Antibiotic therapy as prevention and treatment of Q fever

By

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Medicine

Warwick Medical School
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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

- Dr Isobel Norville inoculated and monitored the *Galleria mellonella* larvae.
- Dr Stuart Armstrong provided the analysis and interpretation of the antibiotic pharmacokinetics in AJ mice.
- Q3 Analytical Ltd, Porton Science Park performed the liquid chromatography-mass spectrometry (LC-MS) analysis of antibiotic levels in blood from AJ mice.
- Assistance in carrying out the aerosol challenge, animal husbandry, antibiotic dosing, euthanisation and tissue processing for AJ mouse antibiotic efficacy studies 1 and 2 was provided by PHE, Porton, members of staff under the Animals (Scientific Procedures) Act 1986.
- The aerobiology team at Dstl performed the aerosol challenge for AJ mouse efficacy study 3 and 4.
- Assistance in carrying out animal husbandry and antibiotic dosing for AJ mouse antibiotic efficacy studies 3 and 4 was given by animal technicians and scientists at Dstl under the Animals (Scientific Procedures) Act 1986.
Abstract

Q fever was first identified as a problem for the military during World War 2 when thousands of troops were affected (1). British military personnel developed Q fever during the recent campaign in Afghanistan, which in approximately 20% of cases led to Q fever fatigue syndrome (QFS), and in some cases resulted in discharge from military service. This highlighted the requirement to prevent disease acquisition or reduce the severity of disease in order to limit progression to QFS.

Q fever is caused by the intracellular Gram-negative bacterium, *Coxiella burnetii*. Novel *in vitro* and *in vivo* experiments were conducted to evaluate the efficacy of a range of antibiotics readily accessible to the military against *C. burnetii*. The benefit of using doxycycline, levofloxacin, ciprofloxacin and co-trimoxazole as chemoprophylaxis and treatment were assessed in two *in vivo* models of infection. Analysis of the data from a military Q fever patient cohort was analysed in order to identify any factors that might predict progression to QFS in an acute Q fever patient.

The results suggest that antibiotic initiation around the time of symptom onset following exposure to *C. burnetii* provides the optimum outcome in the AJ mouse model. Chemoprophylaxis does not appear to improve the outcome in these studies, leading instead to a prolonged incubation period and either relapse or dissemination of disease after antibiotics are stopped. From analysis of the patient data it appears that initiation of doxycycline greater than five days after symptom onset may be associated with progression to QFS.

In conclusion, the studies conducted suggest that to improve the clinical outcome for future Q fever patients, timely diagnosis and early treatment at the point of symptom onset needs to be achieved. It is suggested that this will reduce symptom duration, disease severity and the likelihood of developing long term fatigue.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>%</td>
<td>Percent</td>
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<tr>
<td>°C</td>
<td>Degrees centigrade</td>
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<tr>
<td>ACCM</td>
<td>Acidified citrate cysteine media</td>
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<tr>
<td>ALP</td>
<td>Alkaline phosphotase</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>AQF</td>
<td>Acute Q fever</td>
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<td>ALT</td>
<td>Alanine aminotransferase</td>
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<td>bd</td>
<td>Twice daily</td>
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<td>Cognitive behavioural therapy</td>
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<td>Colony forming unit</td>
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<td>Chronic Q fever</td>
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<td>Deoxynucleic acid</td>
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<tr>
<td>Dstl</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>Genome equivalent</td>
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<td>HRP</td>
<td>Horseradish peroxidise</td>
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<td>Integrin associated protein</td>
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<td>Immunofluorescence assay</td>
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<td>Interferon gamma release assays</td>
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<td>IgG</td>
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<td>LCV</td>
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<td>LRI</td>
<td>Leukocyte response integrin</td>
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<td>NM</td>
<td>Nine Mile</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>od</td>
<td>Once daily</td>
</tr>
<tr>
<td>OMP</td>
<td>Outer membrane protein</td>
</tr>
<tr>
<td>p</td>
<td>Probability</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>pH</td>
<td>Potential of hydrogen - measure of hydrogen ions associated with acidity</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetic</td>
</tr>
<tr>
<td>PV</td>
<td>Parasitic vacuole</td>
</tr>
<tr>
<td>QFS</td>
<td>Q fever fatigue syndrome</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RML</td>
<td>Rocky Mountain Laboratories</td>
</tr>
<tr>
<td>ROI/S</td>
<td>Reactive oxygen intermediates/species</td>
</tr>
<tr>
<td>SCV</td>
<td>Small cell variant</td>
</tr>
<tr>
<td>sec</td>
<td>Second(s)</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>UFI</td>
<td>Undifferentiated febrile illness</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v</td>
<td>Volume</td>
</tr>
<tr>
<td>WCC</td>
<td>White cell count</td>
</tr>
<tr>
<td>xg</td>
<td>Centrifugal force</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1. Historical background

