due to failure to isolate *Ureaplasma* DNA using the QIAGEN® RNA/DNA Mini kit (25) columns. A further advantage of using bacterial culture for detecting *Ureaplasma*, versus detection via molecular methods, means that the antimicrobial susceptibilities of isolates can be examined which was crucial for the data presented in the final results chapter (Chapter 5).

The intermittent culture positivity seen for Baby W related to antimicrobial therapy received during the individual's participation within the study period. The infant was treated with erythromycin for 7 days (IV infusion 10-12.5 mg/kg every 6 hours (4 times a day) following the confirmation of the presence of *Ureaplasma*, but when cultures became negative treatment was stopped

leading to a resurgence of infection. Due to macrolide antibiotics, such as erythromycin, being bacteriostatic in action, the clearance of infection is dependent on the patient's immune system being able to clear the growth-inhibited bacteria. The relatively naïve immune status of preterm infants, such as Baby W, may result in a slight impairment in the clearance of infection relative to that of a healthy individual. Of further interest related to antimicrobial therapy in this patient group was the failure of Baby O to clear *Ureaplasma* following a 10 day course of erythromycin. Retrospective examination of the antimicrobial susceptibility of the *Ureaplasma* isolated from this patient, referred to as isolate UHWO10 in chapter 5, was found to be highly resistant to erythromycin with resistance to clarithromycin as well as an elevated minimum inhibitory concentration to the third macrolide, azithromycin. This information is discussed in detail in chapter 5. Baby Q treated for 10 days and cleared the infection at the end of therapy whereas Baby Y was never treated.

4.3.3 Association between infection and development of CLD

In this chapter I examined various clinical samples from term and preterm infants for the presence of infection by detection of 16S rRNA genes as well as selective culture and PCR for the detection *Ureaplasma* spp. Levels of the inflammatory mediators IL-6 and IL-8 in a selection of BAL samples were assayed and correlated with infection status and clinical outcome. The data presented agrees with the consensus of previous studies that perinatal infection is a significant risk factor in the development of CLD, especially if

acquired within the first three days of life (Kotecha *et al.*. 2004; Miralles *et al.*. 2005).

Ureaplasma was detected in 13 out of 35 (37%) babies who developed CLD relative to 9 out of 88 (10%) of RDS babies and 9 out of 61 (15%) term infants. The day at which samples became positive related to development of CLD. Significance between *Ureaplasma* colonisation and development of CLD was lower when comparing those babies who gave positive PCR results after the first three days of life relative to those who acquired postnatally within three days (Table 4.4a).

The presence of bacterial conserved 16S rRNA genes in DNA extracted samples was used to study the relationship between other bacterial infections and development of CLD. Of the 35 babies who developed CLD and were examined for 16S rRNA, 27 were positive (77%) relative to the 35 out of 73 (48%) babies who developed RDS and 14 out of 34 (41%) who were born at term. As with *Ureaplasma* positivity, presence of the 16S rRNA gene and development of CLD was strongly significant in those babies who acquired the infection within the first three days of life (Table 4.4b). Samples within the first three days of life were twice as likely to be positive in both CLD and RDS infants (50% and 39% of total, respectively) than in samples on days 4 to 6 (26% and 18% of total, respectively) (Figure 4). By sequencing the 16S rRNA products from samples which correlated with peaks in IL-6 and IL-8 from RDS and CLD infected patients, the species of organisms was identified. Gram-negative organisms such as *E. coli, H. influenzae, Enterobacter* Spp and *P.*

aeruginosa were more frequently isolated from CLD patients compared with RDS. The pro-inflammatory nature of the Gram-negative endotoxin LPS has been shown in a preterm lamb model to contribute to lung damage (Kramer *et al.*. 2002). Ventilated lambs, born to pregnant ewes which had received intra-amniotic LPS, had elevated inflammatory markers and fewer alveoli compared with ventilated controls. These data suggest that the species of organism acquired may influence the outcome of disease. *S. epidermidis* was prevalent in a number of both RDS and CLD babies.

4.3.4 Relationship between IL-6 and IL-8 with infection and clinical outcome

Inflammatory mediators IL-6 and IL-8 were quantified in BAL samples from a selection of term, RDS and CLD patients as well as those who died. Peak levels of both cytokines positively correlated with the presence of infection in both <3 and >3 day groups. When the cytokine data was separated on clinical outcome, babies who developed CLD had significantly higher levels when compared to RDS. This association between IL-6 and IL-8 with 16S rRNA positivity and development of CLD has previously been shown by Miralles *et al.*. who examined tissue samples and BAL fluids from a collection of 41 preterm infants (Miralles *et al.*. 2005). Similarly, Kotecha *et al.*. showed that levels of IL-6 and IL-8 were significantly higher within the first day of life in *Ureaplasma* infected babies relative to those who were negative (Kotecha *et al.*. 2004). The significance of high IL-8 levels in BAL samples could result in the recruitment of neutrophils, the predominant inflammatory cell in CLD lungs

(Kotecha *et al.*. 1995). Studies within our laboratories have shown that lavage fluids contain high levels of neutrophil elastase in CLD samples relative to RDS therefore potentially contributing lung damage and development of CLD (Davies *et al.*. 2009).

4.3.5 Relationship between Ureaplasma infection and gestational age

The relationship between *Ureaplasma* colonisation of neonates and gestational age is known to be inversely proportional (Sanchez and Regan, 1990; Kafetzis *et al.*. 2004). My findings support this data by giving an in-depth week by week break down of colonisation rates from 23 weeks gestation to term (Figure 4.6). An interesting observation was 41 % (5/12) of infants born at 25 weeks were colonised with *Ureaplasma*, all of which developed CLD, whereas 39 % (7/18) of those born at 26 weeks were colonised but only 17 % (3/18) developed CLD. This may represent a specific time point in the development of the neonate whereby birth after this week of gestation allows the infant to manage infection in a way that it is limited to being acute and not progresses to a chronic state.

4.4 Conclusion

This chapter has added further evidence for the role of bacterial infection as a risk factor in development of CLD with specific relevance being attached to bacteria from genus *Ureaplasma* as well as Gram-negative organisms. My studies highlight the significance of antenatal acquisition of infection (as judged by detection of microbial 16S rRNA gene presence) within the first three days of life and are supported by the high levels of inflammatory mediators IL-6 and IL-8 for these patients. I have additionally shown that culture based methods are the most reliable means for detection of *Ureaplasma* relative to molecular based techniques following the extraction of DNA and allows for antimicrobial susceptibility testing therefore allowing treatment to be tailored to the isolate in question.

Chapter 5

Antibiotic resistance in *Ureaplasma spp.*

5.1 Introduction

Antibiotic resistance among human pathogens continues to have a major impact on patient morbidity, mortality and economic cost to the health service (Lode, 2009). Due to the limited number of antibiotic effective against *Ureaplasma* as well as the further treatment limitations in neonates and immune compromised patients, the emergence of resistant strains can further complicate treatment.

Due to the technical difficulties in cultivation of *Ureaplasma*, susceptibility testing is predominantly carried out by the microbroth dilution technique using a standardised 10^4-10^5 CCU. In clinical isolates tetracycline resistance results from the expression of the horizontally acquired *tetM* gene, whereas mutations in the quinolone resistance determining regions (QRDRs) of type II topoisomerase genes, predominately encoding a D112E substitution in GyrA protein and/or Ser83Leu, Ala125Thr Ala136Thr substitutions in ParC protein, gives a fluoroquinolone resistant phenotype. To date the molecular mechanisms of macrolide resistance in clinical *Ureaplasma* isolates has not been determined; although laboratory derived strains have shown mutations in domain V of the 23S rRNA as well as changes in the L4 and L22 proteins to (Pereyre *et al.* 2007).

This final results chapter focuses on the potential difficulties encountered as a result of antibiotic resistant strains of *Ureaplasma*. I aim to develop a method of determining antibiotic resistance without prior knowledge of cell number.

Using this methodology examine the incidence of antibiotic resistance among a retrospective cohort of archived samples and subsequently deduce the molecular mechanisms of resistance accounting to any resistant phenotypes.

5.2 Results

5.2.1 Species distribution of screened Ureaplasma isolates between UHW and HPA.

Seventy-five percent (15/20) of the archived UHW samples were successfully revived from -80°C storage, while only 59.7% (46/77) of archived HPA samples were successfully revived. This may reflect the relatively short storage time for the former samples (less than 18 months) compared to that of the latter samples (up to 4 years), although differences in isolate preparation prior to freezing may have equally contributed. For all 61 revived samples, 49 were found to belong to *U. parvum* (80%) and 12 to *U. urealyticum* (20%). A minor difference in species distribution was identified between HPA and UHW collections, but this was not deemed significant by Fisher's exact test analysis (78% *U. parvum* versus 87% *U. parvum*, respectively). A selection of *U. parvum* isolates were further serotyped by genetic methods described by Teng *et al.* (Teng *et al.* 1994). From 16 isolates serotyped, four were classified as SV1, eight were classified as SV3, two as SV6 and two as SV14.

5.2.2 Interpretation of results from adapted 96-well micro-broth technique and breakpoint analysis.

An example showing strains resistant to erythromycin, ciprofloxacin, and tetracycline is shown in Figure 5.1. Non-turbid, dark red wells (which appear dark gray in the Figure) are indicative of positive *Ureaplasma* growth in the selective medium. If the last well showing growth in the 10-fold dilution (in the top row, lacking antibiotics) is considered to have 1 CCU, then four columns to the left represents 10^3 CCU, and five columns to the left represents 10^4 CCU (isolated by the dotted box). Following previously published recommendations that *Ureaplasma* resistance needs to be assessed using an input of 10^4 CCU (Waites *et al.* 2001), resistance-to-threshold antibiotic concentrations (4 mg/L erythromycin, 4 mg/L ciprofloxacin, or 2 mg/L tetracycline) were identified by comparison of wells within the dotted box. Figure 5.1a shows breakpoint identification of an erythromycin-resistant clinical SV1 isolate (UHWO10), a ciprofloxacin-resistant clinical SV1 isolate (HPA18, Figure 5.1b), and a tetracycline-resistant clinical SV6 isolate (HPA23, Figure 5.1c). While 10^4 CCU has been used to assess antibiotic resistance, using 10^3 to 10^1 CCU would be equally valid, and no difference in our MICs was found when reading either the 10^3 or 10^4 CCU.

For those isolates identified by breakpoint analysis to have MICs greater than the breakpoint, the exact MIC for a single antibiotic was determined using a range of concentrations with a full-plate single antibiotic assay (Figure 5.2). Using the same method used for determining which column contained 10^4 CCU (isolated by dotted box), examples of an erythromycin-susceptible

clinical isolate (MIC 1 mg/ L) (Figure 5.2a) and the highly resistant clinical isolate (MIC >64 mg/L) (Figure 5.2b) are shown.

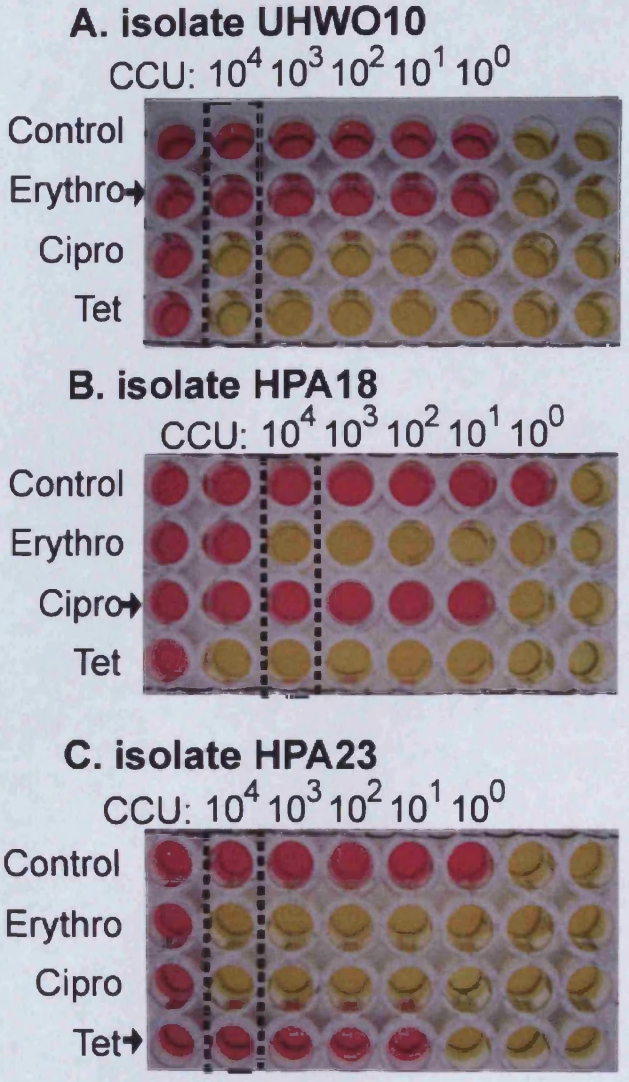


Figure 5.1. Photographs of antibiotic breakpoint investigation showing an erythromycin (Erythro)-resistant strain (UHWO10) (A), a ciprofloxacin (Cipro)-resistant strain (HPA18) (B), and a tetracycline (Tet)- resistant strain (HPA23) (C). Dark red wells indicate growth of *Ureaplasma* while orange-yellow wells represent no *Ureaplasma* growth. Columns containing 10^4 CCU are identified by a dotted box, and comparison of growth in the absence of antibiotics (control) was used to determine resistance to 4 mg/L erythromycin, 4 mg/L ciprofloxacin, or 2 mg/L tetracycline.

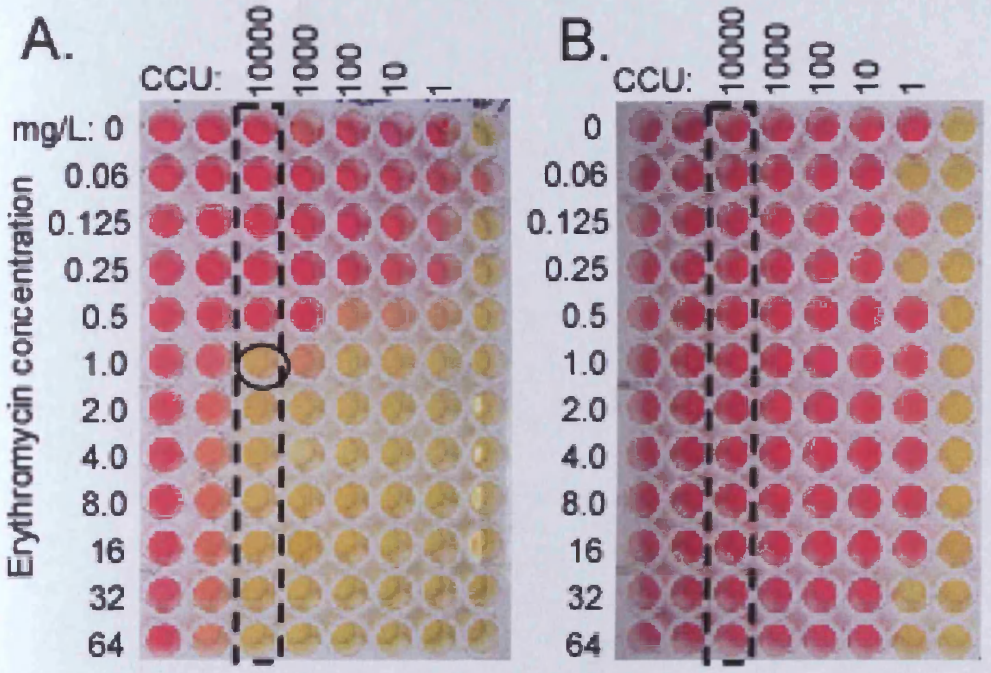


Figure 5.2. Full-plate determination of MIC for erythromycin for a susceptible SV1 isolate (A) and a resistant (UHWO10) isolate (B). Plates contain a gradient of antibiotic from 64 mg/liter to 0.06 mg/liter. Dark red wells indicate growth of *Ureaplasma* while orange-yellow wells represent no *Ureaplasma* growth. Columns representing 10^4 CCU growth in the absence of antibiotic are shown by the dotted box, and for the susceptible strain, a circle shows the first concentration of erythromycin to inhibit growth (MIC = 1 mg/L). Since the resistant isolate (UHWO10) grew even in the presence of 64 mg/L erythromycin, the MIC is determined to be >64 mg/L.

5.2.3 Control isolates

To ensure that conditions for MIC determination, such as temperature and atmospheric conditions, were standardised control isolates recommended by the British Society for Antimicrobial Chemotherapy (BSAC) were examined using the methodology described. As seen in Table 5.1 all isolates were within a 2-fold dilution of that set out by BSAC recommendations (Andrews, 2001).

	Erythromycin		Tetracycline		Ciprofloxacin	
	BSAC	Beeton	BSAC	Beeton	BSAC	Beeton
S. aureus (ATCC 29213)	0.25	0.5	0.5	0.5	0.5	0.25
E. coli (25922)	>32	>32	2	2	0.015	<0.03
E. faecalis (29212)	4	4	16	16	1	0.5

Table 5.1 Comparison of BSAC MIC results for control strains versus that of results obtained in this study

5.2.4 Incidence of antibiotic resistance in Ureaplasma isolates from UHW and HPA.

A total of 61 *Ureaplasma* isolates consisting of 46 from the HPA, Colindale, and 15 from UHW were examined for the presence of antibiotic resistance to erythromycin, tetracycline and ciprofloxacin with the isolates of interest summarized in Table 5.2. Six isolates were identified to have an MIC greater than the MIC range previously published in Cumitech 34 (0.02 to 4 mg/L) (Waites *et al.* 2001), giving the isolates a prevalence of 9.8%, although only one of these isolates (UHWO10) was likely to represent true antibiotic resistance with an MIC of >64 mg/L. Among the erythromycin-resistant isolates, azithromycin and clarithromycin MICs were comparable to those of susceptible controls, with the exception of the highly resistant UHWO10, which was additionally resistant to clarithromycin (MIC 4 mg/L), but susceptible to azithromycin albeit slightly raised (normal MIC ranges, 0.5 to 4 mg/L for azithromycin and 0.004 to 2 mg/L for clarithromycin). A single tetracycline-resistant clinical isolate, HPA23, was identified with tetracycline and doxycycline MICs of 64 and 16 mg/L, respectively (1.6% prevalence). A single ciprofloxacin-resistant clinical isolate, HPA18, was also identified with an MIC of 8 mg/L (1.6% prevalence).

Isolate	Species	MIC					
		Ery	Azi	Cla	Tet	Dox	Cip
HPA3	*U. urealyticum*	4	0.5	<0.125	2	0.5	4
HPA6	*U. urealyticum*	4	<0.25	<0.125	2	0.5	4
HPA12	*U. urealyticum*	**8**	<0.25	<0.125	2	0.5	4
HPA17	*U. urealyticum*	**8**	0.5	<0.125	1	0.25	4
HPA18	*U. parvum*	**8**	<0.25	<0.125	2	0.25	**8**
HPA20	*U. urealyticum*	**8**	<0.25	<0.125	2	0.5	4
HPA23	*U. parvum*	4	<0.25	<0.125	**64**	**16**	<2
HPA32	*U. parvum*	**8**	<0.25	<0.125	1	0.25	<2
UHWJM	*U. parvum*	4	<0.25	<0.125	2	0.5	4
UHWO10	*U. parvum*	**>64**	2	**4**	1	0.25	4
UHWP2	*U. parvum*	4	<0.25	<0.125	2	0.5	4
UHWQ3	*U. parvum*	4	<0.25	<0.125	1	0.25	4

Table 5.2. MIC results for resistant and control *U. parvum* and *U. urealyticum* strains. MIC results from a selection of resistant (in bold) and susceptible isolates as determined by growth comparison at 10^4 CCU. Ery - erythromycin, Azi - azithromycin, Cla - clarithromycin, Tet - tetracycline, Dox - doxycycline, Cip - Ciprofloxacin.

5.2.5 Macrolide resistance

Mutations in domain V of the 23S rRNA genes (two separate operons) and associated L4 or L22 protein from the prokaryotic ribosomal complex have previously been reported to be associated with macrolide resistance (Pereyre *et al.* 2007). These genes were sequenced from the highly resistant clinical isolate UHWO10 as well as HPA17 and HPA32, MIC of 8 mg/L, as well as the fully susceptible Q3 and compared with the published sequences (Glass *et al.* 2000). No mutations were found in either the 23S rRNA operon (Figure 5.3a and 5.3b) or the L22-encoding gene of any of the isolates (Figure 5.4a) within the area previously reported contain mutations; however, a 6-bp deletion (AAGACA) was identified in the L4 protein gene from the highly resistant UHWO10 resulting in a two amino acid deletion of an arginine and glutamine (ΔR66Q67) (Figure 5.4b).

A.

Isolate	Spp.	Nucleotide alignment
U. parvum^{S}	U.p	^{2046}GTGATTGGACGGAAAGACCCCATGAAG^{2072}
UHWQ3^{S}	U.p	^{2046}GTGATTGGACGGAAAGACCCCATGAAG^{2072}
HPA17^{I}	U.u	^{2046}GTGATTGGACGGAAAGACCCCATGAAG^{2072}
HPA32^{I}	U.p	^{2046}GTGATTGGACGGAAAGACCCCATGAAG^{2072}
UHWO10^{R}	U.p	^{2046}GTGATTGGACGGAAAGACCCCATGAAG^{2072}

B.

Isolate	Spp.	Nucleotide alignment
U. parvum^{S}	U.p	^{2046}GTGATTGGACGGAAAGACCCCATGAAG^{2072}
UHWQ3^{S}	U.p	^{2046}GTGATTGGACGGAAAGACCCCATGAAG^{2072}
HPA17^{I}	U.u	^{2046}GTGATTGGACGGAAAGACCCCATGAAG^{2072}
HPA32^{I}	U.p	^{2046}GTGATTGGACGGAAAGACCCCATGAAG^{2072}
UHWO10^{R}	U.p	^{2046}GTGATTGGACGGAAAGACCCCATGAAG^{2072}

Figure 5.3. DNA sequence alignments from the area of interest within the genes encoding domain V of the 23S rRNA operon 1 (A) and operon 2 (B) from isolates which were macrolide susceptible (^{S}), intermediately resistant (^{I}) and resistant (^{R}). *U. parvum* sequence from isolate ATCC 700970 (Glass *et al.* 2000). The region which has previously been associated with macrolide resistance corresponding to A2058 (*E. coli* numbering) is highlighted in yellow. U.p corresponds to *U. parvum* while U.u corresponds to *U. urealyticum*. Superscript numbering corresponds to nucleotide numbering.

A.

Isolate	***Spp.***	**L22 Amino acid alignment**
U. parvum^{S}	U.p	^{76}VANQGPTMKRTLPRAKGSADQLFKRTTHL^{104}
Q3^{S}	U.p	^{76}VANQGPTMKRTLPRAKGSADQLFKRTTHL^{104}
HPA17^{I}	U.u	^{76}VANQGPTMKRTLPRAKGSADQLFKRTTHL^{104}
HPA32^{I}	U.u	^{76}VANQGPTMKRTLPRAKGSADQLFKRTTHL^{104}
UHWO10^{R}	U.p	^{76}VANQGPTMKRTLPRAKGSADQLFKRTTHL^{104}

.

B.

Isolate	***Spp.***	**L4 Amino acid alignment**
U. parvum^{S}	U.p	^{58}GEVRGGGKKPWRQKHTGKARTGSTRNPHW^{83}
Q3^{S}	U.p	^{58}GEVRGGGKKPWRQKHTGKARTGSTRNPHW^{83}
HPA17^{I}	U.u	^{58}GEVRGGGKKPWRQKHTGKARTGSTRNPHW^{83}
HPA32^{I}	U.u	^{58}GEVRGGGKKPWRQKHTGKARTGSTRNPHW^{83}
UHWO10^{R}	U.p	^{58}GEVRGGGKKPW--KHTGKARTGSTRNPHW^{83}

Figure 5.4. Partial alignments of amino acid sequence from (A) L22 and (B) L4 ribosomal protein from isolates which were macrolide susceptible (^{S}), intermediately resistant (^{I}) and resistant (^{R}). *U. parvum* sequence from isolate ATCC 700970 (Glass *et al.* 2000). U.p corresponds to *U. parvum* while U.u corresponds to *U. urealyticum*. Superscript numbering corresponds to nucleotide numbering.

5.2.6 Tetracycline resistance

From the 61 isolates screened for tetracycline resistance, all isolates, but 1, had an MIC <2 μg/ml and were deemed as sensitive. Only one *U. parvum* clinical isolate (HPA23) was found to be tetracycline and doxycycline resistant (Table 5.2), coincident with the identification of the *tetM* gene by PCR (Figure 5.5). PCR screening of all 61 isolates identified a *tetM*-positive *U. urealyticum* clinical isolate (HPA6) that was susceptible to both tetracycline and doxycycline (Table 5.2). The entire *tetM* gene was sequenced and compared to resistant reference strain SV9 (Vancouver) and HPA23 and susceptible strain HPA6 (Figure 5.6). No mutations in the coding region of the *tetM* gene were found for HPA6 that could account for the inability of a resultant protein to mediate tetracycline resistance, nor was any alteration to the endogenous *tetM* gene promoter found that may have stopped TetM protein expression. Therefore, there was no explanation for the susceptibility of this *tetM*-positive isolate. Interestingly, three amino acid polymorphisms (H209Q, V216L, N223S) were found in HPA23 that did not affect function and were identical to those originally reported for a Tet^r strain isolated in Seattle, Washington, in 1988 (Sanchez-Pescador *et al.* 1988).

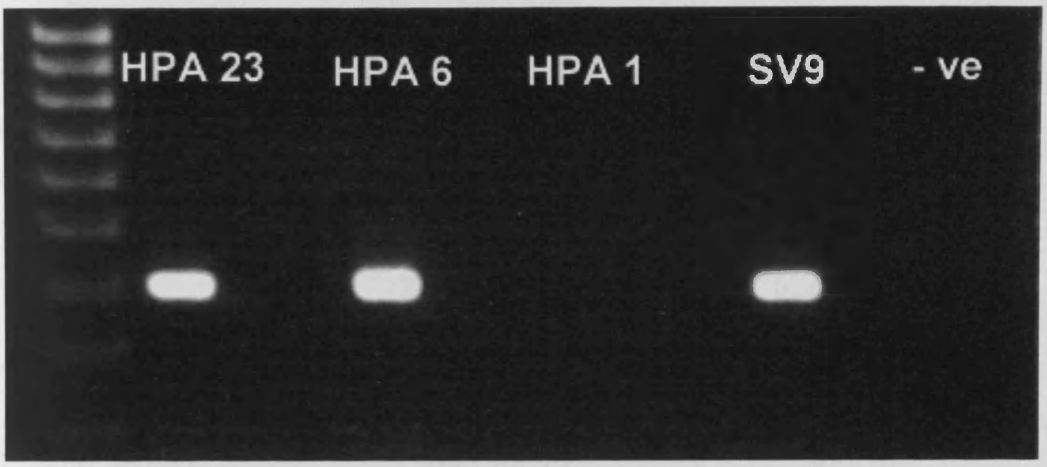


Figure 5.5. Agarose gel showing the results of a PCR looking for the presence of the 397 bp fragment of the *tetM* gene in isolates HPA 23 (tet^R) (lane 2) HPA 6 (tet^S) (lane 4). The *tetM* is gene is present in the positive control (SV9) (lane 4) and absent in the negative controls HPA1 (tet^S) (lane 3) and no DNA (lane 5).

Isolate	Spp.	Amino acid alignment
Seattle^r	N.D	^{202}QEESIRF**H**NCSLFP**V**YHGSAK**N**NIGIDN^{229}
HPA23^r	U.p	^{202}QEESIRF**H**NCSLFP**V**YHGSAK**N**NIGIDN^{229}
HPA6^s	U.u	^{202}QEESIRF**Q**NCSLFP**L**YHGSAK**S**NIGIDN^{229}
SV9 Vancouver^r	U.u	^{202}QEESIRF**Q**NCSLFP**L**YHGSAK**S**NIGIDN^{229}

Figure 5.6. Amino acid alignments demonstrating the amino acid sequence diversity between four different TetM proteins isolated from various tetracycline resistant (^{r}) and susceptible (^{s}) *Ureaplasma*. U.p corresponds to *U. parvum* while U.u corresponds to *U. urealyticum*. Not determined (N.D). Superscript numbering corresponds to nucleotide numbering.

5.2.7 Ciprofloxacin resistance

A single ciprofloxacin-resistant (Cipr) clinical isolate, HPA18, was identified with an MIC of 8 mg/L. The QRDRs of the bacterial gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes, of this isolate as well as sensitive *U. parvum* (UHWQ3) and *U. urealyticum* (UHWW11) controls were sequenced and amino acid sequences were aligned (Figure 5.7). A single base pair change at position 244 (G244A) in the *parC* gene was identified resulting in a codon change and subsequent substitution of aspartic acid for asparagine (D82N) relative to those of other *U. parvum* sequences from susceptible controls from both species. Additional differences were seen between the *U. urealyticum* susceptible control, UHWW11, and both the resistant and susceptible *U. parvum* isolates. Differences between the *U. parvum* and *U. urealyticum* isolates were a D112E substitution in the GyrA protein and a A125T as well as A136T substitutions in the ParC proteins, respectively.

A.

Isolate	Spp.	Amino acid sequence - GyrA
HPA18^{R}	U.p	^{96}PHGDQAVYQTIVRMAQDFSMRYLLVDGHGNFGSIDGDS^{133}
UHWQ3^{S}	U.p	^{96}PHGDQAVYQTIVRMAQDFSMRYLLVDGHGNFGSIDGDS^{133}
UHWW11^{S}	U.u	^{96}PHGDQAVYQTIVRMAQEFSMRYLLVDGHGNFGSIDGDS^{133}

B.

Isolate	Spp.	Amino acid sequence – ParC
HPA18^{R}	U.p	^{81}GNSSIYEAMVRMSQD^{95}-----^{123}TEARLSKIASVMLAN^{137}
UHWQ3^{S}	U.p	^{81}GDSSIYEAMVRMSQD^{95}-----^{123}TEARLSKIASVMLAN^{137}
UHWW11^{S}	U.u	^{81}GDSSIYEAMVRMSQD^{95}-----^{123}TETRLSKIASVMLTN^{137}

Figure 5.7.. Amino acid alignments of GyrA (A.) and ParC (B.) proteins from a ciprofloxacin resistant SV1 (HPA18) and susceptible SV1 (UHWQ3) and *U. urealyticum* (UHWW11) controls. Alignments demonstrate the resistance determining substitution Asp82Asn in HPA18 (highlighted in yellow) which was not present in the sensitive controls, but also the newly identified species-specific differences at positions 112 in GyrA and 125/136 in ParC (filled circle) which had previously been reported as resistance determinants. U.p = *U. parvum*, U.u = *U. urealyticum*. A hyphen indicates a break in amino acid sequence. Superscript numbering refers to *Ureaplasma* protein sequence numbering.

5.2.8 Misreporting of ciprofloxacin resistance in Ureaplasma

5.2.8.1 Phylogenetic analysis of *gyrA, gyrB, parC and parE gene* sequences

From the phylogenetic trees in Figure 5.8, it is clear that a number of species-specific nucleotide polymorphisms occur among the *gyrA*, *gyrB* and *parC* genes causing defined clustering of *U. parvum* and *U. urealyticum*. Within each cluster there is little in the way of intra-species differences with exception to *parC* which contains unique serovar-specific substitutions. The species-specific clustering trend is less clear when examining the tree constructed from *parE* sequences (Figure 5.8d). There is greater sequence homology between *U. urealyticum* SV2, 5, 7, 8, 9 and 11 with *U. parvum* than with the remaining *U. urealyticum* isolates.

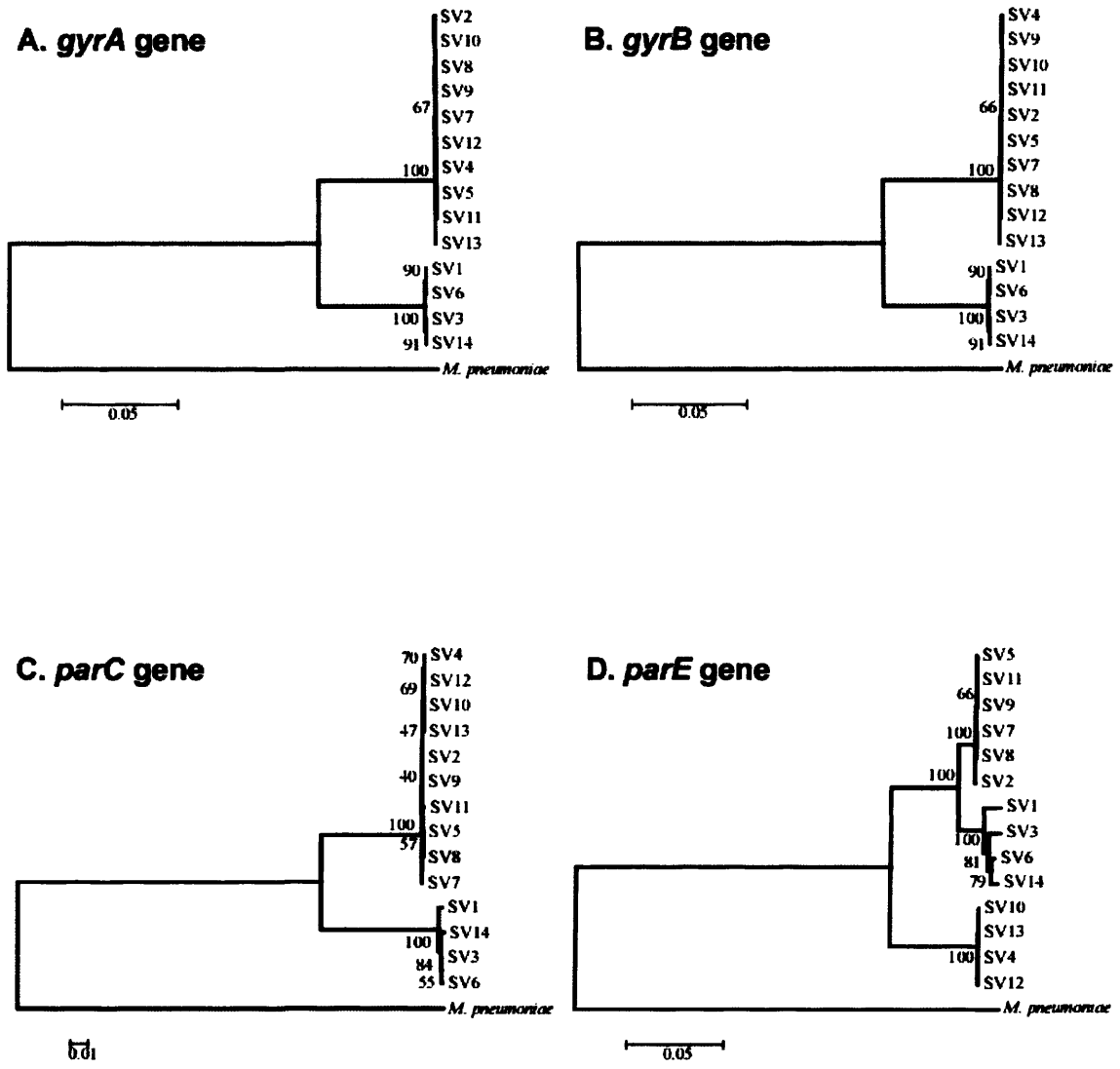


Figure 5.8. Evolutionary relationship of *gyrA* (a), *gyrB* (b), *parC* (c) and *parE* (d) genes of 14 human associated *Ureaplasma* serovars. Sequences were aligned with CLUSTALW and distances calculated using maximum likelihood. The evolutionary history was inferred using the Neighbour-Joining method and the optimal trees after 500 bootstraps. Repeat testing using Jukes Cantor and Tamura Nei distance calculations gave identical tree topology. Trees were rooted using respective gyrase or topoisomerase genes from *Mycoplasma pneumoniae*.

5.2.8.2 List of all serovar and species specific differences

Species-specific differences at both the nucleotide and amino acid levels were found in all gyrase and topoisomerase IV genes and proteins, respectively. Tables in the appendix list the amino acid differences with a total of 39 in GyrA, 26 in GyrB, 107 in ParC and 34 in ParE. GyrA contained a total of 39 amino acid differences of which 37 were species-specific polymorphisms. At position 468 all *U. parvum* and SV4, 5, 7, 8, 9, 11 and 12 of *U. urealyticum* encoded a Lys whereas SV2, 10 and 13 encoded a Glu. This pattern was also true at position 834 in which SV2, 10 and 13 encoded a Lys whereas the remaining *U. urealyticum* encoded a Glu. GyrB contained 26 differences which was the lowest of all the proteins examined.

All GyrB polymorphisms were species restricted with no serovar or intraspecies-specific differences.

A total of 107 differences were noted in the ParC protein, which was the largest number seen within all the proteins, with 102 polymorphisms being species-specific. Deviations from species-specific polymorphism were seen at position 3 for SV3 (Val rather than Asp, which was observed for all *U. urealyticum*), position 735 for SV1 (Thr rather than Ala for *U. parvum* or Met for *U. urealyticum*), Serovar-specific polymorphism were also seen for SV11 (Ala171Val and Ser347Leu) and SV1 (Glu455Lys). All *U. parvum* were also found to be missing Gln835 and Asp836.

ParE encoded a total of 34 differences of which only 9 were species restricted polymorphisms. For 21 of the substitutions there was clustering of *U. parvum* with *U. urealyticum* serovars 2, 5, 7, 8, 9 and 11 whereas serovars 4, 10, 12 and 13 were divergent. A SV14 specific polymorphism was present at Gly106Ser. SV3 contained Asn151, while the remaining *U. parvum* and SV4, 10, 12 and 13 encoded an Asp and the *U. urealyticum* encoded a His. SV1 had two serovar-specific polymorphisms in ParE (Asp249Glu and Ser274Asn) as well as a Val417Thr polymorphism relative to other *U. parvum* SVs, but identical to the 10 *U. urealyticum* sequences.

Table 5.3. GyrA amino acid difference between *Ureaplasma* serovars

	U.p				*U.u*									
Position	1	3	6	14	2	4	5	7	8	9	10	11	12	13
112	D				E									
219	N				D									
220	V				I									
221	T				S									
223	S				P									
228	Y				H									
248	K				N									
287	A				V									
341	P				A									
377	I				V									
383	N				K									
387	K				R									
425	S				A									
428	Q				A									
431	D				G									
464	I				L									
466	V				I									
468	K				E	K	K	K	K	K	E	K	K	E
473	V				I									
475	N				E									
478	N				D									
624	D				E									
660	D				E									
710	N				D									
761	K				R									
774	E				D									
792	K				Q									
827	N				D									
829	-				N									
830	-				D									
831	-				D									
834	-				K	Q	Q	Q	Q	Q	K	Q	Q	K
835	L				T									
836	L				S									
837	Q				H									
838	E				G									
840	D				E									
841	Y				H									
844	K				E									

Amino acids are written by the single letter coding system. Numbering refers to *Ureaplasma* protein number. The amino acid in the first column is conserved to the right until otherwise indicated.

Table 5.4. GyrB amino acid difference between *Ureaplasma* serovars

	U.p				*U.u*									
Position	1	3	6	14	2	4	5	7	8	9	10	11	12	13
8	K				N									
62	I				V									
64	R				K									
67	L				I									
72	S				V									
73	V				I									
74	I				V									
75	E				G									
142	H				Y									
145	Y				H									
161	P				I									
164	N				D									
167	V				I									
215	L				M									
233	T				A									
245	L				A									
256	R				K									
262	E				D									
315	V				I									
342	V				I									
556	A				G									
570	R				K									
575	I				L									
576	S				L									
578	I				T									
632	A				T									

Amino acids are written by the single letter coding system. Numbering refers to *Ureaplasma* protein number. The amino acid in the first column is conserved to the right until otherwise indicated.

Table 5.5. ParC amino acid difference between *Ureaplasma* serovars

	U.p				*U.u*									
Position	1	3	6	14	2	4	5	7	8	9	10	11	12	13
3	D	V	D	D	V									
5	H				Q									
59	Y				H									
125	A				T									
136	A				T									
171	A				A	A	A	A	A	A	A	V	A	A
181	N				S									
205	V				I									
210	E				K									
241	I				V									
245	C				S									
247	D				E									
250	I				T									
251	N				S									
252	D				E									
261	S				N									
300	I				V									
310	I				V									
324	N				S									
345	N				S									
347	S				S	S	S	S	S	S	S	L	S	S
372	I				V									
400	E				D									
408	T				A									
412	V				T									
414	N				A									
440	K				R									
444	Q				E									
445	T				S									
448	D				Q									
449	E				Q									
454	Q				K									
455	K	E	E	E	E									
458	N				K									
477	T				E									
480	F				Y									
481	E				Q									
490	L				F									
491	V				I									
493	V				A									
495	I				V									
505	N				S									
539	I				L									
545	I				V									
551	K				R									
565	V				I									
570	T				S									
571	S				C									
585	V				A									
595	I				V									
599	W				C									
605	I				V									
608	Y				H									
613	A				G									
615	R				K									
625	S				N									
629	I				L									

643	D				E
646	N				G
656	G				D
661	I				V
662	I				V
670	A				V
672	L				M
699	V				I
701	A				S
704	T				V
711	N				S
712	V				I
717	E				D
718	R				Q
720	S				N
724	N				S
735	T	A	A	A	M
738	T				S
739	N				P
742	P				L
743	I				P
747	V				A
749	N				S
757	A				G
758	H				N
767	Q				E
777	S				P
779	P				L
782	T				I
786	T				S
789	S				N
790	I				K
795	Q				L
796	M				I
804	C				S
809	E				S
810	A				V
818	N				S
826	N				S
828	M				L
831	I				T
833	K				E
835	-				Q
836	-				E
837	H				Q
840	S				L
846	D				N
848	E				K
851	K				N
852	K				D

Table 5.5. continued. Amino acids are written by the single letter coding system. Numbering refers to *Ureaplasma* protein number. The amino acid in the first column is conserved to the right until otherwise indicated.

Table 5.6. ParE amino acid difference between *Ureaplasma* serovars

	U.p				*U.u*									
Position	1	3	6	14	2	5	7	8	9	11	4	10	12	13
8	N				N						S			
33	T				A									
39	I				V									
59	N				N						S			
61	I				I						V			
70	V				V						I			
83	S				S						P			
106	S	S	S	G	S									
128	S				S						A			
145	I				I						S			
151	D	N	D	D	H						D			
153	S				S						P			
180	V				V						F			
185	I				I						T			
203	I				I						S			
210	K				K						N			
218	N				N						D			
231	A				T									
240	Y				Y						F			
242	R				K									
243	S				S						N			
244	N				N						T			
249	E	D	D	D	D									
252	I				I						V			
274	N	S	S	S	S									
299	N				N						D			
377	L				L						V			
417	T	V	V	V	T									
542	N				N						T			
588	N				S									
589	N				K									
594	K				R									
595	I				V									
619	K				R									

Amino acids are written by the single letter coding system. Numbering refers to *Ureaplasma* protein number. The amino acid in the first column is conserved to the right until otherwise indicated.

5.2.8.3 Table of past quinolone resistant isolates

A summary of all previous studies reporting fluoroquinolone-resistant *Ureaplasma* strains is presented in Table 5.7. Prior to December 2008, the molecular characterisation of 33 isolates, including HPA18 described here, had been described. Of these isolates, 15 were identified by species: three *U. parvum* isolates and 12 *U. urealyticum* isolates. (Ligon and Kenny, 1991; Blanchard, 1990; Andrews *et al.* 2000; De Silva and Quinn, 1986; De Silva and Quinn, 1991; Smith *et al.* 1993) All *U. urealyticum* isolates described contained the Asp112Glu/Ala125Thr/Ala126Thr polymorphism, while the *U. parvum* isolates did not. For the remaining 18 non-speciated strains, we can predict which species they were likely to be by using the amino acid consensus for GyrA, ParC and ParE proteins. Using this method, we can predict that one quarter were *U. parvum* and the remainder were *U. urealyticum*. If substitutions that have found to be species-specific polymorphisms in the fluoroquinolone-susceptible strains are discounted then, only 22 of the 33 isolates have unique substitutions that could contribute to a fluoroquinolone-resistant phenotype. Therefore, that would mean that no mutation in the topoisomerase and gyrase genes and no potential mechanism of resistance had been identified for the remaining 11 isolates. The unique mutations that would remain candidates for resistance mechanism include Gln100Arg (1) and Gln104Lys (1) in GyrA, Asp82Asn (1), Ser83Leu (19), Glu87Lys (1), Ala88Gly (1), Ser734Gly (1), Ala735Thr (1) in ParC and Asp249Gln (1), Ser274Asp (1), and Ala457Thr (1) in ParE. However, some of these substitutions occur in the same isolate (i.e. all the ParE mutations were

found in the same isolate), which may indicate that multiple substitutions are required to mediate a resistant phenotype.

Table 5.7. Summary of mutations described for fluoroquinolone resistant *Ureaplasmas* in gyrase and topopisomerase genes between 2000 – 2009.

Author	Isolate	*Spp.*	MIC (mg/L)			Amino acid substitution																			
						GyrA			ParC											ParE				
			CIP	OFX	MXF	100	104	112	3	59	82	83	87	88	125	136	301	734	735	151	249	274	417	457
Bebear *et al.*, 2000	UUa	U.u*	64	64	4	***Arg***		Glu							Thr	Thr								
Zhang *et al.*, 2002	19	-	32	16	N/A							***Leu***												
	52	-	≥64	8	N/A			Glu				***Leu***												
	70	-	64	8	N/A			Glu				***Leu***												
	87	-	64	8	N/A			Glu																
	92	-	128	16	N/A			Glu				***Leu***												
	104	-	64	8	N/A			Glu				***Leu***												
	105	-	128	16	N/A			Glu																
	107	-	64	8	N/A			Glu				***Leu***												
	108	-	64	8	N/A							***Leu***												
	131	-	32	8	N/A			Glu																
	176	-	64	8	N/A			Glu				***Leu***												
	215	-	16	8	N/A			Glu																
	224	-	64	8	N/A			Glu				***Leu***												
Bebear *et al.*, 2003	UUe2	U.p	8	8	1							***Leu***												
	UUb	U.u	4-16	4-8	0.5-1			Glu							Thr	Thr								
	UUd	U.u	4-16	4-8	0.5-1			Glu							Thr	Thr								
	UUf1	U.u	4-16	4-8	0.5-1			Glu							Thr	Thr								
	UUg1	U.u	4-16	4-8	0.5-1			Glu							Thr	Thr								
	UUf2	U.u	16	4-8	1			Glu				***Leu***			Thr	Thr								
	UUg2	U.u	16	4-8	1			Glu				***Leu***			Thr	Thr								
	UUg3	U.u	16	4-8	1			Glu				***Leu***			Thr	Thr								
	UUg4	U.u	16	4-8	1			Glu				***Leu***			Thr	Thr								
	UUg5	U.u	16	4-8	1			Glu				***Leu***			Thr	Thr								
	UUc	U.u	8	8	1			Glu					***Lys***		Thr	Thr								
Duffy *et al.*, 2006	48105	U.p	N/A	128	8		***Lys***		Asp			***Leu***		***Gly***			***Gly***	***Thr***	Thr	Asp	Glu	Asp	Thr	***Thr***
Xie *et al.*, 2006	163	-	1	1	N/A																			
	168	-	>2	<1	N/A																		Thr	
	46	-	>2	>4	N/A			Glu				***Leu***			Thr								Thr	
	161	-	>2	>4	N/A							***Leu***												
	7	-	>2	>4	N/A			Glu				***Leu***											Thr	
Geissdorfer *et al.*, 2008	CM239	U.u	>2	1	N/A			Glu		His					Thr	Thr								
Beeton *et al.*, 2009	HPA18	U.p	8	N/A	N/A						***Asn***													
*U. parvum***						Gln	Gln	Asp	Asp[1]	Tyr	Asp	Ser	Glu	Ala	Ala	Ala	Ser	Ala	Ala[2]	Asp[3]	Asp[4]	Ser[5]	Va[6]	Ala
*U. urealyticum****						Gln	Gln	Glu	Val	His	Asp	Ser	Glu	Ala	Thr	Thr	Ser	Ala	Met	His[3]	Asp	Ser	Thr	Ala

* Species determined in the later paper from Bebear *et al.*, 2003. – denotes that the species was not determined. U.u = *U. urealyticum*. U. p = *U. parvum*. N/A represents the antibiotic was not examined. Amino acids in bold and in italics are non-species or serovar specific differences and are potentially the reason for the resistant phenotypes seen. ** The consensus amino acid sequences for all sequenced *U. parvum*. *** The consensus amino acid sequences for all sequenced *U. urealyticum*. [1] SV3 has a Val at this position. [2] SV1 has a Thr at this position. [3] SV3 has Asn at this position; SV1, 6, 14, 10, 12, 13, 4 have Asp at this position; SV 2, 5, 7, 8, 9, 11 have a His at this position. [4] SV1 has a Glu at this position. [5] SV1 has a Asp at this position. [6] SV1 has a Thr at this position.

5.3 Discussion

5.3.1 Species distribution

The species distribution of 80 % *U. parvum* to 20 % *U. urealyticum* seen among the collection of 61 samples was consistent with previous reports (Schelonka and Waites, 2007). From the 16 *U. parvum* isolates serotyped, SV3 was found to be the most common (eight) followed by SV1 (four) with equal numbers of SV2 and SV14 (two each). Previous reports have also found SV3 to be the most commonly isolated serovar (Nelson *et al.* 1998; Cassell *et al.* 1993) and was the basis of choice when undertaking sequencing the first *U. parvum* genome (Glass *et al.* 2000).

5.3.2 Analysis of the method used

The primary advantage to using the modified breakpoint methodology described here, as well as full plate method, is the simultaneous determination of input inoculum for determination of susceptibility at the 10^4 CCU inoculum. The absence of prior inoculum size determination, unlike with previously described methods (Waites *et al.* 1992), meant that the turn around time from isolation of bacteria to sensitivity profile was greatly reduced with a preliminary idea of sensitivity available at 24 hours with full confirmation at 48 hours. This could be of significant clinical importance in cases of *Ureaplasma* meningitis in neonates where rapid administration of effective therapy would be crucial. Although 10^4 CCU were used as the standardised inoculum size to read the MIC, Figures 5.1a-c and 5.2a-b show clearly the difference between resistant

and sensitive isolates. In the breakpoint method seen in Figure 5.1 the resistant isolates were able to grow in antibiotic containing media even at a low inoculum of 10 CCU whereas sensitive isolates caused colour change at $10^5 - 10^6$. Although the full 96 well plate can be seen as seven times more expensive than standard microbroth dilution technique, due to assessing the MIC at multiple bacterial loads, it did produce an accurate and rapid method of determining the antibiotic sensitivity profile of an *Ureaplasma* isolate without prior knowledge of inoculum size. The method was validated by the use of BSAC defined control isolates (Table 5.1) which gave comparable MIC values to that expected. With regards to the breakpoint values set, these were several-fold greater than those recommended for the closet *Ureaplasma* match, *Moraxella catarrhalis*. For example *M. catarrhalis* with an erythromycin MIC of greater than 0.5 µg/ml would be regarded as resistant whereas the breakpoint for erythromycin resistance in *Ureaplasma* was set higher at 4 µg/ml. This was due to the slightly acidic pH of the USM media used (pH 6.6) raising the MIC. A second example was the higher breakpoint for ciprofloxacin resistance in *Ureaplasma* (4 µg/ml) relative to that of *M. catarrhalis* (0.5 µg/ml). Although the breakpoints were higher than suggested by BSAC for organisms such as *M. catarrhalis*, they were in accordance with recommendations by C. M. Bebear (personal communication).

5.3.3 Macrolide resistance

Erythromycin resistance in bacteria can be mediated by a number of different routes including active drug efflux, post-transcriptional modification of the drug target via methylation or by point mutations at or adjacent to residue A2067

(A2058 *E. coli* numbering) in the 23S rRNA. Mutations in the L4 and L22 ribosomal proteins have also been linked to erythromycin resistance. Bacteria with a low number of 23S rRNA operons, such as mycoplasmas, mutations in the 23S rRNA and/or the L4 and L22 ribosomal proteins appear to be the most commonly found (Vester and Douthwaite, 2001).