In 1933 there was an outbreak of an undifferentiated febrile illness (UFI) amongst abattoir workers in Brisbane, Australia and so Dr Edward Derrick, the director of the Laboratory of Microbiology and Pathology at Queensland Health Department, was asked to investigate(2). An UFI is a term used to describe an acute illness (less than 2 weeks) with confirmed fever (>38°C), no coryzal symptoms and no specific organ focus at presentation. There were approximately 20 cases from this outbreak from 1933 to 1935, and Derrick described the clinical course of 9 patients (2,3). The fever was of acute onset and lasted between 7 and 24 days. Initial symptoms included malaise, anorexia, headache, myalgia, fever and patients displayed a relative bradycardia. Other associated symptoms and signs included jaundice, conjunctival congestion, vomiting, diarrhoea or constipation, cough, splenomegaly and abdominal distention. Laboratory tests were often normal, but on occasion showed anaemia or relative and absolute lymphocytosis. Blood cultures and agglutination tests were all negative. Derrick showed that guinea pigs acquired the disease if injected with blood or urine from an affected patient. He also showed that the disease could be transferred to additional guinea pigs by inoculating them with tissue suspensions from the originally infected animals (2). He demonstrated that convalescent guinea pigs from the original experiment did not succumb to further challenges which suggested the outbreak in Brisbane was from the same agent (3). The organism could not be visualised or cultivated and therefore it was assumed to be a virus. Derrick coined the term ‘Q’ or Query fever because the infectious agent could not be identified (4). He sent samples of infected guinea pig liver to Sir MacFarlane Burnet of the Walter and Eliza Hall Institute, Melbourne who was able to replicate the febrile infection seen by Derrick in guinea pigs and mice (5). Burnet saw cytoplasmic micro-colonies of an organism in stained sections of infected mouse spleens. The appearance was consistent with a rickettsia like organism. Derrick suggested naming the organism *Rickettsia burnetii* after Burnet’s discovery (4).

In 1935 Dr Gordan Davis and Dr Herald Cox were also investigating an infectious agent at the Rocky Mountain Laboratory (RML) in Montana that had been isolated in the wood tick, *Dermacentor andersoni*, near Nine Mile Creek in Montana, USA (4,6). The organism was capable of passing through Berkefeld filters N and W
which are usually impermeable to bacteria and Rocky Mountain Spotted Fever (7). Cox was unable to grow the organism on a variety of media, but was able to cultivate the organism in a tissue culture containing chick embryonic tissue (6). The laboratory workers also noticed rickettsia like organisms and named the agent Rickettsia diaporica due to its ability to pass through the bacterial filters, unlike other rickettsiae (4,8).

Dr Rolla Dyer was the Director of the US National Institutes of Health (NIH) from 1942 to 1950, and in 1938 he had supervisory responsibility for the work at the RML. He had been trying unsuccessfully for three years to cultivate rickettsiae in embyonated eggs and did not believe Cox’s report in 1938 of successful cultivation of the Nine-Mile organism in chick embryonated tissue (4). He visited Cox at the RML to discuss his findings. Dyer confirmed that in Cox’s slides rickettisae could be seen in the yolk sac membrane tissue, and he worked with Cox in the RML for several days, assisting with work on the organism causing Nine-Mile fever. Five days after returning home Dyer developed a febrile illness (4). Dyer’s blood was inoculated into a guinea pig and the animal subsequently developed a febrile illness and rickettsia were found in the animal’s spleen (3,4). This organism was named strain ‘X’ and cross immunity was shown with the Nine-Mile strain being worked on in the RML, but not with other rickettsia (4). Dyer had been working on spleens of mice infected with Q fever that Burnet has sent him 6 weeks prior to travelling to the RML to see Cox. He decided to exclude the possibility that his febrile illness was caused by Q fever and decided to challenge 5 guinea pigs that had convalesced from Q fever with strain ‘X’ and found that they all resisted the infectious challenge (3). Dr Dyer’s laboratory acquired infection led to the discovery that the Q fever agent and Nine-Mile agent were the same organism.