This investigation identified one highly resistant erythromycin resistant isolate (MIC, >64 mg/L); however, five additional isolates each consistently showed an MIC of 8 mg/L, which is higher than the range of 0.02 to 4 mg/L listed in the Cumitech 34 reference manual (Waites *et al.* 2001). Interestingly, 4 out of 5 of the less susceptible isolates were *U. urealyticum*, which represented 33% of the total *U. urealyticum* isolates screened, suggesting a possible inherently increased tolerance of *U. urealyticum* isolates to erythromycin relative to that of *U. parvum* isolates. Little information is available about the mechanism of erythromycin resistance in *Ureaplasma* spp. In fact, this is the first full molecular characterization of a clinical *Ureaplasma* isolate with erythromycin resistance. The physiological mechanism of erythromycin resistance was first indicated by Palu *et al.* (Palu *et al.* 1989) almost 20 years ago, who demonstrated reduced radiolabeled erythromycin binding to ribosomes of resistant *Ureaplasma* compared to that of a susceptible strain. However, technical advances since that report have greatly enhanced the ability to investigate molecular mechanisms. Investigation of other macrolide-resistant bacteria has identified mutations that inhibit erythromycin binding to the bacterial 50S ribosomal complex, where it exhibits bacteriostatic activity through inhibition of protein synthesis. Pereyre *et al.* serially passaged a *U.*

parvum reference strain 45 to 50 times in increasing amounts of erythromycin and sequenced associated genes: two 23S rRNA operons and L4 and L22 ribosome-associated proteins (Pereyre *et al.* 2007). They consistently found mutation of the erythromycin-binding site around nucleotide position 2067 (2058 numbering based on homology to *E. coli*) near the peptidyl transferase loop in domain V of one of the 23S rRNA operons and occasionally found additional mutation of the associated L4 or L22 protein. Dongya *et al.* identified a number of point mutations within the 23S rRNA of 18 clinical isolates with various degrees of resistance to the macrolides josamycin, clarithromycin, roxithromycin, and azithromycin; however, they did not investigate resistance to erythromycin (Dongya *et al.* 2008). Interestingly, none of the mutations described were in the region of nucleotide 2067. The investigation carried out here did not find mutations in either the 23S rRNA operon or the L22 protein but did detect a deletion of two adjacent amino acids in the L4 protein for the highly resistant strain (MIC, >64 mg/L). No mutations were found in the isolates with an erythromycin MIC of 8 mg/L, yet compensating mutations outside of the investigated regions, DNA methylation, expression of bacterial proteins that modify macrolides, or increased drug efflux via ion channels may have been responsible for the relative increase in tolerance to erythromycin for these strains. Kenny and Cartwright previously showed that susceptibility to erythromycin was reduced in acidic growth medium; however, medium pH had to fall below 6.5 before an effect was observed (Kenny and Cartwright, 1993). As our USM had a pH of 6.65, the MICs were determined relative to each other in the same batch of USM, and the findings were consistent upon repeated testing. As a result, the reduced

susceptibility of the five isolates was not due to a pH artefact for the USM used. Mutations within the highly conserved region of L4 ribosomal protein have previously been noted in both laboratory-derived and clinically isolated macrolide-resistant pneumococci and *E. coli* strains (Chittum and Champney, 1994; Tait-Kamradt *et al.* 2000b; Tait-Kamradt *et al.* 2000a). Both a substitution mutation (G69C) and an insertion mutation (6-bp insertion between the codons encoding Q67 and K68) were found in two resistant pneumococcus strains (Tait-Kamradt *et al.* 2000b; Tait-Kamradt *et al.* 2000a). Clinical isolates found by the same group made up a distinct clonal cluster which contained a substitution from 69GTG71 to 69TPS71 as well as one isolate which contained a 6-amino-acid insertion, REKGTG, after a glycine at position 71 within the same region (Tait-Kamradt *et al.* 2000b). A similar mutation (K63E) within L4 of *E. coli* has also been found to be associated with resistance to erythromycin (Chittum and Champney, 1994) and was located adjacent to the deletion described here. The resultant three-dimensional alteration to the entire ribosomal complex as a result of the structural L4 mutation must prevent the interaction of the C5 sugar of the macrolide with the residues around A2067 (A2058 *E. coli* numbering) and abrogate the ability of erythromycin to inhibit protein synthesis in the resistant strain (Poehlsgaard and Douthwaite, 2002).

5.3.4 Tetracycline resistance

Currently, the only known mechanism of tetracycline resistance for *Mollicutes* is the presence of the *tetM*-transferable genetic element. It was first described in *Ureaplasma* by Roberts and Kenny in 1986 (Roberts and Kenny, 1986) and

has been the focus of investigations by several groups since then. Unlike the studies by Robertson *et al.* (Robertson *et al.* 1988), who found that all 26 of their Tet^r isolates were also resistant to erythromycin at or above 2 mg/L, our control SV9 Tet^r and clinical Tet^r isolates were susceptible to erythromycin (MIC, 4 mg/L). Blanchard *et al.* (Blanchard *et al.* 1992) have previously shown that screening *Ureaplasma* isolates by PCR readily identified Tet^r strains. However, we found a tetracycline-susceptible strain (HPA6) that screened *tetM* positive. Recently, Degrange *et al.* identified two *tetM*-positive *Mycoplasma hominis* isolates that were tetracycline susceptible (Degrange *et al.* 2008). One of these *M. hominis* isolates had a 1,260 bp insertion in the leader peptide sequence (likely preventing successful transcription), while no mutations were found within the *tetM* gene or promoter region of the second isolate. Like the latter case for our Tet^s *tetM*_HPA6 strain, we could find no explanation for the susceptibility when comparing the sequence to that of the control Tet^r SV9 (Vancouver) strain. Interestingly though Degrange *et al.*, found that if the Tet^S *tetM* positive isolate was serially sub-cultured in increasing levels of doxycycline the MIC rapidly increased suggesting that doxycycline is a greater inducer of resistance than tetracycline. A further explanation for the lack of expression may be due to gene silencing. A study by Enne *et al.*, demonstrated that in a selection of *E. coli* isolates, all of which containing the pVE46 resistance plasmid, a sub-population were phenotypically sensitive (Enne *et al.* 2006). The plasmid DNA sequence of the sensitive isolates were found to be identical to that of the resistant strains, but interestingly when the plasmid from the sensitive strain were introduced to a different *E. coli* strain, the resistant phenotype was restored. This

suggested that chromosomal difference were to explain these differences in gene silencing and could potentially be the mechanism for *tetM* silencing in HPA6. We also found that our Tet^r strains were resistant to both tetracycline and doxycycline, which is in contrast with the findings of Blanchard *et al.* (Blanchard *et al.* 1992) who identified that from 21 clinical Tet^r *Ureaplasma* isolates 8 were resistant to doxycycline, 2 were intermediate, and 11 were susceptible. It has been proposed that doxycycline is less of an inducer of *tetM* transcription in some organisms than tetracycline. In this study, we demonstrated that our clinical tetracycline-resistant isolate HPA23, which harboured a functional TetM protein, was additionally resistant to doxycycline.

5.3.5 Quinolone resistance

Ciprofloxacin was used as a representative of the bactericidal fluoroquinolone antibiotic family that targets two bacterial topoisomerases, namely topoisomerase IV and DNA gyrase. Numerous mechanisms have been described by which bacteria can mediate resistance to fluoroquinolones, including mutation of the topoisomerase genes, decreased membrane permeability, active drug efflux, modification by a fluoroquinolone-inactivating enzyme, or the presence of a Qnr protein (Robicsek *et al.* 2006). In this study a point mutation was identified which lead to an D82N amino acid substitution, two amino acids downstream from the proposed active site of the ParC protein (Morais Cabral *et al.* 1997). Six separate groups have investigated a total of 32 resistant isolates and reported mutations in *Ureaplasma* genes associated with fluoroquinolone resistance (Bebear *et al.* 2003; Bebear *et al.* 2000; Moser *et al.* 2006; Geissdorfer *et al.* 2008; Xie

and Zhang, 2006; Zhang *et al.* 2002). Nineteen of 32 fluoroquinolone-resistant strains investigated by these groups had the same S83L mutation in the ParC protein (or S80L, if using homology to the *E. coli* position to identify the location); furthermore, similar mutations in ParC have been found in fluoroquinolone-resistant *S. aureus* and *Streptococcus pneumoniae*, which are reported to have MICs similar to those of resistant *Ureaplasma* strains (Ince and Hooper, 2000). The mutation in the Cip^r isolate D82N is adjacent to this region, and this isolate has a ciprofloxacin MIC similar to that of isolate UUc identified by Bebear *et al.* (Bebear *et al.* 2003), who also found an E87K mutation in the near vicinity, indicating this is a dominant target for fluoroquinolone resistance.

5.3.6 Misreporting of polymorphisms associated with resistance

5.3.6.1 Alignment of amino acid sequences

Previously, the combined substitutions of Asp112Glu in the GyrA protein along with Ala125Thr and Ala136Thr in the ParC protein have been highlighted in fluoroquinolone-resistance in *Ureaplasma* (Bebear *et al.* 2000; Bebear *et al.* 2003; Geissdorfer *et al.* 2008; Xie and Zhang, 2006; Zhang *et al.* 2002). The alignment data in Figure 5.7 compared the QRDR of a ciprofloxacin-resistant *U. parvum* (HPA18) with susceptible *U. parvum* (UHWQ3) and *U. urealyticum* (UHWW11) controls. Interestingly both the *U. parvum* isolates, including the ciprofloxacin resistant HPA18, had the profile of previously described sensitive isolates whereas the susceptible *U.*

urealyticum, UHWW11, had the profile which had been previously linked to resistance. This evidence formed the basis for the further investigation of amino acid polymorphisms for all serovars of both *U. parvum* and *U. urealyticum* species.

5.3.6.2 Species- and serovar-specific amino acid differences

Phylogenetic analysis demonstrated distinct clustering of species in three out of the four genes analysed (Figures 5.8 A-D). These data support the decision to separate the human associated *Ureaplasmas* into two distinct species based on previous genotypic traits (Robertson *et al.* 2002). However, an unexpected and unexplainable divergence was noticed between SV4, 12, 10 and 13 from the remaining *U. urealyticum* in the *parE* tree. Additionally serovar-specific substitutions were noted for SV1 and 11 in the ParC and SV1, 3 and 14 in ParE. These species and serovar-specific substitutions will be important for comparison of resistant and susceptible strains in the future to avoid incorrect identification of potential resistance mechanisms.

5.3.6.3 Review of previously described mechanisms of fluoroquinolone resistance in *Ureaplasma*.

Between 2000 and 2009 thirty-two fluoroquinolone resistant *Ureaplasma* strains including the one described here, from 7 different investigations were identified, of which amino acid substitution in the type II topoisomerase

proteins were proposed as the molecular mechanism of resistance (Bebear *et al.* 2000; Bebear *et al.* 2003; Geissdorfer *et al.* 2008; Xie and Zhang, 2006; Zhang *et al.* 2002; Moser *et al.* 2006). The substitutions which were proposed to result in the resistant phenotype are summarised in Table 5.7 and fully described as follows.

In 2000 Bebear *et al.*,. (Bebear *et al.* 2000) described the molecular characterisation of a ciprofloxacin resistance in *Ureaplasma*. The sequence from isolate UUa, which was later to be determined to be *U. urealyticum*, was compared with the published SV3 genome (ATCC 700970) (Glass *et al.* 2000) and identified to contain four amino acid transitions which were proposed to be related to resistance: Gln100Arg and Asp112Glu in GyrA and Thr125Ala and Thr136Ala in ParC. The evidence presented in here suggests that only Gln100Arg could potentially be responsible for the ciprofloxacin MIC of >128 mg/L with the remaining three differences all being species-specific polymorphisms.

A report by Zhang *et al.*, (Zhang *et al.* 2002) found a total of 13 resistant isolates with varying degrees of resistance to fluoroquinolones. Nine of the thirteen isolates had a Ser83Leu substitution within the ParC protein. Unfortunately, they did not determine the species of the isolates, but based on the GyrA sequence data it can be speculated that 11 out of 13 isolates were *U. urealyticum* with 2 out 13 being *U. parvum*. The Ala125Thr and Ala136Thr mutations were not reported in the suspected *U. urealyticum* isolates, but it is unsure if this is because the investigators did not sequence this portion of the

gene. As the primers used were not included in the manuscript, it has not been possible to speculate further on this point. Interestingly the authors not only compared the resistant isolate sequences with the SV3 genome, but also with *U. urealyticum* controls from SV4 (ATCC27815) and SV8 (ATCC27618) with the intention of ruling out species-specific polymorphism. Twelve base differences within *gyrA* and 10 within *parC* were identified, but the deduced amino acid sequences were quoted as being identical between serovars.

Subsequent investigations by Bebear *et al.* in 2003 examined a further 11 resistant isolates and compared sequence data with the SV3 genome and a sensitive control UUe1.(Bebear *et al.* 2003) As would be predicted, resistant *U. urealyticum* isolates were consistently found to have the species-specific polymorphism at amino acid 112 in GyrA and 125 and 136 in ParC. The two *U. parvum* isolates examined, one resistant (UUe2) and the sensitive control (Uue1), did not contain these substitutions. Six of the eleven isolates contained the Ser83Leu mutation with a single isolate containing a unique mutation in the ParC protein only 5 amino acids downstream for isolate UUc (Glu87Lys).

The first molecular characterisation of fluoroquinolone resistance in the United States was provided by Duffy *et al.*, who reported a *U. parvum* isolate designated 48105 with an ofloxacin MIC of 128 mg/L.(Moser *et al.* 2006) This isolate was documented as having 12 mutations resulting in the resistant phenotype including Ser83Leu, but from the data in Table 5.5, six of these substitutions can be ruled out as species or serovar-specific polymorphisms.

The Val3Asp mutation in ParC was proposed as a resistant determinant but this substitution turns out to be a serovar-specific substitution for SV3 (which was used as the comparator for their sequence); consensus sequence for SV1, 6 and 14 shows Asp at this position (Table 5.5). Although the serovar of this isolate was not determined it can be hypothesised that it was likely to be SV1 as they also identified the SV1-specific polymorphisms found at position 735 in ParC and positions 249, 274, 417 in ParE (Tables 5.5 and 5.6, respectively).

Investigations of resistance in *Ureaplasma* isolates from China between 1999 and 2004 was reported by Xie *et al.* (Xie and Zhang, 2006) They sequenced the QRDR region from the resistant isolates and three of the five isolates contained the common Ser83Leu resistance-associated mutation. However, comparison of their reported sequences do not align fully with the determined consensus sequences stated in this project. For example isolates 161, 163 and 168 all show the polymorphisms that identify them as *U. parvum* (Asp112 in GyrA and Ala125 and Ala136 in ParE) and isolate 168 also had Thr417 in ParE suggesting that it was an SV1. However, isolates 7 and 46 had Glu112 in GyrA suggesting they were *U. urealyticum*, but isolate 46 was had Ala125 and Ala136 in ParE consistent with *U. parvum* consensus sequences and isolate 7 had Thr125 but Ala136 giving a mixture of the consensus sequences in ParE. We are unable to explain this mixing of consensus sequences. However, isolates 163 and 168 do not have any mutations that are not also found in susceptible *U. urealyticum* or *U. parvum* isolates.

Geissdorfer *et al.* characterised isolate CM239 from a 38 year old male who developed meningitis following kidney transplantation.(Geissdorfer *et al.* 2008) Comparison of the reported sequence against the consensus Tables 5.5 – 5.8 identifies the isolate as belonging to *U. urealyticum*, but no unique resistance candidate mutations remain after species-specific polymorphisms are taken into account.

By critically examining the ciprofloxacin resistant isolate in this study only a single and unique Asp82Asn mutation within the ParC protein was identified. This was immediately adjacent to the commonly found Ser83Leu mutation and is therefore a strong candidate as a resistance-mediating mutation.

This review of the literature highlights that 75% of the fluoroquinolone resistant strains identified to date were *U. urealyticum*, even though this species is isolated much less frequently from patients (20% of patient isolates in this study, which is consistent with other group's findings). (Schelonka and Waites, 2007) Furthermore, the ParC Ser83Leu mutation was found in 58% of the fluoroquinolone resistant *Ureaplasma* strains, which is homologous to the resistance-mediating mutation identified in many other fluoroquinolone resistant bacteria including *S. aureus* and *S. pneumoniae*. (Piddock, 1999) Fluoroquinolones are known to have preferential sites of action: the gyrase proteins for Gram-negative bacteria and the topoisomerase IV proteins for Gram-positive bacteria. (Hooper, 1999) The proposed evolution of *Ureaplasmas* from Gram-positive progenitors may be responsible for the higher incidence of mutations within the topoisomerase IV proteins relative to

gyrase proteins. As a result of this analysis the molecular mechanism of fluoroquinolone resistance in 11 out of the 33 isolates is still undetermined. Resistance in these isolates may have been due to mutation outside of the sequenced regions or through alternative mechanisms such as altered membrane permeability. (Hooper, 1999)

5.4 Summary

In conclusion, a reliable method to determine antimicrobial susceptibility of *Ureaplasma* isolates without prior knowledge of inoculum size is now available. Although this is the second molecular investigation of macrolide resistance in clinical *Ureaplasma* isolates, more erythromycin resistant strains are required to determine the prevalence of mutations in the relevant genes associated with resistance. Screening for the *tetM* gene can identify tetracycline-resistant strains; however, the finding of a false-positive strain, raises a note of caution for the use of PCR screening for antibiotic resistance. It appears that mutations within an eight amino-acid region of the topoisomerase IV protein is commonly found in fluoroquinolone-resistant *Ureaplasma*, with S83L being the dominant mutation. Additionally this chapter highlights the importance of determining the species of *Ureaplasma* and the caution which should be taken when comparing future sequence data for both fluoroquinolone resistance and other genes potentially associated with antibiotic resistance. To avoid similar misinterpretation, sequences should be compared with the appropriate species and in some circumstances the appropriate serovar.

Chapter 6

General discussion

6.1 Discussion

6.1.1 General discussion

This thesis has addressed and answered a diverse range of novel research questions with the central theme revolving around bacteria from the genus *Ureaplasma*. The first results chapter identified the essential role for the classical complement activation pathway for the killing of *U. parvum* in an *in vitro* system. The second chapter examined the consequences behind a failure to clear *Ureaplasma* in a neonatal population and development of the disease chronic lung disease of prematurity (CLD). In addition the role of general bacterial infection was also examined. Finally, this thesis addressed the potential complications in treatment of *Ureaplasma* infection, such as CLD, with relation to antibiotic resistance.

Ureaplasma have been linked to disease such as non-gonococcal ureithritis as well as septic arthritis in hypogammaglobulinemic patients, but the patient group which has been the focus of this thesis have been preterm neonates. *Ureaplasma* have been strongly linked to an infectious cause of premature birth where the organism can additionally be transmitted from mother to child either *in utero*, as a result of an ascending infection from the mother's urogenital tract, or acquisition during birth. Premature birth is the largest risk factor for the development of CLD, a disease whereby the immature status of the neonates lung results in a dependence upon artificial ventilation which extends past the time at which the neonate should have been born (36 weeks).

The rates of lung colonisation by *Ureaplasma* as well as development of CLD has, on numerous occasions, been shown to be inversely proportional to gestational age (Chua *et al.* 1998; Izraeli *et al.* 1991). My data from a cohort of 192 neonates agreed with theses finding as seen in Figure 4.7 of chapter 4. Those infants born at 23 – 26 weeks gestational age were more likely to have lung colonisation by *Ureaplasma* relative to those born nearer to term gestation of 37 weeks as well as more likely to develop CLD. All neonates born between 23 and 25 weeks who were colonised by *Ureaplasma* developed CLD, but although the rate of colonisation at 26 weeks was comparable with those at 25 weeks, the number of those who went on to develop CLD was lower at less than 20 % compared with 100 %. Admittedly some infants born at 26 weeks died before a diagnosis of CLD could be made, but this data may represent some form of gestational-dependent developmental factor which allows the infant to cope with the inflammatory stimuli of the bacteria, or ventilation-induced damage, at 26 weeks relative to that of 25. The role of other bacterial species was not overlooked. The data presented in this thesis demonstrated a role of Gram negative bacteria in the development of CLD. As progression of CLD is driven by an uncontrolled and self-harming immune response, my data suggested a role in CLD for pro-inflammatory LPS containing Gram negative bacteria (chapter 4, Table 4.5). These data were supported by my finding of a statistically significant association between inflammatory cytokines IL-6 and IL-8 with the presence of infection and development of CLD (chapter 4, Figure 4.3).

Although data, such as that which has been presented in this thesis, implicates a role for *Ureaplasma* in the causation of CLD, the associated is still highly debated. One means to define *Ureaplasma* as a causative agent of CLD is to run a randomised clinical trial examining the effects of antibiotics on eradicating the presence of *Ureaplasma* in the lungs of preterm neonates and examining the impact on disease development. Two factors exist which could potentially confound the results of such a trial. Firstly the antibiotics of choice, the macrolides, have known anti-inflammatory properties (Jaffe and Bush, 2001). If *Ureaplasma* are not contributing a role to the development of CLD, this would be missed as the dampening down of the inflammatory response, the driver of disease progression, would be a result of the drug and not the bacteria. The second confounding factor would be the presence of antibiotic resistant clones. If *Ureaplasma* are a major factor in the development of CLD, the presence of resistant strains, which would not be cleared by therapy, would affect the results obtained. To help over come this second confounding factor I have, as presented in chapter 5, developed a method to rapidly identify the presence of resistant strains to a selection of antibiotics as well as determining the molecular mechanisms of resistance to resistant strains found. From this study of 61 strains a single highly resistant erythromycin strain (UHWO10) was isolated from a premature baby (O10). The resistant phenotype was a result of a two amino acid deletion within the L4 ribosomal protein, which was surprising as resistance was hypothesised to result from a point mutation within a region close to the nucleotide at position 2058 in the 23S rRNA as seen in other bacteria with low numbers of ribosomal operons. The L4 deletion resulted in resistance to erythromycin and clarithomycin, but

retained sensitivity to azithromycin. So from these data the issue of azithromycin resistant *Ureaplasma* impacting on a clinical trail is unlikely, but this is not to say that azithromycin resistant strains do not exist. Several laboratory generated resistant strains have been produced with levels of resistance ranging from 8 to >2048 μg/ml (Pereyre *et al.* 2007). Although the antibiotics from the tetracycline and fluoroquinolone families are unlikely to be used in neonatal therapy, the incidence and mechanisms of resistance were still examined. As with the macrolides only a single ciprofloxacin resistant isolate (HPA18) and a single tetracycline resistant isolate (HPA23) were found. Although the mutation leading to the ciprofloxacin resistant phenotype was at a unique position not described previously (D82N), the region which it was found in was not surprising as mutations within the *parC* genes is a common means for resistance in both *Ureaplasma* as well as numerous Gram positive bacteria (Gellert *et al.* 1977; Fournier and Hooper, 1998). What was interesting was the finding that previously described mutations which had been associated with fluoroquinolone resistance were a species specific difference and not related to resistance as demonstrated by my sequencing of sensitive control isolates from each species (chapter 5, Table 5.7). This finding was overlooked by previous investigators due to comparing *U. urealyticum* sequences with that of the published genome of *U. parvum* (Glass *et al.* 2000). To avoid any further misreporting of resistant genotypes a complete series of amino acid tables for each serovar of each species has been constructed so that not only the correct species can be compared, but in some circumstance the correct serovar (chapter 5, Tables 5.3 - 5.6). The only mechanism of tetracycline resistance described in *Ureaplasma* results from

the presence of a functional *tetM* gene with the tetracycline resistant isolate HPA23 being no exception. An interesting finding relating to tetracycline resistance was the presence of a *tetM* positive, but tetracycline susceptible isolate (HPA6). Examination of the *tetM* sequence did not provide any clues to the lack of function, but did argue the need to phenotypically determine the susceptibility of an organism as genotypic resistance does not necessarily mean phenotypic resistance. Fortunately the work presented in chapter 4 found that culture was the most reliable method for detecting *Ureaplasma* which would allow the phenotypic demonstration of resistance (chapter 4, Table 4.1).

The issue of antibiotic resistance in *Ureaplasma*, in the context of a neonatal population, is not likely to be significant due to the mechanisms of resistance in *Ureaplasma* to antibiotics used. The drugs of choice, the macrolides, have good selective toxicity in these patients and as shown in this study with the isolate UHWO10, the mechanism of resistance was due to a deletion within the L4 ribosomal protein. For this reason the spread of macrolide resistance in *Ureaplasma*, such as UHWO10, would be by clonal expansion which is much less of a threat than resistance conferred by a transferable element, such as *tetM*, which has the ability to disseminate throughout a population of bacteria. Additionally no plasmids have been described in Ureaplasma therefore transmission of resistance would be further impeded. Ultimately the greatest risk factor to antibiotic resistance in *Ureaplasma* isolated from neonates is the presence of resistant clones in the mother. Therefore the incidence of antibiotic resistance in GUM populations could be a predictor for

incidence of resistance in neonatal populations as the mother, or the farther if the mother has acquired the organism, is the source of infection.

Although a clinical trail examining the ability of antibiotics to reduce the rate of CLD by clearance or *Ureaplasma* may prove a causative role, ultimately prevention of acquisition of the organism would be better than attempted cure. Additionally the non-prudent use of antibiotics is frowned upon as it can lead to the emergence of resistant strains, not only of *Ureaplasma* but potentially more virulent organisms. Therefore by understanding the mechanisms of immune clearance of *Ureaplasma* and potentially developing a humanised *Ureaplasma* specific antibody it would be possible to implement a more "natural" way to control an infection.

To try and further understand how the body prevents infection by *Ureaplasma* an *in vitro* system was developed to asses the role of membrane attack complex mediated (MAC) killing of the complement system using the serum from 12 healthy adult volunteers of unknown colonisation status. A significant finding was the critical role for the classical pathway in MAC mediated killing of all *Ureaplasma* examined. The pivotal experiment demonstrating this finding was achieved by the blocking of the first complement component C1q by a function blocking antibody (chapter 3, figure 3.9). In the absence of functional C1q, no killing was present suggesting an essential role for the classical pathway, but a negligible role for the alternative and lectin pathways. These finding were unexpected due to the alternative pathway being activated by foreign surfaces, such as bacteria, as well as the previous data by other

investigators demonstrating the ability of MBL to bind *Ureaplasma* (Benstein *et al.* 2004). These data were subsequently backed up by additional experiments (chapter 3, figure 3.3 and 3.6, respectively).

A further interesting finding was the antibody sensitivity of the four serovars examined. SV1, 6 and 14 killing was solely dependent upon the presence of IgG. Using serum which had been depleted of IgG subclasses 1, 2 and 4 or of all IgG subclasses by either a protein A or protein G sepharose columns, respectively; it was possible to conclude that the killing of SV1, 6 and 14 was reliant on IgG1 subclass of antibodies (chapter 3, Graphs 3.5 and 3.10). Further supporting this data were western blots with multiple intense bands which correlated with high levels of killing (chapter 3, Figures 3.4 and 3.3, respectively). Data on SV3 on the other hand was less clear cut. Depletion of IgG1, 2 and 4 did not remove killing whereas the removal of all subclasses removed killing in half of the cases examined. This data suggested that SV3 was unique in that it generated an IgG3 specific response in a number of individuals, but questioned the western blot data regarding the serological status of the designated sero-negative volunteers VxF1 and VxF9. VxF1 for example produce no bands by western blot for either anti-*Ureaplasma* IgG or IgM, but killing was removed in the absence of all IgG suggesting an IgG3 response. In addition VxF9 was also negative by western blot for IgG and IgM, but in the absence of total IgG killing was retained. In both cases the data suggested that western blot was either not sensitive enough to detect low levels of either IgG or IgM, or that the epitope recognised by these antibodies

were confirmationally dependent and as a result of linearization by sample preparation for western blot, binding was prevented.

This finding on the critical dependence upon antibodies for the clearance of *Ureaplasma* is further supported in the clinical field by the association of *Ureaplasma* infection in CVID patients who are deficient in IgG. Although this work was carried using adult serum, it can now be directly transferred to using neonatal serum and potentially exploring the ability of preterm cord blood to kill *Ureaplasma*. These sets of experiments would aid in testing the previous hypothesis of a gestational-dependent developmental ability to clear *Ureaplasma* at 27 weeks relative to that at 26 weeks.

6.1.2 Scope for future lines of investigation

The data presented in this thesis has answered multiple questions, but at the same time has produced many more. With regards to the data on the role of complement in the killing of *Ureaplasma* it would be of interest to examine the prevalence of different invasive serotypes of *Ureaplasma* infecting hypogammaglobuneamic patients. Due to the antibody deficient status of these individuals, and the potentially low titre of anti-*Ureaplasma* antibodies in the pooled serum used to produce the IVIG administered potentially bias the serovars which are able to lead to invasive disease due to the antibody-dependent nature of killing required whereas susceptible serovars, such as SV3, would not disseminate systemically due to the high serum susceptibility?

The alternative pathway had little effect upon killing of any serovar studied. In chapter 3 it was suggested that recruitment of complement regulators such as fH could be the reason for this lack of killing (Figure 6.1). As this is not an uncommon method utilized by bacterial pathogens to evade the immune system it is a plausible hypothesis. Methods by which this could be investigated would either use western blot analysis to look for binding of whole fH molecules or synthetically produced short consensus repeats which could be used to determine which regions of the molecule were required for binding. Alternatively neuraminidase treatment of *Ureaplasma* may cleave off sialic acid and prevent recruitment of fH as been demonstrated in *N. gonorrhoeae* (Ram *et al.* 1998), but this would be dependent on sialic acid being the ligand for fH as well as being present upon *Ureaplasma*.

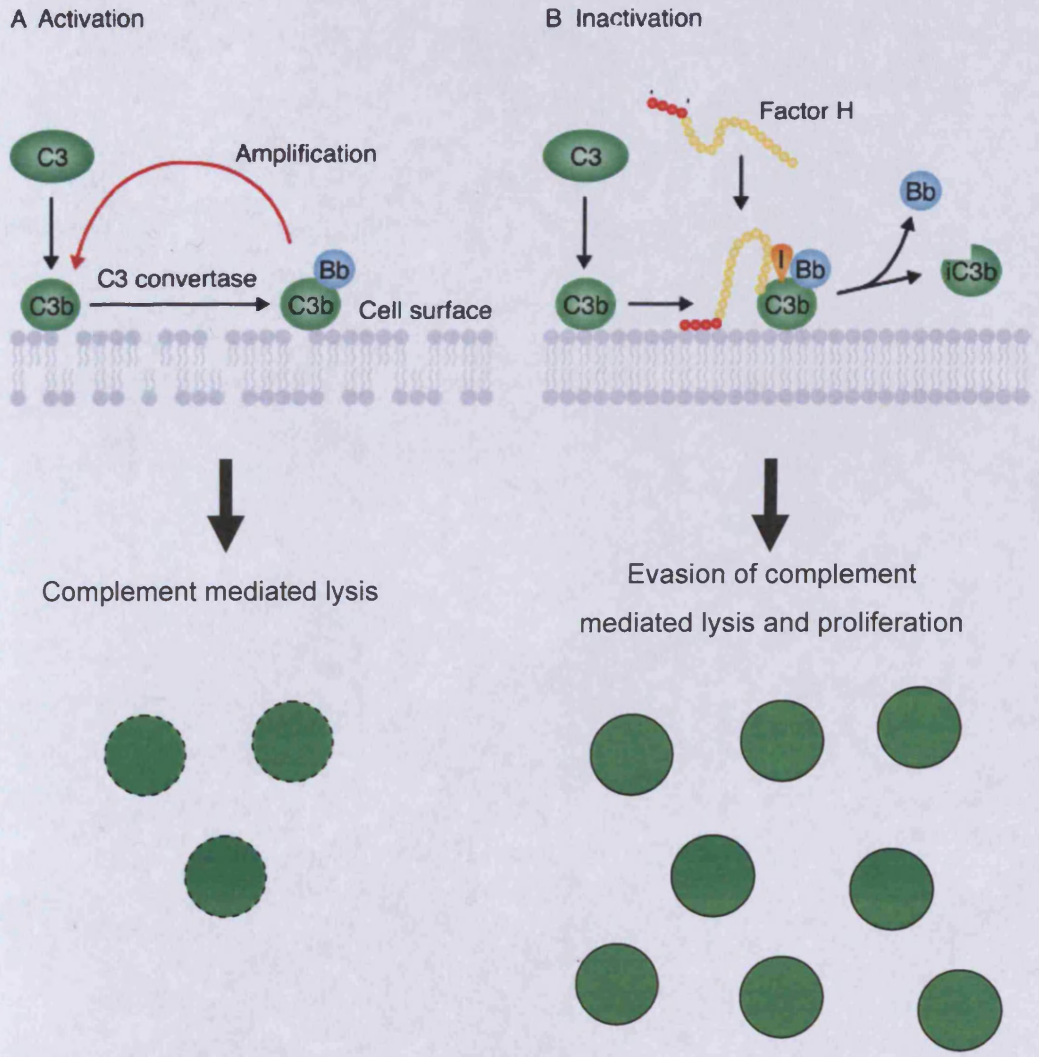


Figure 6.1. A diagrammatic explanation of how the binding of factor H to *Ureaplasma* can protect from complement mediated lysis. Panel A shows binding of C3b to a non-factor H protected cell and subsequent production of the C3 convertase (C3bBb). This leads to the continued cascade of the complement system resulting in *Ureaplasma* lysis (green spheres). Panel B shows that factor H bound to *Ureaplasma* acts as a co-factor for factor I mediated degradation of the C3 convertase leading the subsequent survival of *Ureaplasma* and further proliferation. Adapted from Atkinson and Goodship 2007.

MBL has been previously shown to bind *Ureaplasma*, but ability to result in MAC formation was not provided (Benstein *et al.* 2004). The results presented here suggest that MBL has little role in the MAC-mediated killing of *Ureaplasma*. As mentioned in Chapter 3 this is not to suggest that MBL does not bind and contribute to clearance of *Ureaplasma* by the cell-mediated immune system. It is most likely that MBL would bind due to the carbohydrate composition of external surface (Whitescarver *et al.* 1975; Smith, 1985). A possible reason to why bound MBL does not result in MAC formation maybe due to the recruitment of complement regulators which allow binding, but prevent cascade from continuing such as C4 binding protein of the classical pathway (Gadjeva *et al.* 2001). An argument against this theory would be if this was so then how come the classical pathway would still be effective as the regulator controls both lectin and classical pathways? Alternatively, there may be lectin pathway specific regulators as yet not described. To address the question of the role of MBL opsonisation of *Ureaplasma* a simple experiment could be carried out whereby *Ureaplasma* are incubated with and without MBL and then fed to phagocytes to see if there is difference in uptake due to opsinisation.

An unfortunate draw back of this study was the unknown colonisation status of the individuals examined. It would have been of interest to correlate colonisation status, as well as species if not serovar of isolates with the ability to individual sera to kill the representative serovars. In addition, if individuals were colonised a further interesting question would have been how serum from the colonised volunteer dealt with the organism isolated. Would those

who were colonised be able to kill the matched isolate, or would the reason for the persistent colonisation be a result of the infecting organism to continually evade killing? Previous studies have shown that the presence of antibody can promote the switching off of the MBA gene so to evade killing (Monecke *et al.* 2003; Zimmerman *et al.* 2009). These experiments have solely relied on the animal raised antibodies for the selection process. Using the sero-positive serum identified in this study it would be interesting to see if this switching off of gene expression occurred as a result of selection by human antibody in the presence of human serum as this would represent a more accurate picture of human infection (Figure 6.2). From the work by Zimmerman *et al.*, two genes were proposed which alternately express depending on antibody selective pressure. These may not be the only genes which are alternately expressed under antibody selection. By using techniques to look at global gene expression, such as micro-array, a variety of antibody/complement challenges could be used to fish out other possible candidate genes and increase our knowledge of surface expressed *Ureaplasma* antigens. For strategy of antigen variation to be an evolutionary adaptive one, sub-populations within a community of *Ureaplasma* must exist expressing different surface antigens. This would allow for those variants to which an antibody response is not raised against to proliferate due to the non-selective pressure. Zheng *et al.*, showed that from a pure culture of a single clinical SV3 isolate multiple bands could be detected by western blot using a monoclonal antibody. They found that this was a result of subpopulations within the culture expressing different sized antigens. This finding could have accounted for the different sized bands present in the lanes of the western blots shown in Chapter 3. To

determine if this was so, plating of cultures upon solid media and subsequent propagation of individual colonies would allow identification of proposed sub-populations.

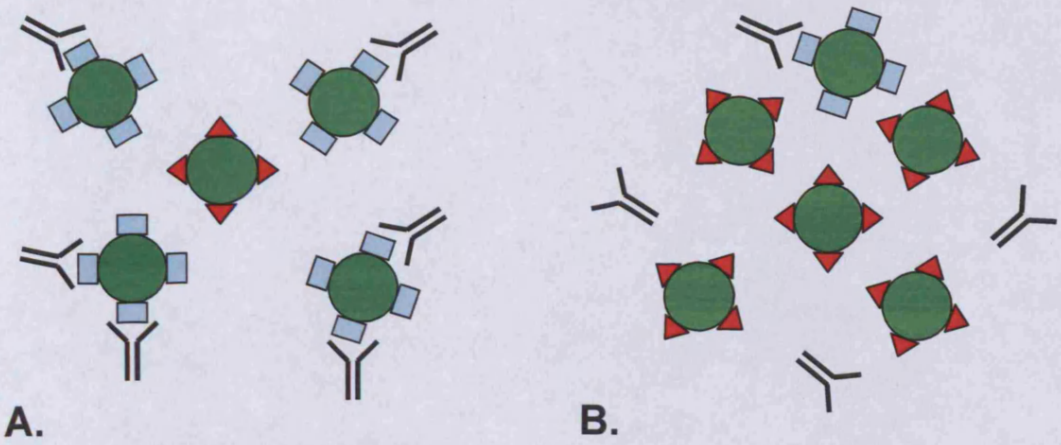


Figure 6.2. An explanation of how sero-positive serum could be used to demonstrate antigenic shift in *Ureaplasma*. By incubating *Ureaplasma* with matched sero-positive serum identified in this thesis it would be interesting to see if it would be possible to shift the predominant antigenic variants within a population. In panel A. the predominant *Ureaplasma* variants are expressing blue antigens to which an antibody response is present. By selection with this serum would the predominant variation then shift to that seen in panel B. with variants expressing red antigens? This would be identified by the lack of killing in the complement assay described in this thesis.

This project has only examined the susceptibilities of four representatives of the species *U. parvum*. Follow on work would repeat this work to see if there are firstly any differences within the 10 serovars of *U. urealyticum*. These results may account for the difference in prevalence between the two species. As this thesis has been examining the role of *Ureaplasma* in neonatal disease, a logical progression would also examine the ability of serum from neonates to kill *U. parvum* using this assay. These results may help to elucidate why preterm neonates are more susceptible to Ureaplasma infection relative to those born at term.

With reference to the data on tetracycline resistance in *Ureaplasma* it would be exciting to demonstrate that the *tetM* gene of HPA23 was not permanently fixed within the genome and could be transferred to another isolate such as HPA18 where the combination of ciprofloxacin and tetracycline could be used to select for double resistant strains. If this was possible then the transposon which the element is located upon could be engineered to incorporate a marker such as fluorescence. A luciferase expressing *Ureaplasma* would be an invaluable tool in quantifying the ability of neutrophils to phagacytose complement coated *Ureaplasma* relative to non-coated controls. This tool would also address the hypothesis that *Ureaplasma* can survive within neutrophils and use this to aid in dissemination around the body to locations such as joints in hypogammaglobumic patients (Webster *et al.* 1988).

If more time was available then further work could be carried out upon understanding the failure of the *tetM* gene in HPA6 to produce a resistant phenotype. By isolating mRNA from actively growing HPA6, cDNA could be produced by reverse transcription to identify if *tetM* was transcribed. Additionally the effects of doxycycline on inducing resistance in HPA6 could be examined as previously shown by Degrange *et al* on *M. hominis* (Degrenge *et al.* 2008). Also if it was possible to transfer the *tetM* gene from HPA6 to another *Ureaplasma* strain it would be interesting to examine the possibility of gene silencing as described in *E. coli* (Enne *et al.* 2006). Finally the identification of the presence of *tetM* upon a plasmid within *Ureaplasma* would be a novel finding. By making a single cut within the *Ureaplasma* chromosome with an endonuclease and running the DNA on a pulse-field gel the small plasmid DNA would run ahead of that chromosome. Using souther-blot a *tetM* probe could then be used to identify if the *tetM* gene is upon the chromosome or upon a plasmid (Figure 6.3).

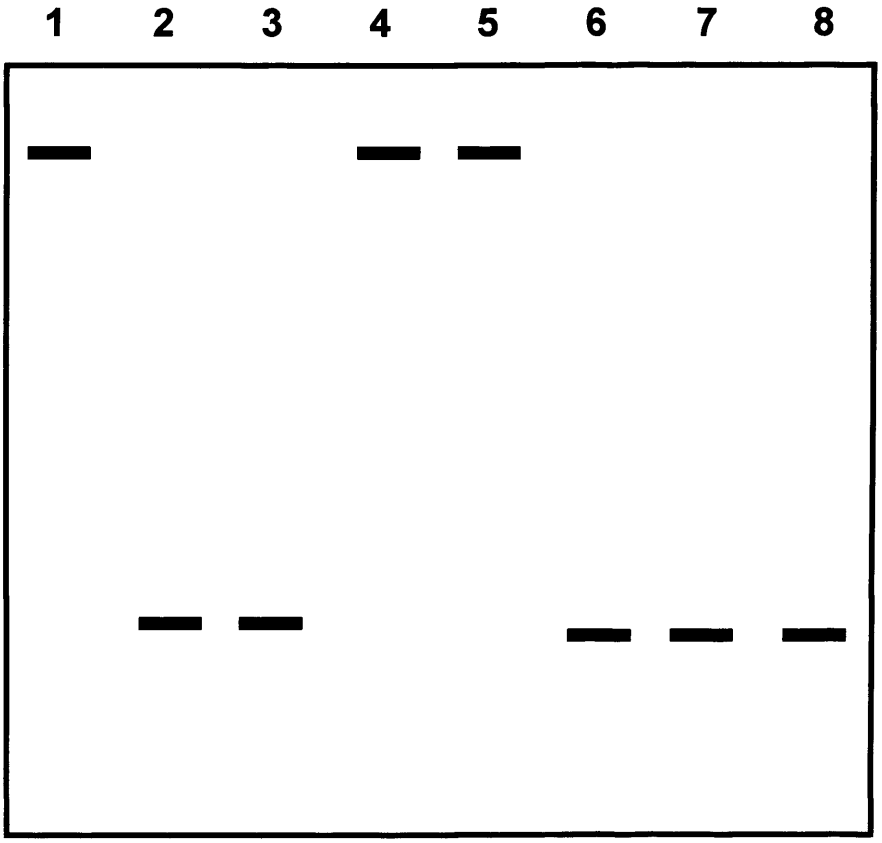


Figure 6.3. A hypothetical southern blot of a range of *tetM* positive *Ureaplasma* following pulse-field gel electrophoresis. Numbers from 1 – 8 represent various *tetM* positive *Ureaplasma* with strains 1, 4 and 5 encoding a chromosomal *tetM* (larger fragments) whereas strains 2, 3, 6, 7 and 8 harbour *tetM* upon a plasmid (smaller fragments).

Further interesting and novel lines of research which could be followed up from the work on antibiotic resistance include determining how the two amino acid deletion in the L4 ribosomal protein produces a macrolide-resistant phenotype and what are the mechanism(s) of fluoroquinolone resistance in the 11 out of 33 isolates now known to have no mechanisms of resistance. The mutation in the L4 protein was unique and has not been described before. By cloning and expressing the mutant protein in *E. coli* it would be possible to determine the crystal structure of the protein. Using this data it would be possible to model how the mutation affects the three-dimesional structure of the protein. Technical difficulties of such a project would include the usage of the UGA codon to encode tryptophan within *Ureaplasma* which acts as a stop codon in other organisms (three present within the L4 gene). To determine the mechanisms of resistance in the 11 resistant isolates with unknown mechanisms, whole genome sequencing can be used. By comparative genomics, resistant and susceptible isolates can be compared for common single nucleotide polymorphisms (SNPs) which may relate to resistance. These could be in any of the topoisomerase genes, within the MATE pumps described (Glass *et al.* 2000), or in other membrane proteins responsible for entry of drug.

Finally, from the data examining the incorrectly identified fluoroquinolone resistant mutations it was noticed that a great deal of variation occurs at the DNA level among the various serovars of *Ureaplasma*. Variations in housekeeping genes, such as the topoisomerases, are used to characterise isolates into groups by the technique known as multilocus sequence typing

(MLST). By using the topoisomerase genes, as well as other housekeeping genes it would be possible to group *Ureaplasma* isolates in to specific clusters and track the epidemiology of strains both at a national and international level as well as potentially correlate strains with disease outcome.

6.2 Concluding statement

The innate immune system may play a substantial role in preventing colonisation of *Ureaplasma* from progressing to full infection. In cases where the balance is tipped in favour of the pathogen, such as immune immature preterm neonates, infection can result in significant morbidity (CLD) and potentially mortality. The ability to treat such morbidities and reduce mortalities is dependent upon rapid and effective antimicrobial therapy, but may be compromised due to resistant strains.

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Appendix I

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2009, p. 2020–2027 Vol. 53, No. 5
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Concurrent Titration and Determination of Antibiotic Resistance in *Ureaplasma* Species with Identification of Novel Point Mutations in Genes Associated with Resistance▽†

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Antibiotic resistance determination of *Ureaplasma* spp. (*Ureaplasma parvum* and *Ureaplasma urealyticum*) usually requires predetermination of bacterial titer, followed by antibiotic interrogation using a set bacterial input. This 96-well method allows simultaneous quantification of bacteria in the presence and absence of antibiotics. A method for determining precise MICs and a method for screening against multiple antibiotics using breakpoint thresholds are detailed. Of the 61 *Ureaplasma*-positive clinical isolates screened, one (1.6%) was resistant to erythromycin (MIC, >64 mg/liter) and clarithromycin (MIC, 4 mg/liter), one to ciprofloxacin (1.6%), and one to tetracycline/doxycycline (1.6%). Five isolates were also consistently found to have an elevated MIC of 8 mg/liter for erythromycin, but this may not represent true antibiotic resistance, as no mutations were found in the 23S rRNA operons or ribosome-associated L4 and L22 proteins for these strains. However, two amino acids (R66Q67) were deleted from the L4 protein of the erythromycin-/clarithromycin-resistant strain. The *tetM* genetic element was detected in the tetracycline-resistant clinical isolate as well as in the positive control Vancouver strain serotype 9. The *tetM* gene was also found in a fully tetracycline-susceptible *Ureaplasma* clinical isolate, and no mutations were found in the coding region that would explain its failure to mediate tetracycline resistance. An amino acid substitution (D82N) was found in the ParC subunit of the ciprofloxacin-resistant isolate, adjacent to the S83L mutation reported by other investigators in many ciprofloxacin-resistant *Ureaplasma* isolates. It is now possible to detect antibiotic resistance in *Ureaplasma* within 48 h of positive culture without prior knowledge of bacterial load, identifying them for further molecular analysis.

Ureaplasmas and mycoplasmas are eubacteria belonging to the class *Mollicutes*. These unique organisms are the smallest self-replicating cells that lack a cell wall. Lack of a rigid cell wall prevents reactivity with Gram staining and makes them insusceptible to antibiotics that target bacterial cell walls (i.e., β-lactams and glycopeptides), while imparting a frailty that mostly limits them to a parasitic existence in association with the eukaryotic cells of their host (39). Shepard first described ureaplasmas, or T-mycoplasmas, in the 1950s, following isolation from a male patient with nongonococcal urethritis (31). This remains the most widely recognized patient group; however, they are also commonly found as commensals in the genital tract in as many as 80% of women of child-bearing age (30). Although the factors leading to intrauterine infection are unclear, detection of *Ureaplasma* in the amniotic fluid is frequently associated with chorioamnionitis, spontaneous abortion, and premature birth (13, 14, 40). It has been suggested that the rate of vertical transmission is inversely proportional to gestational age at time of delivery (1). Furthermore, the presence of *Ureaplasma* in the lungs of very premature neonates has been associated with the development of chronic lung disease (or bronchopulmonary dysplasia) and long-term hospitalization (7, 19, 29, 30).

Ureaplasma spp. are susceptible to bacteriostatic agents such as protein synthesis-inhibiting tetracyclines and macrolides as well as bactericidal agents, including fluoroquinolones. For premature neonatal patients, however, the removal of a microbe must be balanced against the potential (and often unknown) toxicity of the antibiotics in these patients (38). For this reason, physicians routinely favor macrolide antibiotics such as erythromycin when deciding to treat *Ureaplasma* infections in premature neonates.

Only a limited number of reports have been made regarding the trends in resistance among *Ureaplasma* isolates from neonates (18, 34, 37), and all of these comment on the lack of a standardized methodology, which hinders comparison of results. Factors such as inoculum size, pH of media, and time of incubation are known to dramatically alter the MIC, and standardized means of detecting resistance are needed.

Here, we describe a modified breakpoint analysis in a 96-well broth microdilution format that enables a concurrent determination of bacterial load in a sample simultaneously with the determination of resistance without prior knowledge of bacterial load. For isolates showing resistance within the breakpoint concentration, full MICs can be determined using a similar full-plate methodology. The breakpoint method was

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used to screen 15 strains isolated during 2006 and 2007 from bronchoalveolar lavage samples from neonates of various gestational ages at the University Hospital of Wales (UHW), Cardiff, United Kingdom, in addition to 46 frozen isolates from a reference repository of samples submitted to the Health Protection Agency at Colindale (London, United Kingdom) from 2003 to present. Within these 61 isolates, we identified one erythromycin-/clarithromycin-resistant, one ciprofloxacin-resistant, and one tetracycline-resistant *Ureaplasma* strain. Further PCR and sequence analyses were performed to determine the mechanism of resistance.

MATERIALS AND METHODS

USM and culture conditions. Ureaplasmas were grown in commercially available ureaplasma selective medium (USM) purchased from Mycoplasma Experience Ltd. (Surrey, United Kingdom). While the exact formula of the commercially obtained medium is proprietary, the simple broth base medium was supplemented with yeast extract, 1 g/liter urea, and 10% porcine serum, with phenol red as the pH indicator (starting pH, 6.65) and with 2.5 μg/ml amphotericin B and 0.25 mg/ml ampicillin (neither of which inhibit the growth of ureaplasmas). The adapted microbroth technique was carried out in flat-bottom 96-well plates covered with adhesive sealing tape (Elkay, Basingstoke, United Kingdom) in a humidified tissue culture incubator set at an ambient CO_2 concentration at 37°C. The sealing tape does not allow gas exchange, and the humidified incubator ensures that the integrity of the outermost wells is the same as those that are more central to the plate.

Sample collection. Bronchoalveolar lavage (BAL) samples were collected from 20 preterm and term neonates requiring mechanical ventilation on the neonatal ward at the UHW (Cardiff, United Kingdom). Samples were initially screened for *Ureaplasma* infection by addition of 25 μl BAL fluid to 2 ml USM and incubation at 37°C for 1 week. Samples that turned a clear red color (indicating *Ureaplasma* growth in the absence of contaminating bacteria) following incubation were confirmed by PCR using the *Ureaplasma*-specific primers U4 and U5, which selectively amplify the urease gene (see below). Positive samples were diluted 1:100 and 1:1,000 in USM, incubated overnight, divided into aliquots, and frozen at −80°C for later batch analysis. Fifteen clinical *Ureaplasma* isolates were successfully archived at −80°C in USM and used for later susceptibility testing. An additional 46 archived *Ureaplasma* isolates were revived from a potential 77 samples submitted for testing to the Health Protection Agency (HPA) at Colindale. The HPA clinical laboratory confirmed that no samples used were contaminated with mycoplasma using specific culture methods. Known serovar (SV) isolates used as reference strains were also obtained from HPA, although SV1 (DKF-1) and SV9 (Vancouver isolate) were originally obtained from the Institute of Medical Microbiology, University of Aarhus, Denmark.

Screening of HPA isolates using a modified breakpoint method. Erythromycin was purchased from Sigma-Aldrich (Dorset, United Kingdom) as a 1-mg/ml stock solution, while azithromycin, clarithromycin, ciprofloxacin, doxycycline, and tetracycline (also purchased from Sigma-Aldrich) were prepared as 1-mg/ml stocks dissolved in dimethyl sulfoxide or ethanol as directed by the manufacturer. Working stocks of antibiotics consisted of freshly diluted antibiotics at concentrations of 64 mg/liter in USM. An adapted breakpoint analysis was used to screen for the presence of resistant mutants within the 61 isolates. Although no official breakpoint values are available for *Ureaplasma*, we determined the following values based on the normal ranges of MICs reported for *Ureaplasma*, as stated in Cumitech 34 (36). As seen in Fig. 1, the bottom row of wells contained 2 mg/liter tetracycline, the next row up contained 4 mg/liter ciprofloxacin, the second row contained 4 mg/liter erythromycin, and the top row did not contain an antibiotic for a growth control. Two milliliters of USM was inoculated with either growing or frozen *Ureaplasma* spp. the night before titration was to be performed. Twenty microliters of this culture of unknown color-changing units (CCU) was then added to each well from A1 to A4, and serial 10-fold dilutions were performed from columns A to H. In this manner, the 96-well plate was organized to investigate the susceptibility of three different strains to three antibiotics per plate by repeating the layout for wells A5 to H8 and A9 to H12 as described above; but using different test strains of *Ureaplasma*. Plates were sealed and incubated at 37°C in a humidified cell culture incubator with ambient CO_2 for 48 h, at which time color change within the growth control rows had ceased. From the titration of the antibiotic-free row, it was possible to calculate back through the titration from 1 CCU, the final well in which color change occurred, to the recommended 10^4 CCUs. Any strain which showed growth in antibiotic-containing media at this level was subsequently fully investigated using the full-plate method (see below), with additional MIC determinations for the macrolides clarithromycin and azithromycin for suspected erythromycin-resistant isolates and doxycycline for suspected tetracycline-resistant isolates. SV9 (Vancouver strain) was used as a positive control for tetracycline resistance; however, no known characterized resistant strains were available to use as controls for erythromycin and ciprofloxacin resistance. USM (with and without antibiotics) was also incubated in the absence of added *Ureaplasma* spp. to serve as a negative color-changing control. Validation of this technique was performed by determining the antibiotic MICs for ATCC (American Type Culture Collection, Bethesda, MD) isolates of *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), and *Escherichia coli* (ATCC 25922). MICs determined in Mueller-Hinton medium for erythromycin, ciprofloxacin, and tetracycline were within the accepted MIC ranges for these bacteria.

Modified broth microdilution technique for determination of antibiotic MICs. In a 96-well plate, wells A1 to H1 received 360 μl of 64 mg/liter antibiotic in USM, and 180 μl sterile USM was added to the remaining wells (A2 to H12) of the plate. Using a multichannel pipette, rows of doubling dilutions were made by transferring 180 μl from row A1 to H11 (and an excess of 180 μl was discarded from row A11 to H11; see the instructional diagram in Fig. S1 in the supplemental material). Rows A12 to H12 remained free of antibiotic for unrestricted growth comparison. Thus, an antibiotic gradient was created from 64 mg/liter to 0.0625 mg/liter for the antibiotic in question. Twenty microliters of *Ureaplasma* from the overnight culture of unknown CCU was added to each well in the columns A1 to A12 (1:10 dilution). A 10-fold dilution curve of bacteria was then titrated at 90 degrees across the antibiotic gradient (i.e., A1 to -12 to H1 to -12). Plates were sealed and incubated at 37°C in a humidified cell culture incubator with ambient CO_2 for 48 h, at which time color change within the growth control had ceased (see Fig. 2). The MIC was defined as the lowest concentration of antibiotic that prevented a color change after 48 h when read at 10^4 CCU (relative to growth in the antibiotic-free medium). USM (with and without antibiotics) was also incubated in the absence of added *Ureaplasma* isolates to serve as a negative color-changing control.

Speciation of *Ureaplasma* isolates. Confirmation of *Ureaplasma* was determined by amplification of the *Ureaplasma*-specific urease gene (a 430-bp DNA product) using Blanchard and coworkers' (5) U4 and U5 primers (Table 1). Clinical isolates were further separated into species based on PCR primers which amplify a region of the multiple-banded antigen (MBA), as published by Teng et al. (35), also listed in Table 1. Primer set UM-1 for *Ureaplasma parvum* yielded a 403-bp product, with a 448-bp product for *Ureaplasma urealyticum*. For antibiotic-resistant *U. parvum* strains, SVs were determined by sequencing the UM-1 primer set amplicon and comparing SV-specific base compositions at nucleotides −54 to −56 and −82 to −84 (17, 35). Bacterial DNA from a 1.5-ml overnight culture was released by boiling lysis (95°C for 10 min) following centrifugation at 13,000 × *g* for 10 min, removal of all USM, and resuspension in 50 μl sterile water. A 20-μl PCR (containing 1× GoTaq Flexi buffer [Promega], 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.25 μM each primer, and 1.25 units of GoTaq DNA polymerase) was performed on 1 μl (as well as 1 μl of a 1:100 dilution to lessen possible polymerase inhibitor concentrations) for 35 cycles using the annealing temperatures listed in Table 1. To determine the DNA sequence, five 20-μl PCRs were pooled and purified from other reaction components using a Novagen SpinPrep PCR clean-up kit (Merck Chemicals Ltd., Nottingham, United Kingdom) per the manufacturer's instructions and then sequenced using the BigDye Terminator v3.1 cycle sequencing kit and analyzed with an ABI Prism 3130xl genetic analyzer (Applied Biosystems, Warrington, United Kingdom).

Amplification and sequence determination of putative resistance targets. For erythromycin- and ciprofloxacin-resistant isolates, mutations in bacterial genes previously associated with resistance (3, 22) were investigated.

DNA encoding bacterial 23S rRNA gene, L22, and L4 proteins was amplified and sequenced for macrolide-resistant strains; gyrase subunits and topoisomerase subunits were sequenced for ciprofloxacin-resistant strains, and *tetM* genes from both tetracycline-susceptible and -resistant isolates were sequenced. PCR primers and annealing temperatures are outlined in Table 1. Amplicons were purified and sequenced as detailed above.

RESULTS

Species distribution among *Ureaplasma* isolates tested. Seventy-five percent (15/20) of the archived UHW samples were successfully revived from −80°C storage, while only 59.7% (46/77) of archived HPA samples were successfully revived.

TABLE 1. Primers and conditions[a]

Primers used for PCR amplification	Primer sequence	Annealing temp (°C)	Size (bp)	Reference
Ureaplasma-specific urease gene primers				
U4	5′ ACGACGTCCATAAGCAACT 3′	54	430	5
U5	5′ CAATCTGCTCGTGAAGTATTAC 3′			
UM-1				
UMS-125	5′ GTATTTGCAATCTTTATATGTTTTCG 3′	52	403 (*U.p*)	35
UMA226	5′ CAGCTGATGTAAGTGCAGCATTAAATTC 3		448 (*U.u*)	
Erythromycin resistance primers				
23S OP1 domain V				
MH23S-11	5′ TAACTATAACGGTCCTAAGG 3′	56	1,339	22
UP23S-OP1	5′ ACCACCATTCAATGTTTGAC 3′			
23S OP2 domain V				
MH23S-11	5′ TAACTATAACGGTCCTAAGG 3′	56	1,427	22
UP23S-OP2R2	5′ CGTATACTTTGCCATAGTGTTGCC 3′			
L4				
UPL4-U	5′ TCTATTGATGGTAACTTCGC 3′	60	392	22
UPL4-R	5′ GTTGAAGGTGTTTCTAAATCGC 3′			
L22				
UPL22-U	5′ TTCGCACCGTAAAGCTTCTC 3′	60	458	22
UPL22-R	5′ GTTCTGGATCAACGTTTTCG 3′			
Tetracycline resistance primers				
TetM primers for screening				
TetMF	5′ TTATCAACGGTTTATCAGG 3′	48	397	4
TetMR	5′ CGTATATATGCAAGACG 3′			
TetM primers for sequencing				
TetMF-78	5′ GTATACCTATGGTTATGC 3′	48	901	
TetMR	5′ CGT ATA TAT GCA AGA CG 3′*			
TetMF	5′ TTATCAACGGTTTATCAGG 3′	54	1,715	
TetMR 2123	5′ GCATTTCGGACAATAGAGGGGG 3′*			
Ciprofloxacin resistance primers				
GyrA				
gyrA-1	5′ TTGCTGCTTTCGAAAACGG 3′	50	336	3
gyrA-2	5′ CTGATGGTAAAACACTTGG 3′			
GyrB				
gyrB-3	5′ CCTGGTAAATTAGCTGACTG 3′	55	310	3
gyrB-4	5′ TTCGAATATGACTGCCATC 3′			
ParC				
parC-5	5′ ACGCAATGAGTGAATTAGG 3′	55	309	3
parC-6	5′ CACTATCATCAAAGTTTGGAC 3′			
ParE				
parE-7	5′ ATGGGCGGAAAATTAACGC 3′	55	313	3
parE-8	5′ CTTGGATGTGACTACCATCG 3′			

[a] PCR primers used for the amplification and sequencing of genes associated with resistance to respective antibiotics. Annealing temperatures and predicated product sizes are indicated. *U. p*, *U. parvum*; *U. u*, *U. urealyticum*; *, primers which were designed for this study.

This may reflect the relatively short storage time for the former samples (less than 18 months) compared to that of the latter samples (up to 4 years), although differences in isolate preparation prior to freezing them may have equally contributed. For all 61 revived samples, 49 were found to belong to *U. parvum* (80%) and 12 to *U. urealyticum* (20%). A minor difference in species distribution was identified between HPA and UHW collections, but this was not deemed significant by Fisher's exact test analysis (78% *U. parvum* versus 87% *U. parvum*, respectively).

Examples of breakpoint and detailed MIC determination. Ninety-six-well plates were set out as detailed in Materials and Methods for breakpoint screening of antibiotic resistance. An example showing strains resistant to erythromycin, ciprofloxacin, and tetracycline is shown in Fig. 1. Nonturbid, dark red wells (which appear dark gray in the figure) are indicative of positive *Ureaplasma* growth in the selective medium. If the last well showing growth in the 10-fold dilution (in the top row, lacking antibiotics) is considered to have 1 CCU, then four columns to the left represents 10^3 CCU, and five columns to the left represents 10^4 CCU (isolated by the dotted box). Following previously published recommendations that *Ureaplasma* resistance needs to be assessed using an input of 10^4 CCU (36), resistance-to-threshold antibiotic concentrations (4 mg/liter erythromycin, 4 mg/liter ciprofloxacin, or 2 mg/liter tetracycline) were identified by comparison of wells within the dotted box. Figure 1 shows breakpoint identification of an erythromycin-resistant clinical SV1 isolate (UHWO10; i.e., 10th sample from UHW patient O) (Fig. 1, top), a ciprofloxacin-resistant clinical SV1 isolate (HPA18; i.e., 18th HPA sam-

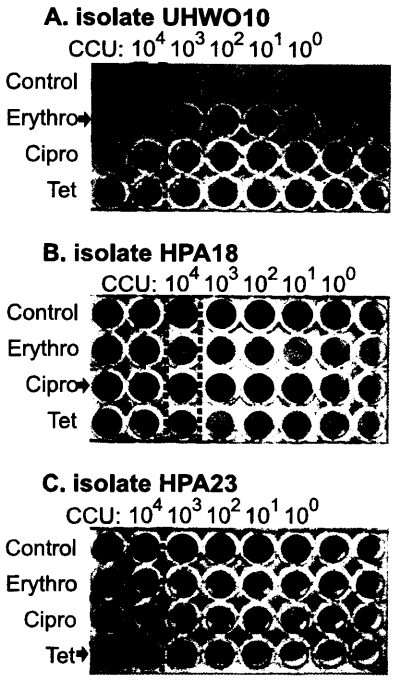

FIG. 1. Photographs of antibiotic breakpoint investigation showing an erythromycin (Erythro)-resistant strain (UHWO10) (A), a ciprofloxacin (Cipro)-resistant strain (HPA18) (B), and a tetracycline (Tet)-resistant strain (HPA23) (C). Dark red wells indicating growth of ureaplasma appear dark gray, while orange-yellow wells representing no ureaplasma growth appear light gray in the grayscale photograph. Columns containing 10^4 CCU are identified by a dotted box, and comparison of growth in the absence of antibiotics (control) was used to determine resistance to 4 mg/liter erythromycin, 4 mg/liter ciprofloxacin, or 2 mg/liter tetracycline.

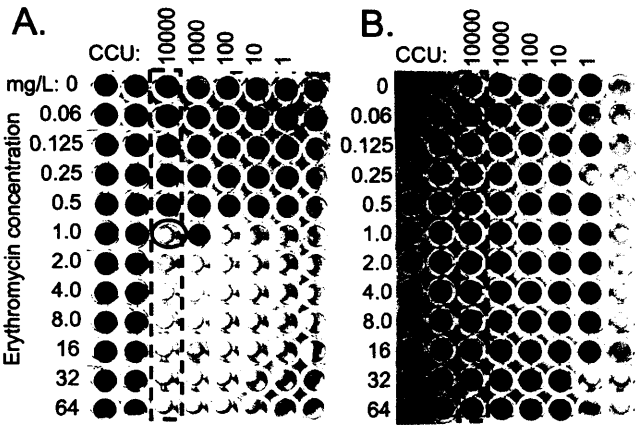

FIG. 2. Full-plate determination of MIC for erythromycin for a susceptible SV1 isolate (A) and a resistant (UHWO10) isolate (B) in plates containing a gradient of antibiotic from 64 mg/liter to 0.06 mg/liter. Dark red wells indicating growth of ureaplasma appear dark gray, while orange-yellow wells representing no ureaplasma growth appear light gray in the grayscale photograph. Columns representing 10^4 CCU growth in the absence of antibiotic are shown by the dotted box, and for the susceptible strain, a circle shows the first concentration of erythromycin to inhibit growth (MIC = 1 mg/liter). Since the resistant isolate (UHWO10) grew even in the presence of 64 mg/liter erythromycin, the MIC is determined to be >64 mg/liter.

ple) (middle), and a tetracycline-resistant clinical SV6 isolate (HPA23) (bottom). While we have used the 10^4 CCU column to assess antibiotic resistance, using 10^3 CCU would be equally valid, and no difference in our MICs was found when reading either the 10^3 or 10^4 CCU column.

For those isolates identified by breakpoint analysis, MICs for a single antibiotic were determined using a range of concentrations with a full-plate single antibiotic assay (Fig. 2). Using the same method used for determining which column contained 10^4 CCU (isolated by dotted box), examples of an erythromycin-susceptible clinical isolate (HPA18; MIC, 1 mg/liter) (Fig. 2A) and the highly resistant clinical isolate (UHWO10; MIC, >64 mg/liter) (Fig. 2B) are shown.

Summary of MICs among resistant isolates. All isolates were examined for resistance to erythromycin, ciprofloxacin, and tetracycline with the resistant isolates summarized in Table 2. Six isolates were identified to have an MIC greater than the normal MIC range previously published in Cumitech 34 (0.02 to 4 mg/liter) (36), giving the isolates a prevalence of 9.8%, although only one of these isolates (UHWO10) is likely to represent true antibiotic resistance with an MIC of >64 mg/liter. Among the erythromycin-resistant isolates, azithromycin and clarithromycin MICs were comparable to those of susceptible controls, with the exception of the highly resistant UHWO10, which was additionally resistant to clarithromycin (MIC = 4 mg/liter) but susceptible to azithromycin (normal MIC ranges, 0.5 to 4 mg/liter for azithromycin and <0.004 to 2 mg/liter for clarithromycin) (36). A single tetracycline-resistant clinical isolate, HPA23, was identified with tetracycline and doxycycline MICs of 64 and 16 mg/liter, respectively (1.6% prevalence). A single ciprofloxacin-resistant clinical isolate, HPA18, was also identified with an MIC of 8 mg/liter (1.6% prevalence).

Molecular characterization of macrolide resistance. Mutations in the 23S rRNA genes (two separate operons) and associated L4 or L22 protein from the prokaryotic ribosomal complex have previously been reported to be associated with macrolide resistance (22). These genes were sequenced from the highly resistant clinical isolate UHWO10 as well as the five isolates with an erythromycin MIC of 8 mg/liter and a selection of the susceptible isolates. No mutations were found in either the 23S rRNA operon or the L22-encoding gene of any of the isolates; however, a 6-bp deletion was identified in the L4 protein gene from the highly resistant UHWO10. This in-frame deletion resulted in the loss of arginine and glutamine residues at residue numbers 66 and 67, respectively (ΔR66Q67) (Table 3). This region was sequenced in clinical isolates representing susceptible SV1 strains (representing an SV control) and other *U. parvum* strains. All were identical to the published SV3 genome sequence (accession number AF222894) (12). Furthermore, this region was also conserved in the SV8 reference strain and a *U. urealyticum* clinical strain (Table 3). Comparison of all sequences found three species-specific conserved nucleotide polymorphisms (all silent) in the L4 protein gene (*U. parvum* T309, G357, and C373 compared to *U. urealyticum* C309, A357, and T373). While 80% of all isolates were found to belong to *U. parvum*, four out of five of the isolates with MICs of 8 mg/liter belonged to *U. urealyticum*.

TABLE 2. MICs for resistant and control *U. parvum* and *U. urealyticum* strains

Isolate	Species (SV)	MICs with indicated antibiotics[a]					
		Ery	Azi	Cla	Tet	Dox	Cip
HPA3	*U. urealyticum*	4	0.5	<0.125	2	0.5	4
HPA6	*U. urealyticum*	**8**	<0.25	<0.125	2	0.5	4
HPA12	*U. urealyticum*	**8**	<0.25	<0.125	2	0.5	4
HPA17	*U. urealyticum*	**8**	0.5	<0.125	1	0.25	4
HPA18	*U. parvum* (SV1)	4	<0.25	<0.125	2	0.25	**8**
HPA20	*U. urealyticum*	**8**	<0.25	<0.125	2	0.5	4
HPA23	*U. parvum* (SV6)	4	<0.25	<0.125	**64**	**16**	<2
HPA32	*U. parvum* (SV14)	**8**	<0.25	<0.125	1	0.25	<2
UHWJM	*U. parvum*	4	<0.25	<0.125	2	0.5	4
UHWO10	*U. parvum* (SV1)	**>64**	2	**4**	1	0.25	4
UHWP2	*U. parvum*	4	<0.25	<0.125	2	0.5	4
UHWQ3	*U. parvum* (SV1)	4	<0.25	<0.125	1	0.25	4

[a] MIC results from a selection of resistant isolates (in boldface) and susceptible isolates, as determined by growth comparison at 10^4 CCU. Ery, erythromycin; Azi, azithromycin; Cla, clarithromycin; Tet, tetracycline; Dox, doxycycline; Cip, ciprofloxacin.

Therefore, 33% of the all *U. urealyticum* isolates identified were found to be less susceptible to erythromycin (but susceptible to clarithromycin and azithromycin) (Table 2).

Molecular characterization of tetracycline resistance. Tetracycline-resistant bacterial strains are often found to have the transferable *tetM* genetic element encoded in their genome. The SV9 (Vancouver) reference strain, known to contain the *tetM* gene (25), demonstrated a tetracycline-resistant (Tet^r) phenotype, with tetracycline and doxycycline MICs of >64 mg/liter and 64 mg/liter, respectively. Only one *U. parvum* clinical isolate (HPA23) was found to be Tet^r and doxycycline resistant (Table 2), coincident with the identification of the *tetM* gene by PCR (data not shown). PCR screening of all 61 isolates identified a *tetM*-positive *U. urealyticum* clinical isolate (HPA6) that was susceptible to both tetracycline and doxycycline (Table 2). The entire *tetM* gene was sequenced and compared to resistant strains SV9 (Vancouver) and HPA23 and susceptible strain HPA6 (Table 3). No mutations in the coding region of the *tetM* gene were found for HPA6 that could account for the inability of a resultant protein to mediate tetracycline resistance, nor was any alteration to the endogenous *tetM* gene promoter found that may have stopped TetM protein expression. Therefore, we have no explanation for the susceptibility of this *tetM*-positive isolate. Interestingly, three amino acid polymorphisms (H209Q, V216L, N223S) were found in HPA23 that did not affect function and were identical to those originally reported for a Tet^r strain isolated in Seattle, Washington, in 1984 (Table 3) (28).

TABLE 3. Partial protein sequence alignments of antibiotic-resistant and -susceptible isolates

Isolate tested with indicated antibiotic[a]	Sequence[b]
Erythromycin	
L4 isolates	
$SV3^s$	^{63}KPWRQKHT^{70}
$UHWO10^r$	^{63}KPW--KHT^{70}
Tetracycline	
TetM isolates	
SV9 $Seattle^r$	^{209}**H**NCSLFPVYHGSAKN**N**IGID^{228}
$HPA23^r$	^{209}**H**NCSLFPVYHGSAKN**N**IGID^{228}
$HPA6^s$	^{209}QNCSLFPLYHGSAKS**N**IGID^{228}
SV9 $Vancouver^r$	^{209}QNCSLFPLYHGSAKS**N**IGID^{228}
Fluoroquinolone	
ParC isolates	
$Consensus^s$	^{78}HPHGDSSIYE^{87}
$HPA18^r$	^{78}HPHG<u>N</u>SSIYE^{87}
Bebear $UUg2\text{-}5^r$	^{78}HPHGD<u>L</u>SIYE^{87}
Bebear UUc^r	^{78}HPHGDSSIY<u>K</u>^{87}

[a] Superscript letters (r and s) indicate resistance and susceptibility, respectively, to the indicated antibiotics.

[b] Boldfaced amino acids identify areas of sequence divergence within a variable region of the protein. The sequence of TetM of the Vancouver strain was determined at the UHW. Underlined amino acids identify areas of sequence divergence relative to the sequences of susceptible strains. Previous S83L amino acid substitutions arising from mutation and E87K substitutions associated with resistance as described by Bebear et al. (2) are also shown. –, deletion of amino acids R and Q.

Molecular characterization of ciprofloxacin resistance. A single ciprofloxacin-resistant (Cip^r) clinical isolate, HPA18, was identified with an MIC of 8 mg/liter. Mutations in the subunits of the bacterial gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes, known as the quinolone resistance-determining regions, have previously been associated with resistance to other fluoroquinolone antibiotics (2, 3, 10, 11, 41, 42). The sequences of these genes were determined from the Cip^r HPA18 isolate and several susceptible clinical isolates and compared to both known SV reference strains and the published SV3 sequence (12). A single base pair change at position 244 (G244A) in the *parC* gene was identified (Table 3), resulting in a codon change and subsequent substitution of aspartic acid for asparagine (D82N) relative to those of other *U. parvum* sequences from susceptible controls from both species.

DISCUSSION

Summary of mutations found. This study describes a modified breakpoint analysis used to screen for antibiotic resistance among a large number of clinical *Ureaplasma* samples of unknown initial CCU titration. *U. parvum* strains resistant to ciprofloxacin and tetracycline as well as one highly erythromycin-resistant strain were identified. The species of all clinical isolates were determined by PCR analysis of the gene encoding the MBA protein, and in many cases, the SV was determined through analysis of the amplicon sequence in this region. Po-

tential underlying molecular mechanisms of antibiotic resistance were investigated, based on target genes identified by previous investigators. A unique deletion of ΔR66Q67 was identified in the L4 protein of the bacterial 50S ribosomal complex in an isolate with high erythromycin resistance, a unique point mutation of D82N in the ParC subunit of bacterial topoisomerase in the ciprofloxacin-resistant isolate, and the presence of the *tetM* element in the tetracycline-resistant isolate.

Advantages of the breakpoint method. The primary advantage to using the modified breakpoint methodology described here is the simultaneous determination of input inoculum for determination of susceptibility at the 10^4 CCU inoculum. Using this methodology, the investigator also has the option of determining the amount of CCU reduction at a defined antibiotic concentration. By defining the final well in which color change occurred in the growth control as 1 CCU, comparison of growth in the column representing an initial inoculum of 10^4 CCU (as indicated in Fig. 1) identifies resistant organisms to be further interrogated by the full-plate, single-antibiotic method. As seen in Fig. 1, the presence of a resistant isolate is readily noticeable: 10-fold reduction (or less) in growth was observed at a 2 or 4 mg/liter concentration of antibiotic. Further investigation with the full-plate method found that less than 10-fold reduction in growth was seen for UHWO10 when grown in up to 64 mg/liter erythromycin (Fig. 2).

Ciprofloxacin resistance. Ciprofloxacin was used as a representative of the bactericidal fluoroquinolone antibiotic family that targets two bacterial topoisomerases, namely topoisomerase IV and DNA gyrase. Numerous mechanisms have been described by which bacteria can mediate resistance to fluoroquinolones, including mutation of the topoisomerase genes, decreased membrane permeability, active drug efflux, modification by a fluoroquinolone-inactivating enzyme, or the presence of a Qnr protein (27). We have identified a point mutation leading to an amino acid substitution which was two amino acids downstream from the proposed active site of the ParC protein (20). Six separate groups have investigated a total of 32 resistant isolates and reported mutations in *Ureaplasma* genes associated with fluoroquinolone resistance (2, 3, 10, 11, 41, 42). Nineteen of 32 fluoroquinolone-resistant strains investigated by these groups have the same mutation in the *parC* gene, S83L (or S80L, if using homology to the *E. coli* position to identify the location) (Table 3); furthermore, similar mutations in ParC have been found in fluoroquinolone-resistant *S. aureus* and *Streptococcus pneumoniae*, which are reported to have MICs similar to those of resistant *Ureaplasma* strains (15). The mutation in the Cip^r isolate D82N is adjacent to this region, and this isolate has a ciprofloxacin MIC similar to that of isolate UUc identified by Bebear et al. (2), who also found an E87K mutation in the near vicinity, indicating this is a dominant target for fluoroquinolone resistance. (Table 3).

Erythromycin resistance. This investigation also identified one highly resistant erythromycin resistant isolate (MIC, >64 mg/liter); however, five additional isolates each consistently showed an MIC of 8 mg/liter, which is higher than the range of 0.02 to 4 mg/liter listed in the Cumitech 34 reference manual (36). Interestingly, 4/5 of the less susceptible isolates were *U. urealyticum*, which represented 33% of the total *U. urealyticum* isolates screened, suggesting a possible inherently increased tolerance of *U. urealyticum* isolates to erythromycin relative to that of *U. parvum* isolates. There is much less information available about the mechanism of erythromycin resistance in *Ureaplasma* spp. In fact, this is the first full molecular characterization of a clinical *Ureaplasma* isolate with erythromycin resistance. The physiological mechanism of erythromycin resistance was first indicated by Palu et al. (21) almost 20 years ago, who demonstrated reduced radiolabeled erythromycin binding to ribosomes of resistant *Ureaplasma* compared to that of a susceptible strain. However, technical advances since that report have greatly enhanced the ability to investigate molecular mechanisms. Investigation of other macrolide-resistant bacteria has identified mutations that inhibit erythromycin binding to the bacterial 50S ribosomal complex, where it exhibits bacteriostatic activity through inhibition of protein synthesis. Pereyre et al. serially passaged a *U. parvum* reference strain 45 to 50 times in increasing amounts of erythromycin and sequenced associated genes: two 23S rRNA operons and L4 and L22 ribosome-associated proteins (22). They consistently found mutation of the erythromycin-binding site around nucleotide position 2067 (2058 numbering based on homology to *E. coli*) near the peptidyl transferase loop in domain V of one of the 23S rRNA operons and occasionally found additional mutation of the associated L4 or L22 protein. Dongya et al. identified a number of point mutations within the 23S rRNA of 18 clinical isolates with various degrees of resistance to the macrolides josamycin, clarithromycin, roxithromycin, and azithromycin; however, they did not investigate resistance to erythromycin (9). Interestingly, none of the mutations described were in the region of nucleotide 2067. Our investigation did not find mutations in either the 23S rRNA operon or the L22 protein but did detect a deletion of two adjacent amino acids in the L4 protein for the highly resistant strain (MIC, >64 mg/liter). No mutations were found in the isolates with an erythromycin MIC of 8 mg/liter, yet compensating mutations outside of the investigated regions, DNA methylation, expression of bacterial proteins that modify macrolides, or increased drug efflux via ion channels may have been responsible for the relative increase in tolerance to erythromycin for these strains. Kenny and Cartwright previously showed that susceptibility to erythromycin was reduced in acidic growth medium; however, medium pH had to fall below 6.5 before an effect was observed (16). As our USM had a pH of 6.65, the MICs were determined relative to each other in the same batch of USM, and the findings were consistent upon repeated testing. As a result, we do not feel that the reduced susceptibility of these five isolates was due to a pH artifact for the USM used.

Mutations within the highly conserved region of L4 have previously been noted in both laboratory-derived and clinically isolated macrolide-resistant pneumococci and *E. coli* strains (6, 32, 33). Both a substitution mutation (G69C) and an insertion mutation (6-bp insertion between the codons encoding Q67 and K68) were found in two resistant pneumococcus strains (32, 33). Clinical isolates found by the same group made up a distinct clonal cluster which contained a substitution from 69GTG71 to 69TPS71 as well as one isolate which contained a 6-amino-acid insertion within the same region (33). A similar mutation (K63E) within L4 of *E. coli* has also been found to be associated with resistance to erythromycin (6) and was located adjacent to the deletion described here. The resultant three-

dimensional alteration to the entire ribosomal complex as a result of the structural L4 mutation must prevent the interaction of the C5 sugar of the macrolide with the residues around A2067 (A2058 *E. coli* numbering) and abrogate the ability of erythromycin to inhibit protein synthesis in the resistant strain (23).

Tetracycline resistance. Currently, the only known mechanism of tetracycline resistance for mollicutes is the presence of the *tetM*-transferable genetic element. It was first described in *Ureaplasma* by Roberts and Kenny in 1986 (24) and has been the focus of investigations by several groups since then. Unlike the studies by Robertson et al. (26), who found that all 26 of their Tet^r isolates were also resistant to erythromycin at or above 2 mg/liter, our control SV9 Tet^r and clinical Tet^r isolates were susceptible to erythromycin (MIC, 4 mg/liter). Blanchard et al. (4) have previously shown that screening *Ureaplasma* isolates by PCR readily identified Tet^r strains. However, we found a tetracycline-susceptible strain (HPA6) that screened *tetM* positive with these strains. Recently, Degrange et al. identified two *tetM*-positive *Mycoplasma hominis* isolates that were tetracycline susceptible (8). One of these *M. hominis* isolates had a 1,260-bp insertion in the leader peptide sequence (likely preventing successful transcription), while no mutations were found within the *tetM* gene or promoter region of the second isolate. Like the latter case for our Tet^s *tetM*$^+$ HPA6 strain, we could find no explanation for the susceptibility when comparing the sequence to that of the control Tet^r SV9 (Vancouver) strain. We also found that our Tet^r strains were resistant to both tetracycline and doxycycline, which is in contrast with the findings of Blanchard et al. (4). They identified 21 clinical Tet^r *Ureaplasma* isolates and found that 8 were resistant to doxycycline, 2 were intermediate, and 11 were susceptible. It has been proposed that doxycycline is less of an inducer of *tetM* transcription in some organisms than tetracycline. In this study, we demonstrated that our clinical tetracycline-resistant isolate HPA23, which harbored a functional TetM protein, was additionally resistant to doxycycline.

Conclusion. In conclusion, we have developed a reliable method to determine antimicrobial susceptibility of *Ureaplasma* isolates without prior knowledge of inoculum size. Although this is the second molecular investigation of macrolide resistance in clinical *Ureaplasma* isolates, more erythromycin-resistant strains are required to determine the prevalence of mutations in the relevant genes associated with resistance. Screening for the *tetM* gene could identify tetracycline-resistant strains; however, we found one false-positive strain, which raises a note of caution for the use of PCR screening for antibiotic resistance. It appears that mutation within an 8-amino-acid region of topoisomerase IV *parC* is commonly found in fluoroquinolone-resistant *Ureaplasma*, with S83L being the dominant mutation, but molecular screening methods would have to employ more-complex methods, such as capillary electrophoresis-based single-strand conformation polymorphism, to screen for all potential mutations.

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Comparison of full *gyrA*, *gyrB*, *parC* and *parE* gene sequences between all *Ureaplasma parvum* and *Ureaplasma urealyticum* serovars to separate true fluoroquinolone antibiotic resistance mutations from non-resistance polymorphism

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***Objectives*: To determine the role of amino acid substitutions in *Ureaplasma* GyrA, GyrB, ParC and ParE proteins in mediating fluoroquinolone resistance.**

***Methods*: Nucleic acid sequences from *gyrA*, *gyrB*, *parC* and *parE* genes from all 14 *Ureaplasma* serovars were aligned. Full genome sequences for serovars 1, 3–7, 9 and 11–14 were available from the National Center for Biotechnology Information database and we sequenced the full topoisomerase genes from ciprofloxacin-susceptible reference strains of serovars 2, 8 and 10. Phylogenetic trees were constructed to analyse nucleotide sequence similarity. Deduced amino acid sequences were compared with all 33 previously reported fluoroquinolone-resistant strains to clarify true fluoroquinolone-resistance-associated substitutions.**

***Results*: Non-resistance-associated polymorphisms were identified in GyrA (39), GyrB (26), ParC (107) and ParE (34) proteins. Phylogenetic analysis demonstrated species clustering for all genes, except *parE* in which serovars 4, 12, 10 and 13 formed a separate cluster more similar to *Ureaplasma parvum* than the remaining *Ureaplasma urealyticum* serovars. Examination of all previously reported fluoroquinolone-resistant strains found that one-third of identified residue substitutions could be attributed to normal species polymorphism; therefore, the mechanism of resistance for these strains is still undetermined. In particular, Glu or Asp at position 112 in GyrA and Ala or Thr at 125/136 in ParC were substitutions identified when *U. urealyticum* strain sequences were previously aligned with the published serovar 3 genome sequence.**

***Conclusion*: Combining analysis of the recently available *Ureaplasma* genomes with sequences from the additional serovars has enabled us to clarify which substitutions found by previous investigators could potentially be responsible for fluoroquinolone resistance.**

Keywords: genetic polymorphisms, ciprofloxacin, DNA gyrase, topoisomerase IV

Introduction

Ureaplasmas are among the smallest and simplest self-replicating organisms known. They belong to the order Mycoplasmatales, family Mycoplasmataceae, but are distinguished from the genus *Mycoplasma* by their ability to hydrolyse urea as their main energy source. They have been associated with a range of pathology, including non-gonococcal urethritis, reactive arthritis, neonatal pneumonia and chronic lung disease in pre-term neonates.[1]

Prior to 2002, a single human-associated species, *Ureaplasma urealyticum*, was recognized, consisting of two biovars that were subsequently further differentiated into 14 serovars (SVs). However, the combination of sequence analysis of 16S rRNA genes, the 16S–23S rRNA intergenic region and the urease gene together with DNA–DNA hybridization experiments led to the reclassification of the two biovars as two independent species, designated *Ureaplasma parvum* (SVs 1, 3, 6 and 14) and *U. urealyticum* (SVs 2, 4, 5 and 7–13).[2]

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Treatment of *Ureaplasma* infections is limited to tetracyclines, macrolides and fluoroquinolones.[3] Resistance to all three antibiotic classes has been documented in clinical *Ureaplasma* isolates, with unique nucleic acid substitutions identified as potential molecular mechanisms for each.[4–6] To date, a total of 33 clinical isolates of fluoroquinolone-resistant *Ureaplasma* have been described. Substitutions of Ser83Leu in the ParC protein, or a triple substitution of Asp112Glu in GyrA protein along with Ala125Thr and Ala136Thr in ParC protein, all within the quinolone resistance determining regions (QRDRs), being most commonly noted.[5–11] Here we describe that the latter triple mutation is not related to a resistance phenotype, but is the result of species-specific polymorphism, which is found in all *Ureaplasma* SVs. We also review the previous literature on mutations reported in fluoroquinolone-resistant *Ureaplasma* isolates and discuss the evidence that supports this hypothesis.

Materials and methods

Ureaplasma strains and ciprofloxacin susceptibilities

Isolates UHWQ3 (ciprofloxacin-susceptible *U. parvum*) and UHWW11 (ciprofloxacin-susceptible *U. urealyticum*) were collected from lung lavage fluids from pre-term neonates at the University Hospital of Wales (UHW). The ciprofloxacin-resistant isolate HPA18 (SV1) and ciprofloxacin-susceptible control strains of SVs 2, 8 and 9 were kindly provided by the Health Protection Agency (Colindale, UK). Susceptibility to ciprofloxacin was determined as described previously.[5]

Sequencing of gyrB/A and parE/C genes

PCR for the QRDRs of HPA18, UHWQ3 and UHWW11 were carried out as described by Bebear *et al.*[6] PCR primers for amplification of the whole *gyrA/B* and *parC/E* genes for SVs 2, 8 and 9 were designed from consensus sequences of known *U. urealyticum* and are listed in Table 1. PCR reaction conditions consisted of 1×GoTaq® Flexi Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM each primer and 1.25 units of GoTaq DNA polymerase. PCR was performed on 1 μL of DNA for 35 cycles at 50–55°C. Five 20 μL PCR reactions were pooled and amplicons purified using a Novagen spin-prep PCR clean-up kit™ (Merck Chemicals Ltd, Nottingham, UK), as per the manufacturer's instructions, sequenced using the BigDye Terminator v3.1 cycle sequencing kit and analysed by an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Warrington, UK).

Collection of gyrA, gyrB, parC and parE sequences from Entrez genome database

DNA and protein sequences were retrieved from the Entrez genome database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome) for SV1 (ATCC 27813), SV3 (ATCC 700970), SV4 (ATCC 27816), SV5 (ATCC 27817), SV6 (ATCC 27818), SV7 (ATCC 27819), SV9 (ATCC 33175), SV10 (ATCC 33699), SV11 (ATCC 33695), SV12 (ATCC 33696), SV13 (ATCC 33698) and SV14 (ATCC 33697). At the time of collation, *gyrA*, *gyrB*, *parC* and *parE* sequences for SV2, SV8 and SV10 as well as *parC* and *parE* for SV9 were not available, and were sequenced at the UHW as described above.

Table 1. Primers used to sequence *gyrB/A* and *parE/C* operons from *Ureaplasma* SVs

PCR primer name	Primer sequence	Size (bp)	
		U. parvum	*U. urealyticum*
gyrB-98F	5′-GAAAATAGACGTGGTCG-3′	1668	1668
gyrB-4[a]	5′-TTCGAATATGACTGCCATC-3′		
gyrB-3[a]	5′-CCTGGTAAATTAGCTGACTG-3′	1249	1249
gyrA-2[a]	5′-CTGATGGTAAAACACTTGG-3′		
gyrA-1[a]	5′-TTGCTGCTTTCGAAAACGG-3′	1456	1456
gyrA 1655R	5′-CCTTGTACACCTACACCAC-3′		
gyrA 1504F	5′-GGTGATGAGCGTCGTACAG-3′	**1258**	**1270**
gyrA 2743R	5′-CCTGCAACTTCACCAATTG-3′		
parE-54	5′-CAGCAACCATTTTATGCCGATCC-3′	1576	1576
parE-8[a]	5′-CTTGGATGTGACTACCATCG-3′		
parE-7[a]	5′-ATGGGCGGAAAATTAACGC-3′	1182	1182
parC-6[a]	5′-CACTATCATCAAAGTTTGGAC-3′		
parC-5[a]	5′-ACGCAATGAGTGAATTAGG-3′	1397	1397
par 1545R	5′-GTATCCATCACGACTTATTAC-3′		
parC 1367F	5′-GCTAAAACGCTACAAGAACG-3′	**1318**	**1324**
parC 2553R	5′-CAACGTTGGCATAAATTGG-3′		

Bold font denotes product size variation between species.
[a]Taken from Bebear *et al.*[6]

Alignment of sequences and construction of phylogenetic trees

Sequences were aligned with CLUSTALW and distances calculated using maximum likelihood. The evolutionary history was inferred using the neighbour-joining method and the optimal trees after 500 bootstraps are shown (Figure 2). Repeat testing using Jukes–Cantor and Tamura–Nei distance calculations gave identical tree topology.[12] Trees were rooted using respective gyrase or topoisomerase genes from *Mycoplasma pneumoniae*.

(a)

Isolate	sp.	Amino acid sequence - GyrA
HPA18^{R}	U.p	^{96}PHGDQAVYQTIVRMAQDFSMRYLLVDGHGNFGSIDGDS^{133}
UHWQ3^{S}	U.p	^{96}PHGDQAVYQTIVRMAQDFSMRYLLVDGHGNFGSIDGDS^{133}
UHWW11^{S}	U.u	^{96}PHGDQAVYQTIVRMAQEFSMRYLLVDGHGNFGSIDGDS^{133}

(b)

Isolate	sp.	Amino acid sequence – ParC
HPA18^{R}	U.p	^{81}G**N**SSIYEAMVRMSQD^{95}-----^{123}TEARLSKIASVMLAN^{137}
UHWQ3^{S}	U.p	^{81}GDSSIYEAMVRMSQD^{95}-----^{123}TEARLSKIASVMLAN^{137}
UHWW11^{S}	U.u	^{81}GDSSIYEAMVRMSQD^{95}-----^{123}TETRLSKIASVMLTN^{137}

Figure 1. Amino acid alignments of GyrA (a) and ParC (b) proteins from a ciprofloxacin-resistant SV1 (HPA18) isolate, and susceptible SV1 (UHWQ3) and *U. urealyticum* (UHWW11) controls. Alignments demonstrate the resistance-determining substitution Asp82Asn in HPA18 (highlighted and in bold), which was not present in the susceptible controls, and also the newly identified species-specific differences at positions 112 in GyrA and 125/136 in ParC (filled circles), which had previously been reported as resistance determinants. U.p, *U. parvum*; U.u, *U. urealyticum*. A hyphen indicates a break in the amino acid sequence. Superscript numbering refers to *Ureaplasma* protein sequence numbering.

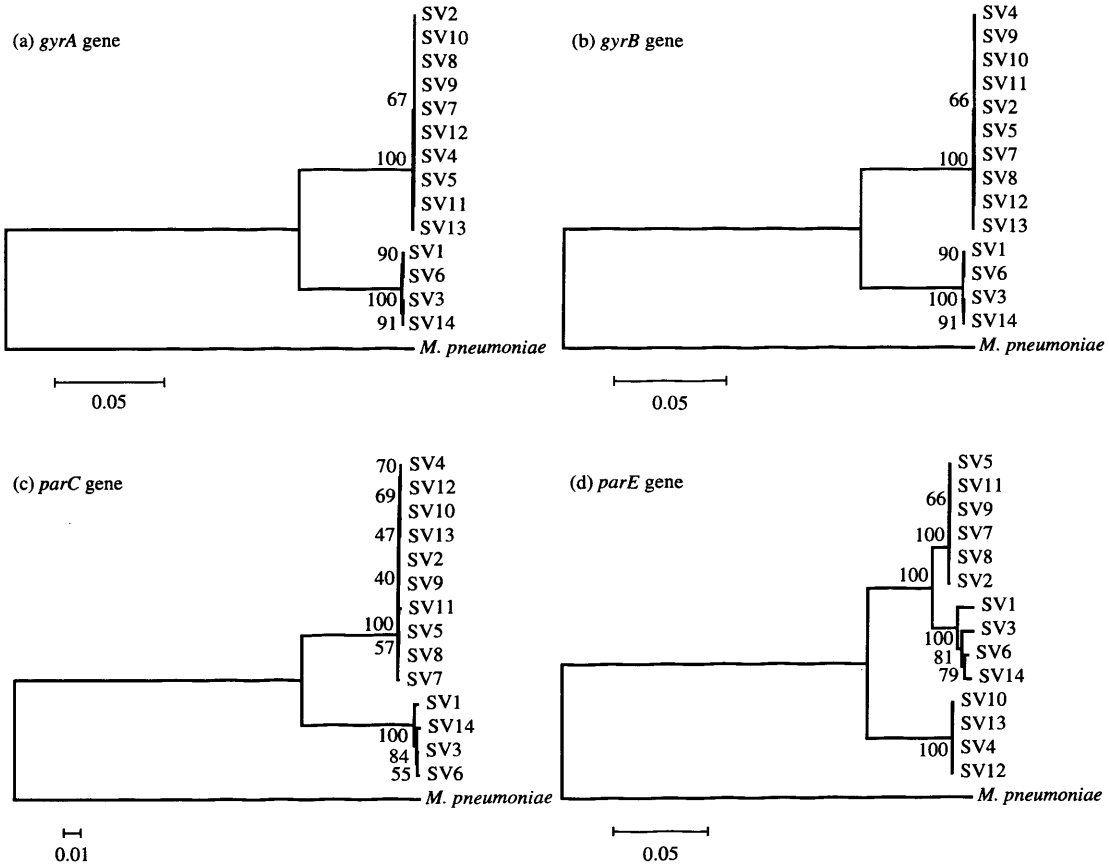

Figure 2. Evolutionary relationship of *gyrA* (a), *gyrB* (b), *parC* (c) and *parE* (d) genes of 14 human-associated *Ureaplasma* SVs. Sequences were aligned with CLUSTALW and distances calculated using maximum likelihood. The evolutionary history was inferred using the neighbour-joining method and the optimal trees after 500 bootstraps are shown. Repeat testing using Jukes–Cantor and Tamura–Nei distance calculations gave identical tree topology. Trees were rooted using respective gyrase or topoisomerase genes from *M. pneumoniae*.

Results

Alignment of amino acid sequences from UHW isolates

Figure 1 shows the amino acid alignments of the QRDRs of GyrA and ParC proteins from the ciprofloxacin-resistant (MIC=8 mg/L) *U. parvum* HPA18 (SV1) isolate as well as susceptible *U. parvum* UHWQ3 (SV1) and *U. urealyticum* UHWW11 controls. The susceptible *U. urealyticum* control had a Glu residue at position 112 of the GyrA protein, whereas the resistant and susceptible *U. parvum* isolates (HPA18 and UHWQ3, respectively) contained an Asp residue. When looking at positions 125 and 136 of ParC, UHWW11 was again unique in that it encoded Thr at both positions, whereas both HPA18 and UHWQ3 encoded an Ala. We found the Asp82Asn substitution to be unique to the resistant strain, which supports our previous hypothesis that this mutation mediates fluoroquinolone resistance.[5]

Phylogenetic analysis of gyrA, gyrB, parC and parE sequences

From the phylogenetic trees in Figure 2, it was clear that a number of species-specific nucleotide polymorphisms occur among the *gyrA*, *gyrB* and *parC* genes, causing defined clustering of *U. parvum* and *U. urealyticum*. Within each cluster there is little in the way of intraspecies differences, with the exception of *parC*, which contains unique SV-specific substitutions. The species-specific clustering trend was less clear when examining the tree constructed from *parE* sequences (Figure 2d). There was greater sequence homology between *U. urealyticum* SVs 2, 5, 7–9 and 11 with *U. parvum* than with the remaining *U. urealyticum* isolates.

List of all SV- and species-specific differences

Species-specific differences at both the nucleotide and amino acid levels were found in all gyrase and topoisomerase IV genes and proteins, respectively. Tables 2–5 list the amino acid differences, with a total of 39 in GyrA, 26 in GyrB, 107 in ParC and 34 in ParE. Of the 39 amino acid differences in GyrA, 37 were species-specific polymorphisms. At position 468 all *U. parvum* and SVs 4, 5, 7–9, 11 and 12 of *U. urealyticum* encoded Lys, whereas SVs 2, 10 and 13 encoded Glu. This pattern was also true at position 834, in which SVs 2, 10 and 13 encoded Lys, whereas the remaining *U. urealyticum* encoded Glu. GyrB contained 26 differences which was the lowest of all the proteins examined. All GyrB polymorphisms were species-restricted, with no SV- or intraspecies-specific differences.

Among the 107 differences noted in the ParC protein, 102 were species-specific. Unique serovar-specific polymorphisms were seen at position 3 for SV3 (Val rather than Asp, which was observed for all *U. urealyticum*) and position 735 for SV1 (Thr rather than Ala for *U. parvum* or Met for *U. urealyticum*). Additional serovar-specific polymorphisms were also seen for SV11 (Ala171Val and Ser347Leu) and SV1 (Glu455Lys). All *U. parvum* were also found to be missing Gln835 and Asp836.

ParE exhibited a total of 34 differences, of which only 9 were species-restricted polymorphisms. For 21 of the substitutions there was clustering of *U. parvum* with *U. urealyticum* SVs 2, 5, 7–9 and 11, whereas SVs 4, 10, 12 and 13 were divergent. A SV14-specific polymorphism was present at Gly106Ser. SV3 contained Asn151, while the remaining *U. parvum* and SVs 4, 10, 12 and 13 encoded Asp and *U. urealyticum* encoded His. SV1 had two SV-specific polymorphisms in ParE (Asp249Glu and Ser274Asn) as well as a Val417Thr polymorphism relative to other *U. parvum* SVs, but identical to the 10 *U. urealyticum* sequences.

Table 2. GyrA amino acid difference between *Ureaplasma* serovars

	U. parvum				*U. urealyticum*									
Position	1	3	6	14	2	4	5	7	8	9	10	11	12	13
112	D				E									
219	N				D									
220	V				I									
221	T				S									
223	S				P									
228	Y				H									
248	K				N									
287	A				V									
341	P				A									
377	I				V									
383	N				K									
387	K				R									
425	S				A									
428	Q				A									
431	D				G									
464	I				L									
466	V				I									
468	K				E	K	K	K	K	K	E	K	K	E
473	V				I									
475	N				E									
478	N				D									
624	D				E									
660	D				E									
710	N				D									
761	K				R									
774	E				D									
792	K				Q									
827	N				D									
829	—				N									
830	—				D									
831	—				D									
834	—				K	Q	Q	Q	Q	Q	K	Q	Q	K
835	L				T									
836	L				S									
837	Q				H									
838	E				G									
840	D				E									
841	Y				H									
844	K				E									

Amino acids are written by the single letter coding system. Numbering refers to *Ureaplasma* protein number. The amino acid in the first column is conserved to the right until otherwise indicated. A dash denotes no respective sequence in *U. parvum*.

Comparison of all previously characterized fluoroquinolone-resistant isolates

A summary of all previous studies reporting fluoroquinolone-resistant *Ureaplasma* strains is presented in Table 6. Of 33

Table 3. GyrB amino acid difference between *Ureaplasma* serovars

	U. parvum				*U. urealyticum*								
Position	1	3	6	14	2	4	5	7	8	9	10	11	12	13
8	K				N									
62	I				V									
64	R				K									
67	L				I									
72	S				V									
73	V				I									
74	I				V									
75	E				G									
142	H				Y									
145	Y				H									
161	P				I									
164	N				D									
167	V				I									
215	L				M									
233	T				A									
245	L				A									
256	R				K									
262	E				D									
315	V				I									
342	V				I									
556	A				G									
570	R				K									
575	I				L									
576	S				L									
578	I				T									
632	A				T									

Amino acids are written by the single letter coding system. Numbering refers to *Ureaplasma* protein number. The amino acid in the first column is conserved to the right until otherwise indicated.

Table 4. ParC amino acid difference between *Ureaplasma* serovars

	U. parvum				*U. urealyticum*									
Position	1	3	6	14	2	4	5	7	8	9	10	11	12	13
3	D	V	D	D	V									
5	H				Q									
59	Y				H									
125	A				T									
136	A				T									
171	A				A	A	A	A	A	A	A	V	A	A
181	N				S									
205	V				I									
210	E				K									
241	I				V									
245	C				S									
247	D				E									
250	I				T									
251	N				S									
252	D				E									
261	S				N									
300	I				V									
310	I				V									
324	N				S									
345	N				S									
347	S				S	S	S	S	S	S	S	L	S	S
372	I				V									
400	E				D									
408	T				A									
412	V				T									
414	N				A									
440	K				R									
444	Q				E									
445	T				S									
448	D				Q									
449	E				Q									
454	Q				K									
455	K	E	E	E	E									
458	N				K									
477	T				E									
480	F				Y									
481	E				Q									
490	L				F									
491	V				I									
493	V				A									
495	I				V									
505	N				S									
539	I				L									
545	I				V									
551	K				R									
565	V				I									
570	T				S									
571	S				C									
585	V				A									
595	I				V									
599	W				C									
605	I				V									
608	Y				H									

Continued

isolates described prior to June 2009, 15 were identified to species and comprised 3 *U. parvum* and 12 *U. urealyticum*.[5–11] All *U. urealyticum* isolates described contained the Asp112Glu/Ala125Thr/Ala126Thr polymorphism, while the *U. parvum* isolates did not. Using the amino acid consensus for GyrA, ParC and ParE proteins, it was predicted that of the remaining 18 non-speciated isolates, one-quarter were likely to be *U. parvum* and the remainder *U. urealyticum*. Discounting the substitutions found to be species-specific polymorphisms in the present study, only 22 of the 33 isolates have unique substitutions that could contribute to a fluoroquinolone-resistant phenotype. Thus, no mutation in the topoisomerase and gyrase genes, and no potential mechanism of resistance were identified for the remaining 11 isolates. The unique mutations that remain as candidates for the resistance mechanism include Gln100Arg (1) and Gln104Lys (1) in GyrA, Asp82Asn (1), Ser83Leu (19), Glu87Lys (1), Ala88Gly (1), Ser734Gly (1) and Ala735Thr (1) in ParC, and Asp249Gln (1), Ser274Asp (1) and Ala457Thr (1) in ParE. However, some of these substitutions occur in the same isolate (i.e. all the ParE mutations were found in the same isolate), which may indicate that multiple substitutions are required to mediate a resistant phenotype.

Table 4. *Continued*

	U. parvum				*U. urealyticum*									
Position	1	3	6	14	2	4	5	7	8	9	10	11	12	13
613	A				G									
615	R				K									
625	S				N									
629	I				L									
643	D				E									
646	N				G									
656	G				D									
661	I				V									
662	I				V									
670	A				V									
672	L				M									
699	V				I									
701	A				S									
704	T				V									
711	N				S									
712	V				I									
717	E				D									
718	R				Q									
720	S				N									
724	N				S									
735	T	A	A	A	M									
738	T				S									
739	N				P									
742	P				L									
743	I				P									
747	V				A									
749	N				S									
757	A				G									
758	H				N									
767	Q				E									
777	S				P									
779	P				L									
782	T				I									
786	T				S									
789	S				N									
790	I				K									
795	Q				L									
796	M				I									
804	C				S									
809	E				S									
810	A				V									
818	N				S									
826	N				S									
828	M				L									
831	I				T									
833	K				E									
835	—				Q									
836	—				E									
837	H				Q									
840	S				L									
846	D				N									
848	E				K									

Continued

Table 4. *Continued*

	U. parvum				*U. urealyticum*									
Position	1	3	6	14	2	4	5	7	8	9	10	11	12	13
851	K				N									
852	K				D									

Amino acids are written by the single letter coding system. Numbering refers to *Ureaplasma* protein number. The amino acid in the first column is conserved to the right until otherwise indicated. A dash denotes no respective sequence in *U. parvum*.

Table 5. ParE amino acid difference between *Ureaplasma* serovars

	U. parvum				*U. urealyticum*									
Position	1	3	6	14	2	5	7	8	9	11	4	10	12	13
8	N				N						S			
33	T				A									
39	I				V									
59	N				N						S			
61	I				I						V			
70	V				V						I			
83	S				S						P			
106	S	S	S	G	S									
128	S				S						A			
145	I				I						S			
151	D	N	D	D	H						D			
153	S				S						P			
180	V				V						F			
185	I				I						T			
203	I				I						S			
210	K				K						N			
218	N				N						D			
231	A				T									
240	Y				Y						F			
242	R				K									
243	S				S						N			
244	N				N						T			
249	E	D	D	D	D									
252	I				I						V			
274	N	S	S	S	S									
299	N				N						D			
377	L				L						V			
417	T	V	V	V	T									
542	N				N						T			
588	N				S									
589	N				K									
594	K				R									
595	I				V									
619	K				R									

Amino acids are written by the single letter coding system. Numbering refers to *Ureaplasma* protein number. The amino acid in the first column is conserved to the right until otherwise indicated.

Table 6. Summary of mutations described for fluoroquinolone-resistant ureaplasmas in gyrase and topoisomerase genes between 2000 and 2009

						Amino acid substitution																		
			MIC (mg/L)			GyrA			ParC												ParE				
Author	Isolate	Species	CIP	OFX	MXF	100	104	112	3	59	82	83	87	88	125	136	301	734	735	151	249	274	417	457
Bebear *et al.*, 2000	UUa	U.u[a]	64	64	4	***Arg***		Glu							Thr	Thr								
Zhang *et al.*, 2002	19	—	32	16	NA							***Leu***												
	52	—	≥64	8	NA			Glu				***Leu***												
	70	—	64	8	NA			Glu				***Leu***												
	87	—	64	8	NA			Glu																
	92	—	128	16	NA			Glu				***Leu***												
	104	—	64	8	NA			Glu				***Leu***												
	105	—	128	16	NA			Glu																
	107	—	64	8	NA			Glu				***Leu***												
	108	—	64	8	NA							***Leu***												
	131	—	32	8	NA			Glu																
	176	—	64	8	NA			Glu				***Leu***												
	215	—	16	8	NA			Glu																
	224	—	64	8	NA			Glu				***Leu***												
Bebear *et al.*, 2003	UUe2	U.p	8	8	1							***Leu***												
	UUb	U.u	4–16	4–8	0.5–1			Glu							Thr	Thr								
	UUd	U.u	4–16	4–8	0.5–1			Glu							Thr	Thr								
	UUf1	U.u	4–16	4–8	0.5–1			Glu							Thr	Thr								
	UUg1	U.u	4–16	4–8	0.5–1			Glu							Thr	Thr								
	UUf2	U.u	16	4–8	1			Glu				***Leu***			Thr	Thr								
	UUg2	U.u	16	4–8	1			Glu				***Leu***			Thr	Thr								
	UUg3	U.u	16	4–8	1			Glu				***Leu***			Thr	Thr								
	UUg4	U.u	16	4–8	1			Glu				***Leu***			Thr	Thr								
	UUg5	U.u	16	4–8	1			Glu				***Leu***			Thr	Thr								
	UUc	U.u	8	8	1			Glu					***Lys***		Thr	Thr								
Duffy *et al.*, 2006	48105	U.p	NA	128	8		***Lys***		Asp			***Leu***		***Gly***			***Gly***	***Thr***	Thr	Asp	Glu	Asp	Thr	***Thr***
Xie *et al.*, 2006	163	—	1	1	NA																			
	168	—	>2	<1	NA																		Thr	
	46	—	>2	>4	NA			Glu				***Leu***			Thr								Thr	
	161	—	>2	>4	NA							***Leu***												
	7	—	>2	>4	NA			Glu				***Leu***											Thr	
Geissdorfer *et al.*, 2008	CM239	U.u	>2	1	NA			Glu		His					Thr	Thr								
Beeton *et al.*, 2009	HPA18	U.p	8	NA	NA						***Asn***													

Continued

Table 6. *Continued*

Author	Isolate	Species	MIC (mg/L)			Amino acid substitution																		
						GyrA				ParC								ParE						
			CIP	OFX	MXF	100	104	112	3	59	82	83	87	88	125	136	301	734	735	151	249	274	417	457
U. parvum[b]						Gln	Gln	Asp	Asp[d]	Tyr	Asp	Ser	Glu	Ala	Ala	Ala	Ser	Ala	Ala[e]	Asp[f]	Asp[g]	Ser[h]	Val[i]	Ala
U. urealyticum[c]						Gln	Gln	Glu	Val	His	Asp	Ser	Glu	Ala	Thr	Thr	Ser	Ala	Met	His[f]	Asp	Ser	Thr	Ala

CIP, ciprofloxacin; OFX, ofloxacin; MXF, moxifloxacin; NA, not applicable (the antibiotic was not examined).
Amino acids in bold and in italics are non-species- or serovar-specific differences and are potentially the reason for the resistant phenotypes seen.
[a]Species determined in the later paper from Bebear *et al.*, 2003 (a dash denotes that the species was not determined; U.u, *U. urealyticum*; U.p, *U. parvum*).
[b]The consensus amino acid sequences for all sequenced *U. parvum*.
[c]The consensus amino acid sequences for all sequenced *U. urealyticum*.
[d]SV3 has a Val at this position.
[e]SV1 has a Thr at this position.
[f]SV3 has Asn at this position; SVs 1, 4, 6, 10, 12, 13 and 14 have Asp at this position; SVs 2, 5, 7–9 and 11 have His at this position.
[g]SV1 has a Glu at this position.
[h]SV1 has a Asp at this position.
[i]SV1 has a Thr at this position.

Discussion

Previously, the combined substitutions of Asp112Glu in the GyrA protein along with Ala125Thr and Ala136Thr in the ParC protein were highlighted in fluoroquinolone-resistance in *Ureaplasma*.[6,7,9–11] In this study, a comparison of the QRDRs of a ciprofloxacin-resistant *U. parvum* (HPA18) with susceptible *U. parvum* (UHWQ3) and *U. urealyticum* (UHWW11) isolates showed that both *U. parvum* isolates had the same profile as previously described susceptible isolates, whereas the susceptible *U. urealyticum* had the profile previously linked to resistance. This finding stimulated further investigation of amino acid polymorphisms for all SVs of both *U. parvum* and *U. urealyticum*.

Phylogenetic analysis demonstrated distinct clustering of species in three out of the four genes analysed, supporting the decision to separate the human-associated ureaplasmas into two distinct species based on previous genotypic traits.[2] However, this study revealed an unexpected divergence of SVs 4, 12, 10 and 13 from the remaining *U. urealyticum* in the *parE* tree. SV-specific substitutions were noted for SVs 1 and 11 in ParC, and SVs 1, 3 and 14 in ParE. These species and SV-specific substitutions will be important for comparison of resistant and susceptible strains in the future, to avoid incorrect identification of potential resistance mechanisms.

Thirty-three fluoroquinolone-resistant *Ureaplasma* isolates from seven different investigations were identified, for which amino acid substitution in the type II topoisomerase proteins was proposed as the molecular mechanism of resistance.[5–11] The substitutions that were proposed to result in the resistant phenotype are summarized in Table 6. In 2000, Bebear *et al.*[6] described the molecular characterization of a ciprofloxacin resistance in *Ureaplasma*. The sequence of an isolate of *U. urealyticum* was compared with the published SV3 genome (ATCC 700970)[13] and identified to contain four amino acid transitions (Gln100Arg and Asp112Glu in GyrA, and Thr125Ala and Thr136Ala in ParC), which were proposed to be related to resistance. The evidence presented in the present study suggests that only Gln100Arg could potentially be responsible for the ciprofloxacin MIC of >128 mg/L, with the remaining three differences all being species-specific polymorphisms. A report by Zhang *et al.*[11] found that 9 of 13 isolates with varying degrees of resistance to fluoroquinolones had a Ser83Leu substitution within the ParC protein. Unfortunately, the authors did not determine the species of the isolates, but based on the GyrA sequence data we can speculate that 11 of the 13 isolates were *U. urealyticum* and 2 were *U. parvum*. The Ala125Thr and Ala136Thr mutations were not reported in the suspected *U. urealyticum* isolates, but it is not known if the investigators sequenced this portion of the gene as the primers used were not reported. Interestingly, the authors not only compared the resistant isolate sequences with the SV3 genome, but also with *U. urealyticum* controls from SV4 (ATCC 27815) and SV8 (ATCC 27618) with the intention of ruling out species-specific polymorphism. Twelve base differences within *gyrA* and 10 within *parC* were identified, but the deduced amino acid sequences were quoted as being identical between SVs.

Subsequent investigations by Bebear *et al.*[7] in 2003 examined a further 11 resistant isolates and compared sequence data with the SV3 genome and a susceptible control, UUe1. As would be predicted, resistant *U. urealyticum* isolates were consistently found to have the species-specific polymorphism at amino acid

112 in GyrA and 125 and 136 in ParC, although two *U. parvum* isolates examined (one resistant and one susceptible) did not contain these substitutions. Six of the eleven isolates contained the Ser83Leu mutation, with a single isolate containing a unique mutation in the ParC protein only five amino acids downstream for isolate UUc (Glu87Lys).

The first molecular characterization of fluoroquinolone resistance in the USA was provided by Duffy *et al.*,[8] who reported a *U. parvum* isolate with an ofloxacin MIC of 128 mg/L. This isolate was documented as having 12 mutations, including Ser83Leu, but from the data in Table 6, six of these substitutions can be ruled out as contributing to resistance as they are species- or SV-specific polymorphisms. The Val3Asp mutation in ParC was proposed as a resistance determinant, but this substitution turns out to be a SV-specific substitution for SV3 (which was used as the comparator for their sequence); consensus sequences for SVs 1, 6 and 14 show Asp at this position (Table 4). Although the SV of this isolate was not determined it can be hypothesized that it was likely to be SV1, as they also identified the SV1-specific polymorphisms we have found at position 735 in ParC, and positions 249, 274 and 417 in ParE (Tables 4 and 5, respectively).

Resistance in *Ureaplasma* isolates from China between 1999 and 2004 was reported by Xie *et al.*,[10] who found the Ser83Leu resistance-associated mutation in the QRDR of three of five resistant isolates. However, their reported sequences do not align fully with our determined consensus sequences. For example, isolates 161, 163 and 168 all show polymorphisms that identify them as *U. parvum* (Asp112 in GyrA, and Ala125 and Ala136 in ParE), and isolate 168 also had Thr417 in ParE suggesting that it was an SV1. However, isolates 7 and 46 had Glu112 in GyrA suggesting they were *U. urealyticum*, but isolate 46 had Ala125 and Ala136 in ParE consistent with *U. parvum* consensus sequences, and isolate 7 had Thr125 but Ala136 giving a mixture of the consensus sequences in ParE. We are unable to explain this mixing of consensus sequences; however, isolates 163 and 168 do not have any mutations that are not also found in susceptible *U. urealyticum* or *U. parvum* isolates.

Geissdorfer *et al.*[9] characterized an isolate from a patient who developed meningitis following kidney transplantation. Comparison of the reported sequence with our consensus Tables identifies the isolate as *U. urealyticum*, but no unique resistance candidate mutations remain after species-specific polymorphisms are taken into account. Finally, we previously described a single ciprofloxacin-resistant isolate from a cohort of 61 *Ureaplasma* isolates from neonates.[5] We identified the isolate as an SV1 strain with a unique Asp82Asn mutation within the ParC protein. This is immediately adjacent to the commonly found Ser83Leu mutation and is therefore a strong candidate as a resistance-mediating mutation.

This review of the literature highlights that 75% of the fluoroquinolone-resistant strains identified to date were *U. urealyticum* (Table 6), even though this species is isolated much less frequently from patients (20% of patient isolates in our previous study,[5] which is consistent with other groups' findings).[1] Furthermore, the ParC Ser83Leu mutation was found in 58% of the fluoroquinolone-resistant *Ureaplasma* strains, which is homologous to the resistance-mediating mutation identified in many other fluoroquinolone-resistant bacteria, including *Staphylococcus aureus* and *Streptococcus pneumoniae*.[14] Fluoroquinolones are known to have preferential sites of action: the gyrase proteins for Gram-negative bacteria and the topoisomerase IV proteins for Gram-positive bacteria.[15] The proposed evolution of ureaplasmas from Gram-positive progenitors may be responsible for the higher incidence of mutations within the topoisomerase IV proteins relative to gyrase proteins. Based on our analysis, the molecular mechanism of fluoroquinolone resistance in 11 out of the 33 isolates is still undetermined. Resistance in these isolates may have been due to mutations outside of the sequenced regions or through alternative mechanisms, such as altered membrane permeability.[15]

This report highlights the importance of determining the species of *Ureaplasma* and the caution that should be taken when comparing future sequence data for both fluoroquinolone resistance and other genes potentially associated with antibiotic resistance. To avoid similar misinterpretation, sequences should be compared with the appropriate species and, in some circumstances, the appropriate SV.

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Transparency declarations

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Relationship of proteinases and proteinase inhibitors with microbial presence in chronic lung disease of prematurity

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ABSTRACT

Background A proteolytic imbalance has been implicated in the development of "classical" chronic lung disease of prematurity (CLD). However, in "new" CLD this pattern has changed. This study examines the longitudinal relationship between neutrophil proteinases and their inhibitors in ventilated preterm infants and their relationship to microbial colonisation.

Methods Serial bronchoalveolar lavage fluid was obtained from ventilated newborn preterm infants. Neutrophil elastase (NE) activity, cell counts, metalloproteinase (MMP)-9, MMP-9/TIMP-1 complex, SerpinB1 concentration and percentage of SerpinB1 and α_1-antitrypsin (AAT) in complex with elastase were measured. The presence of microbial genes was examined using PCR for 16S rRNA genes.

Results Statistically more infants who developed CLD had NE activity in at least one sample (10/20) compared with infants with resolved respiratory distress syndrome (RDS) (2/17). However, NE activity was present in a minority of samples, occurring as episodic peaks. Peak levels of MMP-9, MMP-9/TIMP-1 complex, percentage of AAT and SerpinB1 in complex and cell counts were all statistically greater in infants developing CLD than in infants with resolved RDS. Peak values frequently occurred as episodic spikes and strong temporal relationships were noted between all markers. The peak values for all variables were significantly correlated to each other. The presence of bacterial 16S rRNA genes was associated with the development of CLD and with elevated elastase and MMP-9.

Conclusion NE activity and MMP-9 appear to be important in the development of "new" CLD with both proteinase and inhibitor concentrations increasing episodically, possibly in response to postnatal infection.

INTRODUCTION

Chronic lung disease of prematurity (CLD, also called bronchopulmonary dysplasia, BPD) causes ongoing respiratory morbidity and mortality in preterm infants[1 2] and is associated with large oversimplified alveoli suggesting aberrant lung development (sometimes called "new" CLD/BPD).[3] Inflammation is a hallmark in the development of CLD, with neutrophils and macrophages releasing reactive oxygen species and proteolytic enzymes including the serine proteinase neutrophil elastase (NE)[4] and matrix metalloproteinases including MMP-9.[5 6] Normally, any free proteinase is effectively neutralised by proteinase inhibitors. Tissue inhibitors of matrix metalloproteinases (TIMPs) are regulators of MMPs, which for MMP-9 in the lung is primarily TIMP-1.[5–7] For NE, the best characterised is α_1-antitrypsin (AAT) with which it forms an irreversible covalent 1:1 complex; other proteinase inhibitors include secretory leukoproteinase inhibitor (SLPI), elafin, α_2-macroglobulin and SerpinB1 (originally called monocyte neutrophil elastase inhibitor) which rapidly binds covalently with NE. SerpinB1 has been studied in rat and mouse models and in newborn baboons but not in human preterm infants.

Early studies reported proteolytic imbalance in preterm infants developing classical CLD.[8–10] "New" CLD shows a different pattern with elastase activity being less frequently reported.[11–15] An imbalance between MMP-9 and TIMP-1 has also been described, but the importance of MMP-9 in the development of CLD remains unclear.[5–7 16–18]

The aims of this study were (1) to serially measure NE, AAT, SerpinB1, MMP-9 and MMP-9/TIMP-1 complex in bronchoalveolar lavage (BAL) fluid of preterm infants at risk of developing CLD; (2) to examine the relationships between these variables and their relationship with CLD development; and (3) to correlate enzyme dysregulation with bacterial colonisation as identified by the presence of bacterial 16S ribosomal RNA (rRNA) genes.

METHODS

Patient groups

Three groups of mechanically ventilated infants were studied: (1) preterm infants (<32 weeks gestation) who developed mild, moderate or severe CLD using National Institute of Child Health criteria[1]; (2) preterm infants who developed and recovered from neonatal respiratory distress syndrome (RDS); and (3) a control group comprising term infants who required ventilation for non-respiratory reasons needing ≤28% oxygen throughout the study period. Participants were recruited from the neonatal unit at the University Hospital of Wales, Cardiff between May 2005 and December 2006.

Bronchoalveolar lavage

BAL was performed as previously described.[19 20] BAL fluid was collected daily for the first week of life then twice a week until 28 days of age or until extubation. Two aliquots of 1 ml/kg normal saline were instilled and suctioned immediately. Samples were centrifuged at 1000*g* for 10 min at 4°C within 30 min of collection. The supernatant was aliquoted and stored at −80°C. Pelleted cells were resuspended in phosphate buffered saline, counted with a haemocytometer and cytospins were stained with

Diff-Quik for cell differential counts. The unused cell suspension was recentrifuged and the pellet stored at −80°C.

NE activity

NE activity in BAL fluid was measured by the kinetic conversion of Suc-Ala-Ala-Pro-Val-pNA (Bachem, St Helens, UK), as described previously.[11 14] The specificity of free NE activity was verified by neutralisation with recombinant AAT (Arriva Pharmaceuticals, Alameda, California, USA).

AAT analysis

The protein concentration in BAL fluid was determined using a BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA). BAL fluid AAT was analysed by western blot analysis with equal protein load as described elsewhere[13] using horseradish peroxidase (HRP)-conjugated goat polyclonal anti-human AAT (AB7635; Abcam PLC, Cambridge, UK). Excess purified NE (0.5 μg) was added to BAL fluid for 30 min at room temperature prior to western blot analysis when immunoreactive native AAT was observed coincident with NE activity to test specificity.

SerpinB1 analysis

SerpinB1 was assessed in equal volumes of BAL fluid by western blot analysis using a rabbit polyclonal anti-SerpinB1 primary and an HRP-conjugated goat anti-rabbit secondary antibody.[21] A standard curve (3–30 ng) of recombinant SerpinB1 was included on each gel. SerpinB1 was quantified by densitometry using ImageJ software (http://rsb.info.nih.gov/ij/).

Total MMP-9 and MMP-9/TIMP-1 complex

These were measured by ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Bacterial 16S rRNA analysis

Cell pellet DNA was isolated using the Qiagen RNA/DNA Mini kit (Qiagen, Crawley, UK). PCR was used to amplify the 16s rRNA gene using the primers 27f (5′-AGAGTTTGATC(AC)TGGCTCAG-3′) and 1492r (5′-TACGG(CT)TACCTTGTTACGACTT-3′).[22 23] The integrity of extracted DNA was assessed by amplifying human mitochondrial cytochrome oxidase (HMCO) using specific primers. PCR products were separated on 0.7% agarose gels stained with ethidium bromide and visualised in a BioDoc-IT UV Transilluminator (Ultra-Violet Products, Cambridge, UK).

Sequencing of 16s rRNA genes

Amplicons from samples positive for 16s rRNA genes were purified using a Novagen spin-prep PCR clean-up kit (Merck Chemicals, Nottingham, UK) and sequenced using the BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) and an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Sources of bacterial 16S rRNA genes were determined by amplicon sequence comparison using the BlastN website.

Statistical methods

Statistical analysis was performed using SPSS V.15.0. The Mann–Whitney U test was used for non-parametric data and correlations between groups were performed using Spearman correlation coefficients. The presence of NE or 16s rRNA genes between groups was compared using χ^2 tests. p Values of <0.05 were considered statistically significant.

RESULTS

Patients

Forty-two infants of <32 weeks' gestational age were recruited: 20 developed CLD (5 mild, 12 moderate, 3 severe CLD); 17 recovered from RDS; 5 infants died before they could be classified and were excluded from the analysis; there were also 6 term controls (table 1). Two hundred and twenty BAL fluid samples were collected: 130 from the CLD group, 45 from the RDS group, 22 from infants who died and 23 from term controls. The median BAL fluid yield was 50% (IQR 38–63%).

Cell counts

Median (IQR) peak cell counts in BAL fluid were statistically greater in infants developing CLD (6.75×10^6 cells/ml; $3.54–17.9\times10^6$) than in the RDS group (2.22×10^6 cells/ml; $1.05–5.50\times10^6$, p=0.002) and controls (0.63×10^6 cells/ml; $0.156–1.26\times10^6$, p<0.001). The moderate/severe CLD group (6.30×10^6 cells/ml; $3.97–11.3\times10^6$) had a statistically greater peak cell count than the RDS group (p=0.005). Neutrophils dominated the cell counts but macrophages and some epithelial cells were observed. Peak neutrophil counts were statistically greater in infants developing CLD (3.36×10^6 cells/ml; $1.79–9.93\times10^6$) than in infants with resolved RDS (1.14×10^6 cells/ml; $0.65–2.88\times10^6$, p=0.005) and term controls (0.38×10^6 cells/ml; $0.048–0.63\times10^6$, p=0.001). Infants with moderate/severe CLD (2.56×10^6 cells/ml; $1.91–7.13\times10^6$) had statistically more neutrophils than the RDS group.

Elastase activity

Free NE activity was detected in 26/198 BAL fluid samples (13%). Statistically more infants who developed CLD (10/20) had free NE activity in at least one BAL fluid sample compared with the RDS group (2/17, p=0.013) or controls (0/6, p=0.027). Three of 5 infants with mild CLD had detectable NE in at least one BAL fluid sample. More infants with moderate/severe CLD had free NE detected in BAL fluid than infants in the RDS group (p=0.035). No statistical difference was noted between the RDS and control groups. No sample from the term controls had free NE activity. NE activity was present in 23/129 (18%) lavages from the infants developing CLD and in 3/46 (7%) samples from infants with resolved RDS.

Detection of NE activity was episodic, with the day of first occurrence varying from days 2 to 26 (median 7 days), and NE activity coincided with increased BAL fluid cell counts (figure 1 and online data supplement). In all cases, NE activity was blocked by the addition of exogenous recombinant AAT (data not shown), suggesting an imbalance between proteinases and proteinase inhibitors.

Table 1 Patient characteristics

	Chronic lung disease	Resolved respiratory distress syndrome	Term control
Number of patients	20	17	6
Number of samples	130	45	23
Gestational age (weeks)	27 ($25^{+4}–29^{+3}$)*	28 $^{+6}$ ($27–29^{+2}$)*	Term
Birth weight (g)	835 (695–1025)	1120 (935–1315)	2550 (2090–2825)
M:F	11:9	10:7	3:3
Vaginal:caesarean delivery	10:10	6:11	5:1
Antenatal steroids >24 h	16/20 (80%)	12/17 (71%)	0/6 (0%)
Surfactant therapy	20/20 (100%)	17/17 (100%)	0/6 (0%)
Prolonged rupture of membranes (>24 h)	4/20 (20%)	1/17 (6%)	0/6
Length of ventilation (days)	7 (5.5–11)	1 (1–4.5)	3

* Values are shown as median (IQR).
Superscript numbers indicate the number of days in addition to gestation in weeks.

α_1-Antitrypsin

Western blotting detected free AAT as a 53 kDa band, AAT-proteinase complex at 80 kDa and apparent degraded AAT at 48 kDa (figure 2A). AAT-NE complex was detected in 18/20 (90%) infants who developed CLD, which is statistically more than 8/17 (47%) infants whose RDS resolved (p=0.004). The maximum percentage of complexed AAT as estimated by densitometry was statistically greater for infants in the CLD group (median 22%; IQR 13–32%) than for infants whose RDS resolved (median 0; 0–19%, p=0.012) and term controls (median 0; 0–9%, p=0.003), and between the RDS group and infants with moderate/severe CLD (median 22%; 13.5–33.5%, p=0.013).

Interestingly, BAL fluid collected from some infants with CLD contained both free NE and unbound AAT (53 kDa) (figure 2B). In all cases when a molar excess of NE was incubated with the BAL fluid prior to western blot analysis, the unbound AAT (53 kDa) formed a complex with NE or was degraded.

SERPINB1

The median (IQR) SerpinB1 concentration on the first day of life was 155 ng/ml (64–414), which did not increase significantly over the first 5 days of life; no statistical difference was noted between the CLD and RDS groups over this period. Median (IQR) peak SerpinB1 concentrations were not statistically different between groups (CLD group: 400 ng/ml; 185–2130; RDS group: 205 ng/ml; 113–480, p=0.161) and term controls (163 ng/ml; 78–164, p=0.107). No statistical difference was seen between peak SerpinB1 concentrations in the RDS group and infants with moderate/severe CLD (455 ng/ml; 191.5–2130, p=0.157). Episodic increases in SerpinB1 were observed more frequently in infants developing CLD than in a minority of RDS infants (figure 3), and these increased SerpinB1 episodes corresponded to episodic increases of cell counts, free NE and NE-AAT complexes (figure 1 and online supplement). Statistically more infants with CLD had covalently complexed SerpinB1 in at least one BAL fluid sample (11/20) compared with infants with resolved RDS (3/17), p=0.020. SerpinB1 complex was not observed in control infants.

MMP-9

Median (IQR) peak MMP-9 protein concentrations were statistically greater in infants who developed CLD (763 ng/ml; 200–1790) than in infants in the RDS group (47 ng/ml; 20–352, p=0.004) and term controls (74 ng/ml; 36–250, p=0.007). Infants with moderate/severe CLD (948 ng/ml; 252–2322) also had statistically greater MMP-9 than those with RDS (p=0.005). Peak MMP-9/TIMP-1 complex concentrations were greater in infants who developed CLD (29.9 ng/ml; 13.1–51.3) than in the RDS group (1.5 ng/ml; 0.9–24.1, p=0.004) and than infants with moderate/severe CLD (29.9 ng/ml; 16.5–51.3) than in infants in the RDS group (p=0.017). Differences between CLD and term infants (7.0 ng/ml; 1.4–24.2) were not statistically significant (p=0.068).

Relationship between variables

Strong correlations were observed between most peak values, particularly MMP-9 and MMP-9/TIMP-1 complexes and NE activity relative to the percentage of complexed SerpinB1 (table 2). In addition, there were strong temporal correlations between cell counts, proteinases and anti-proteinases (figure 1 and online supplement).

Bacterial 16S rRNA genes

DNA was extracted from 198 BAL fluid samples and satisfactory DNA integrity was confirmed in 162 by HMCO amplification by PCR (only three infants failed to have a satisfactory sample DNA). Bacterial 16S rRNA genes were detected in 75/162 (46%) of these samples; statistically more infants who developed CLD had 16S rRNA genes in their BAL fluid at some point (14/19, 74%) than infants in the RDS group (5/16 (31%), p=0.012) or term controls (2/5 (40%), p=0.155). Statistically more infants with moderate/severe CLD (11/14, 79%) had 16s rRNA genes detected than infants in the RDS group (p=0.001). Elastase activity was found in greater numbers of 16S rRNA gene-positive

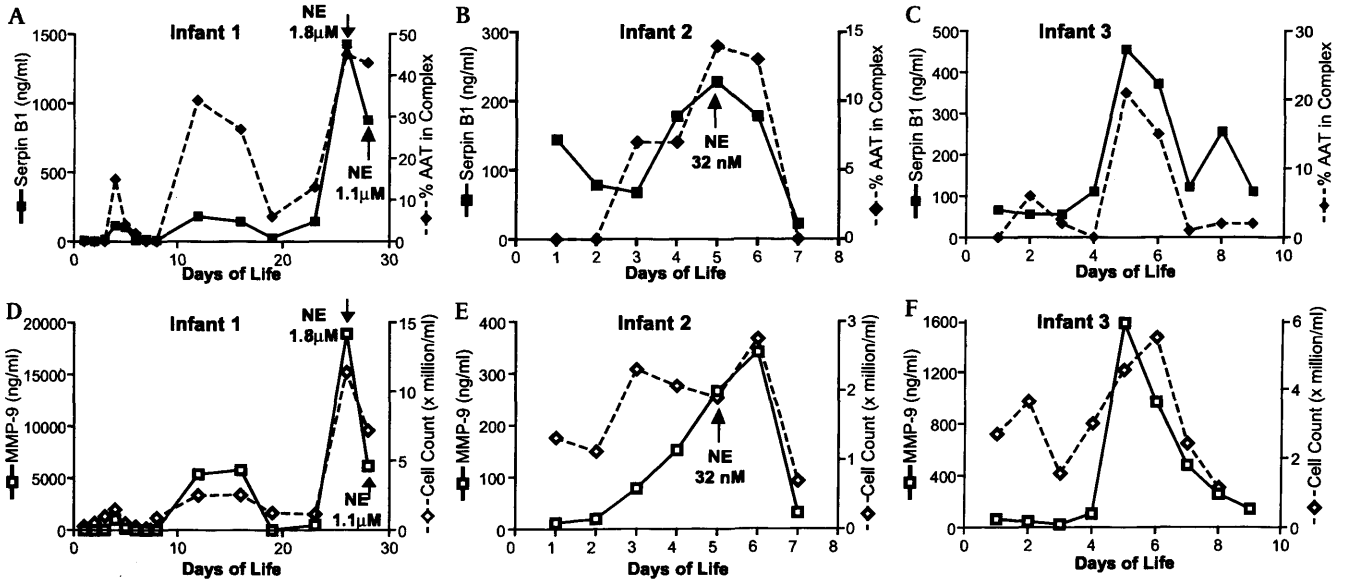

Figure 1 Longitudinal analysis of bronchoalveolar lavage fluid from three infants showing temporal relationships between variables. Graphs A–C show SerpinB1 concentrations (left axis, solid line) and the proportion of α_1-antitrypsin (AAT) in complex with serine protease (right axis, broken line) relative to day of life (x-axis). Graphs D–F show the same infants with matrix metalloproteinase (MMP)-9 concentrations (left axis, solid line) and total cell counts (right axis, broken line) against days of life. The arrows indicate the days when free neutrophil elastase (NE) was detected. No free NE was detected in infant 3.

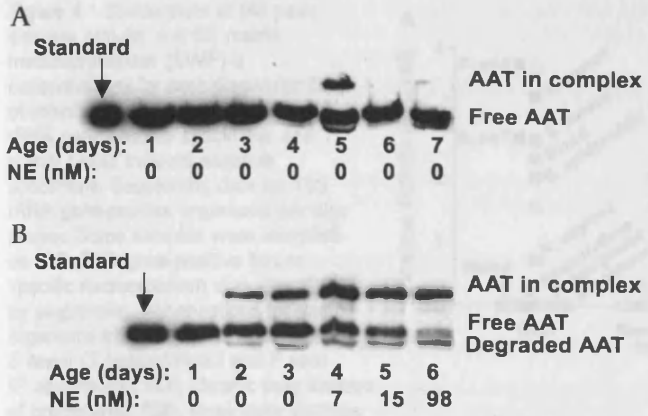

Figure 2 Western blot of α_1-antitrypsin (AAT) species in sequential bronchoalveolar lavage (BAL) fluid specimens for (A) a representative infant from the resolved respiratory distress syndrome group in whom active neutrophil elastase (NE) was not detected and (B) an infant from the chronic lung disease of prematurity group in whom active NE was detected. Patient age and NE concentrations are shown below each lane of the western blot. Equal amounts of protein were loaded for analysis and the left lanes show purified plasma AAT migrating at 53 kDa (standard). Additional bands in BAL fluid specimens are the complex with serine proteases at 80 kDa and degraded AAT at 48 kDa. Note that the day 1 samples for both infants contain free AAT only, while day 5 BAL fluid for patient A and days 2–6 for patient B also contain an additional higher AAT-proteinase complex. BAL fluid from days 2–6 of patient B also contains degraded AAT.

lavages (19/75, 25%) than 16S rRNA gene-negative lavages (5/87 (6%), p=0.009). Furthermore, elastase activity was higher in the 16S rRNA gene-positive lavages than in the negative lavages (although the median value for both was zero, p=0.0018). The median (IQR) total BAL fluid MMP-9 concentration was greater in 16S rRNA gene-positive lavages (211 ng/ml; 64–1047) than in 16S rRNA gene-negative samples (44 ng/ml; 21–115, p<0.001).

PCR amplicon sequencing showed that the samples with the greatest NE activity and total MMP-9 concentrations contained virulent organisms such as *Stegastes aureus* and *E coli* (figure 4A), while the RDS infants with free elastase and the highest MMP-9 concentrations had organisms regarded as being less virulent such as *S epidermidis* (figure 4B).

DISCUSSION

This study provides a detailed profile of NE and its inhibitors, AAT (SerpinA1) and SerpinB1 as well as MMP-9 and MMP-9/

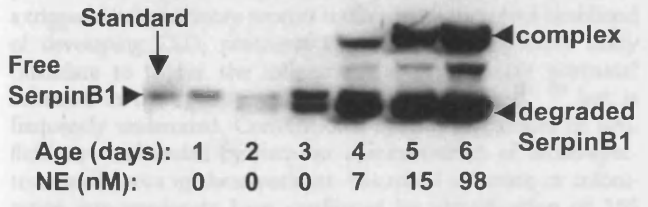

Figure 3 Western blot of SerpinB1 species in sequential bronchoalveolar lavage (BAL) fluid specimens of an infant with chronic lung disease of prematurity in whom active neutrophil elastase (NE) was detected. Equal volumes of BAL fluid were loaded for analysis and the left lane shows purified SerpinB1 (10 ng) migrating at 42 kDa (standard). Other species present in BAL fluid are the 66 kDa complex with serine proteinase, a partially degraded intermediate product and degraded SerpinB1 at 38 kDa. A statistically significant increase in both the total concentration and percentage in the complex is seen, occurring with increased NE activity.

Table 2 Correlation coefficients between maximum values of cell counts, neutrophil elastase (NE) activity, matrix metalloproteinase (MMP)-9 concentrations, SerpinB1 concentrations, percentage α_1-antitrypsin (AAT) and SerpinB1 in complex and MMP-9/TIMP-1 concentrations in BAL fluid from ventilated newborn infants

Maximum value	Maximum value	Spearman's correlation coefficient (p value)
Log_{10} MMP-9	Log_{10} MMP-9/TIMP-1	0.94
Log_{10} elastase	% SerpinB1 in complex	0.88
Log_{10} SerpinB1	% SerpinB1 in complex	0.76
Log_{10} MMP-9	Log_{10} cell count	0.75
Log_{10} elastase	Log_{10} neutrophil count	0.71
Log_{10} neutrophil count	% SerpinB1 in complex	0.70
Log_{10} elastase	Log_{10} SerpinB1	0.68
Log_{10} elastase	Log_{10} cell count	0.65
Log_{10} elastase	Log_{10} MMP-9	0.65
% AAT in complex	% SerpinB1 in complex	0.64
% AAT in complex	Log_{10} elastase	0.62
Log_{10} neutrophil count	Log_{10} SerpinB1	0.62
Log_{10} cell count	Log_{10} SerpinB1	0.59

Statistical analysis for all correlations shown were statistically significant at p<0.001.

TIMP-1 complex in ventilated preterm infants. It identified relationships between proteolytic enzymes and their inhibitors and highlights the importance of postnatal infection in proteinase release and in the development of CLD.

Our study is the most detailed longitudinal analysis to date of NE activity in CLD and includes the previously unstudied SerpinB1. Previous studies examined NE at single time points[11 13 14] or examined a limited number of samples.[12 15] We found that NE activity was episodic and frequently occurred relatively late in the development of CLD, which may cause NE activity to be missed when summary data are presented rather than longitudinally for individual infants. BAL fluid studies of intubated infants prevent comparisons with patients with RDS at equivalent time points. However, our data suggesting that infection may lead to dysregulated inflammation are in agreement with published evidence that prolonged mechanical ventilation commonly results in the development of pneumonia.[24]

Some infants developed CLD without NE activity being detected. It is possible that NE activity occurred between lavages or following extubation. Also, NE activity may be increased in the pericellular environment as suggested by the presence of complexed AAT and SerpinB1. These complexes, which may be regarded as surrogate markers for regulated NE release, were present in far more samples than was free NE, particularly in infants developing CLD, suggesting successful regulation of proteinases. The simultaneous presence of free NE and unbound AAT (53 kDa) was interesting. This may reflect oxidation of the AAT reactive site methionine residue resulting in a decreased rate of NE-AAT complex formation.[25] Thus complex formation occurred after in vitro incubation for a further 30 min with excess NE. Alternatively, increased proteinases including MMP-9 may degrade and inactivate AAT leading to more NE activity. Infection may trigger this dysregulation as it was closely correlated with the increased NE.

SerpinB1 has not been studied in human newborn infants but has been examined in a baboon CLD model.[26] Higher NE activity was reported in baboons with "classical" compared with "new" CLD. Baboons developing "new" CLD had 2.5 times the level of SerpinB1 mRNA in BAL fluid compared with term controls, while "classical" CLD was associated with lower SerpinB1

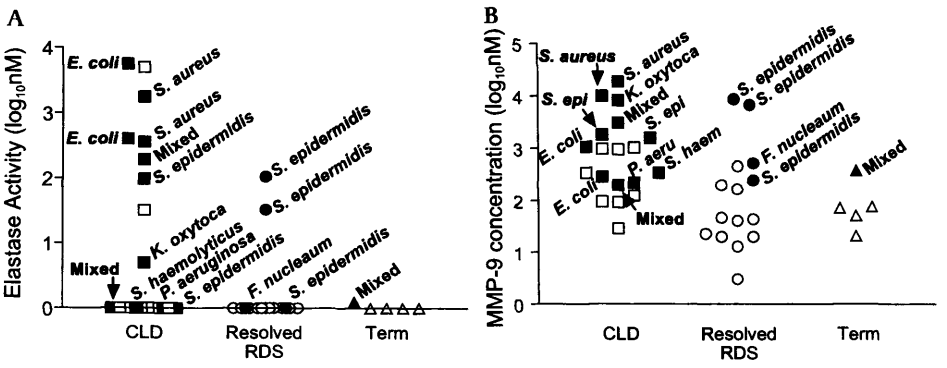

Figure 4 Scatterplots of (A) peak elastase activity and (B) matrix metalloproteinase (MMP)-9 concentrations for each diagnostic class of infant. Filled symbols indicate 16S rRNA gene-positive specimens and empty boxes indicate negative specimens. Sequencing data for 16S rRNA gene-positive organisms are also shown. Some samples were identified as 16S rRNA gene-positive but no specific microorganism was identified by sequencing. Abbreviations for micro-organisms include *S epi* (*S epidermidis*), *S haem* (*S haemolyticus*) and *P aeru* (*P aeruginosa*). CLD, chronic lung disease of prematurity; RDS, respiratory distress syndrome.

concentrations. We thus hypothesised that a higher SerpinB1 content in "new" CLD may reduce NE activity in human patients and we did find a trend towards higher SerpinB1 levels in infants with CLD than in those with RDS and controls. Temporal relationships were noted between SerpinB1 concentration, active NE and the percentage of SerpinB1 complexed with proteinase, which suggests that the SerpinB1 increase was part of a protective mechanism to regulate NE activity.

AAT is an acute phase reactant.[27] Merritt *et al*[8] showed that the AAT concentration almost doubled in lung lavages from infants developing classical CLD. However, we have shown that the SerpinB1 concentration increased locally and rapidly in association with increased NE activity. Moreover, the increase in SerpinB1 was far greater (>10-fold increase for some neonates, figure 3) than has previously been described for AAT[8] and may be more important in neutralising NE activity. The mechanisms of SerpinB1 release are unknown as the protein lacks an N-terminal classical secretion sequence.[28] The BAL fluid concentration of SerpinB1 correlated well with infiltrating inflammatory cells, particularly neutrophils (which contain the highest concentration of SerpinB1); therefore, SerpinB1 may have been released following cell death or by a non-classical mechanism. However, the possibility of SerpinB1 upregulation and release cannot be discounted.[29] Our finding of SerpinB1 increasing in parallel with inflammation is in keeping with those reported in patients with cystic fibrosis where a similar correlation between SerpinB1 levels and neutrophil numbers was also noted.[30]

The simultaneous episodic increases of neutrophils, proteinases (NE and MMP-9) and anti-proteinases (AAT and SerpinB1) indicate a triggered inflammatory process leading to an increased likelihood of developing CLD; postnatal infection was the most likely candidate to trigger the inflammation. The role of postnatal infection in the development of CLD is recognised[31 32] but is frequently underrated. Conventional culture techniques of BAL fluid are confounded by frequent administration of broad-spectrum antibiotics in these patients. Microbial infection or colonisation has previously been confirmed by identification of 16S rRNA genes.[33–35] Our finding of 16S rRNA genes detection in patients developing CLD lends further support to postnatal infection being integral in the pathogenesis of CLD. NE was detected more frequently and in higher levels for BAL fluid samples when 16S rRNA genes were detected. This suggests that infection rather than microbial colonisation of the airways is responsible for episodic spikes of protective proteinases and inhibitors.

Previous studies of MMP-9 have reported conflicting results. Dik *et al*[17] showed an increased MMP-9 concentration on days 2 and 4 of life in infants with RDS compared with those with CLD, but Sweet *et al*[5] and Ekekezie *et al*[6] found no statistical difference in MMP-9 levels between groups although both reported a higher ratio of MMP-9 to TIMP-1 in the BAL fluid of infants with CLD. Danan *et al*[7] showed that MMP-9 concentrations, primarily of inactive pro-MMP-9, were increased early in life, but no difference was noted between infants with CLD and those with RDS. Studies of baboons found higher MMP-9 concentrations in extremely premature animals with CLD.[16] Our longitudinal study of human infants suggests that MMP-9 may be important in the development of CLD and, as with NE, the increased concentrations were episodic. MMP-9/TIMP-1 concentrations in neonatal BAL fluid have not been reported previously. We found a strong correlation between total MMP-9 and MMP-9/TIMP-1 complex concentrations; however, only a small proportion of total MMP-9 appeared to be in complex with TIMP-1. Furthermore, western blot analysis of our BAL fluid (data not shown) showed that more than half of the MMP-9 was present as dimer rather than monomer, which may interfere with TIMP-1 binding, and the mass of the monomer MMP-9 appeared to be mostly present as the pro-enzyme form. Our finding of an increased MMP-9 concentration in 16S rRNA gene-positive samples suggests that the episodic spikes observed in proteinase concentrations is probably due to infective episodes.

The sequencing data suggest that, while a number of organisms may be associated with high peaks of elastase and MMP-9, those infants who develop CLD had more virulent organisms within their BAL fluid. *S aureus* and *E coli* were particularly prominent in infants with CLD with BAL fluid elastase present, while the higher proteinase activity in the BAL fluid of infants whose RDS resolved was associated with *S epidermidis*, which is frequently dismissed as a commensal.

In conclusion, our study gives a detailed longitudinal profile of NE and MMP-9 and their inhibitors AAT and SerpinB1 as well as MMP-9/TIMP-1 concentrations in the lungs of preterm infants. It showed that episodic peaks of both proteinases and their inhibitors occurred, which may be important in the development of CLD, and we suggest that postnatal infection is a crucial trigger for inflammatory infiltration and proteinase release leading to lung injury resulting in CLD development.

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Contributors PLD and OBS contributed equally to the research and preparation of the manuscript and share first co-authorship. All authors listed contributed significantly to the research that resulted in the submitted manuscript.

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Competing interests None.

Ethics approval This study was conducted with the approval of the South Wales local research ethics committee and written informed consent was obtained from the parents of study subjects.

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Appendix II

12th European Meeting on Complement in Human Disease. September 2009

Visegrád, Hungary

Serovar variations in mechanism and susceptibility for complement killing of *Ureaplasma parvum*

Michael L. Beeton, Saliesh Kotecha, O. Brad Spiller.

Dept. Child Health, Cardiff University School of Medicine, Cardiff, U.K.

Oral presentation

Ureaplasma parvum is the most common organism associated with premature birth, causes non-gonococcal urethritis and neonatal meningitis. *U. parvum* consists of serovars (SV) 1, 3, 6 and 14 with SV3 being the most common. We screened for complement killing of clinical isolates of each SV by normal serum, as assessed by measuring survival titres following 1 hour incubation at 37 °C with 50% serum (relative to matched heat-inactivated controls).

No bacterial killing was observed for any SV when serum was diluted in alternative pathway buffer (containing EGTA and magnesium), indicating Ureaplasma does not overtly activate the alternative pathway. When serum diluted in veronal-buffered saline, 5/12 sera killed SV1 by >1000 fold, all sera killed SV3 by >1000 fold, 8/12 sera killed SV6 by 10 – 1000 fold and 4/12 sera killed SV14 by 10 – 1000 fold (only 1 by >1000 fold). IgG-depletion by protein A-sepharose removed the bacteriocidal activity of all serum for SV1, SV6 and SV14, but had no effect on killing of SV3. The antibody dependence of complement-mediated bacteriocidal activity was supported by Western blot analysis, although the molecular mass of bacterial proteins bound by human immunoglobulins varied widely between sera and between strains of the same SV. Two sera with no immunoreactivity to any SV by Western blot were completely MBL-depleted by mannan-agarose, but retained 100% SV3 bacteriocidal activity, while C6- and C7-deficient sera had no bacteriocidal activity.

We found that killing of SV1, SV6 and SV14 was IgG-dependent and the alternative and lectin pathways were not bacteriocidal for *U. parvum*. SV3 killing is calcium-dependent, but antibody- and MBL-independent. Variability of Western blot bands amongst strains of SV suggests hypervariability of dominant immunogens, with potential for escape. Preliminary studies suggest SV3 directly binds C1q leading to complement activation, although the role of ficolin-mediated activation is currently being ruled out.

I^3 IRG annual meeting. July 2009. Gregynog, UK

Serovar variations in mechanism and susceptibility for complement killing of *Ureaplasma parvum*

Michael L. Beeton, Sailesh Kotecha and O. Brad Spiller

Dept. Child Health, Cardiff University School of Medicine, Cardiff, U.K.

Oral presentation

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I[3] IRG annual meeting. July 2008. Gregynog, UK

Identification of antibiotic resistance in *Ureaplasma* species and determination of novel mutations in genes associated with resistance.

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Oral presentation – **3rd prize**

Ureaplasmas represent the smallest free living bacteria which also lack a cell wall conferring an inherent resistance to all beta-lactam antibiotics. The genus Ureaplasma is divided into two species encompassing 14 serovars. U. parvum (U.p) contains serovars 1, 3, 6, & 14 whereas U. urealyticum (U.u) contains serovars 2, 4, 5, 7, 8, 9, 10, 11, 12 and 13. Here we have developed a rapid microbroth dilution assay that quantifies the initial input of bacteria co-incident with determination of the antibiotic minimum inhibitory concentration (MIC). A total of 61 isolates (10/61 U.u, 50/61 U.p) from newborn neonates were screened. 1/61 (1.7%) was found to be resistant to tetracycline (MIC >2mg/L), 1/61 (1.7%) was found to be resistant to ciprofloxacin (MIC >4mg/L), 5/61 (8.2%) were found to be erythromycin resistant (MIC 8 mg/L) with one additional isolate (total of 9.8%) was found to have very high erythromycin resistance (>64 mg/L). Tetracycline resistance was mediated by presence of the transferable tetM resistance gene. Resistance in the highly erythromycin resistant isolate was mediated by a novel 2 amino acid deletion (R66Q67) in the L4 ribosomal protein, which is likely to mediate resistance by altering the ribosome's three-dimensional structure to block erythromycin interacting with its binding site within the 23S rRNA. No mutations were found for the intermediate erythromycin resistant strains, although 4/5 belonged to the U. urealyticum species, suggesting a bias in susceptibility between U.u. and U.p. Ciprofloxacin resistance was found to be mediated by an amino acid substitution (D82N) in the parC topoisomerase gene. However we found species specific mutations in other topoisomerase genes previously incorrectly identified as resistance mutations by other investigators

Society for General Microbiology March 2009. Harrogate, UK

Identification and characterisation of antibiotic resistance in *Ureaplasma* spp.

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Poster presentation

Background: Ureaplasmas represent some of the smallest free living bacteria and have been associated with causing a number of diseases from nongonococcal urethritis to neonatal pneumonia.

Objective: To identify and characterise the molecular mechanism of resistance in Ureaplasma to macrolides, tetracyclines and fluroquinolones.

Methods: An adapted breakpoint methodology was used to identify resistantce. Genes previously associated with resistance to the respective antibiotics were sequenced to identify any amino acid differences which potentially could account for the resistant phenotypes.

Results: From 61 isolates 1 was highly resistant to erythromycin (MIC > 64μg/ml) due to a R66Q67 deletion in the L4 ribosomal protein, 1 resistant to ciprofloxacin (MIC 8μg/ml) with a D82N substitution in the ParC protein and 1 was tetracycline resistant (MIC 64μg/ml) which harboured the *tetM* gene. Additionally we identified 1 isolate which was *tetM* positive but tetracycline susceptible as well as identifying a number of species specific differences in the topoisomerase II proteins which have previously been incorrectly associated with quinolone resistance.

Conclusion: Amino acid differences within specific genes as well as acquisition of the *tetM* gene mediated resistance in *Ureaplasma*. Some previous mutations in the topoisomerase II genes are species specific differences but are not related to resistance.

23rd Annual Postgraduate Research Day. November 2008. Cardiff University, UK

Identification of antibiotic resistance in *Ureaplasma* species and determination of novel mutations in genes associated with resistance.

Michael Beeton[1], Dr Victoria Chalker[2], Dr Nicola Maxwell[1], Prof Saliesh Kotecha[1] and Dr Brad Spiller[1]

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Oral presentation

Ureaplasmas represent some of the smallest free living bacteria with the genus Ureaplasma consisting of two species encompassing 14 serovars. *U. parvum* (U.p) contains serovars 1, 3, 6, & 14 whereas *U. urealyticum* (U.u) contains serovars 2, 4, 5 - 13. Ureaplasma have been associated with causing a number of diseases from nongonococcal urethritis to neonatal pneumonia. The lack of a cell wall confers an intrinsic resistance to all beta-lactam antibiotics rendering treatment to mostly protein synthesis inhibitors. Here we have developed a rapid microbroth dilution assay that quantifies the initial input of bacteria co-incident with determination of the antibiotic minimum inhibitory concentration (MIC). A total of 61 isolates from newborn neonates were screened and the molecular mechanism of erythromycin and ciprofloxacin resistance determined. Resistance in a highly erythromycin resistant isolate (O10) was mediated by a novel 2 amino acid deletion (R66Q67) in the L4 ribosomal protein. Ciprofloxacin resistance was found to be mediated by an amino acid substitution (D82N) in the ParC topoisomerase protein. However we also found species specific mutations in other topoisomerase genes previously incorrectly identified as resistance mutations by other investigators

22rd Annual Postgraduate Research Day. November 2007. Cardiff University, UK

A new method for determining antibiotic resistance in *Ureaplasma* species; A case report on the characterisation of a clinical erythromycin resistance *Ureaplasma*

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Poster presentation – **1st prize**

The role of the bacterium *Ureaplasma* spp. in the development of chronic lung disease of prematurity (CLD) is highly controversial. To determine its role a large multi-centre randomised control trial focusing on the ability of the macrolide antibiotic to eradicate the *Ureaplasma* and subsequent effect on reducing CLD is required. Before this can take place a standardised methodology to determine the presence of macrolide resistance strains is required. Until now the ability to standardise the inoculum size of the organism had not been possible without prior knowledge of cell numbers. We have developed a 96-well plate assay which allows for reproducible inoculum size without prior knowledge of cells numbers. Using this new methodology we examined the antibiotic sensitivities profiles of *Ureaplasma* isolates from patients on the neonatal ward at UHW. A clinically erythromycin resistant strain was isolated from a preterm neonate who, following a course of erythromycin, did not clear *Ureaplasma* colonisation from the lung. Characterisation of the resistant strain was carried out via sequencing of regions known to harbour mutations linked to macrolide resistance in other clinically significant bacteria. A deletion of 6 base pairs resulting in a 2 amino acid deletion was found within a highly conserved region the structural L4 ribosomal protein of the 50S ribosomal subunit. Alignments of this region against phenotypically sensitive strains demonstrated the absence of mutation within sensitive strains. This is the first detailed report of the mechanism of macrolide resistance in clinically isolated *Ureaplasma* strain.

Additional published abstracts

1. Spiller OB, **Beeton ML**, Maxwell NC, Davies PL, Kotecha S. Frequency and mechanism of antibiotic resistance in bacteria associated with premature birth and development of chronic lung disease of prematurity. Perinatal Medicine 2008.

2. Maxwell NC, Davies PL, **Beeton ML**, Spiller OB, Kotecha S. Ureaplasm*a spp.* or bacterial colonisation are associated with the development of chronic lung disease of prematurity. Perinatal Medicine 2008.

3. Maxwell NC, Davies PL, **Beeton ML**, Spiller OB, Kotecha S. The role of infection in the development of chronic lung disease of prematurity. European Respiratory Society Conference 2007.