R. burnetii (R. diaporica) was found to have many differing qualities to other rickettsiae and in 1948 it was proposed that the organism had a separate genus and be called Coxiella burnetii, after Cox and Burnet who first discovered the rickettsia like organisms in the spleen of infected animals (9).

1.2. The bacterium

Initially C. burnetii was thought to be part of the Rickettsiaceae family, but 16s rRNA analysis, later corroborated with whole genome sequencing showed that the Coxiella genus belonged within the gamma subdivision of Proteobacteria, along
with *Legionella pneumophila* and *Francisella tularensis* (10). Until recently it was believed that *C. burnetii* was the only species within the genus but further species have now been identified. *Coxiella cheraxi* was first discovered in crayfish in 2000, and the *Coxiella* like organism “*Candidatus Coxiella avium*” has been found in non-mammalian hosts such as birds (11,12). The importance of these species to humans is not clear but molecular diagnostics may fail to differentiate between the species.

1.2.1. Size variation

The bacterium can exist as either a large cell variant (LCV) or small cell variant (SCV) (10,13-16). The LCV (length > 0.5 μm) is the intracellular, replicating form, whereas the SCV (length 0.2 – 0.4 μm) is the extracellular, non-replicating form and is considered to be “spore-like” in view of its environmental stability (10). Both variants have typical Gram-negative cell walls, consisting of 2 layers separated by periplasmic space. However, in the SCV this space is filled with a dense material consisting of proteins and peptidoglycans, which probably accounts for its environmental stability (10). The SCV is resistant to osmotic pressure, chemicals such as 5% sodium hypochlorite or ammonium chloride, low or high pH changes, heat and desiccation (10,15). It requires more than 10% formalin for prolonged periods of time (greater than 12 hours) to kill *C. burnetii* (10). With lower concentrations viable bacteria can still be isolated (15). LCVs will develop into SCVs if left in axenic acidified cysteine citrate medium 2 (ACCM-2) or vero cells for prolonged periods of time (greater than 21 days) (17).

1.2.2. Phase variation

*C. burnetii* exists as two different phases, phase I (PI) is the virulent form isolated from humans, animals and arthropods infected with Q fever. Phase II (PII) *C. burnetii* is the avirulent form that is found after serial passage in cell cultures or embryonated egg cultures (10). Phase II cannot revert back to phase I (18,19). Phase I and phase II *C. burnetii* can be cultured in axenic media. The phase variation is due to a change in the lipopolysaccharide (LPS). In phase II the LPS is truncated because the O antigen has been deleted from the core sugar in the LPS (10,14). The LPS of the Phase I strain contains sugars such as virenose, dihydrohydroxystreptose, and galactosamine uronyl (1/6)-glucosamine, which are
absent in the Phase II strain (10). A third strain of *C. burnetii*, Nine-Mile crazy strain, was found with an intermediate LPS structure (20). LPS is the predominant surface component that varies between strains and it has been shown using these three phases of the nine mile strain, that the greater the degree of truncation the greater the loss of virulence (21).

1.2.3. Intracellular lifecycle

*C. burnetii* enters host cells through a passive endocytic process (22). The two phases enter the cell via different mechanisms. Phase II bacteria bind to the CR3 receptor to enter the human macrophage, whereas phase I bacteria block the CR3 receptor and enter via the leukocyte response integrin $\alpha_v\beta_3$ (LRI) and integrin associated protein (IAP) (19). Uptake of phase I bacteria is poor but it can survive within the macrophage and monocyte, whereas phase II internalises well but is easily eliminated by the phagolysosome (9,22). Once internalised the growth kinetics of both phases in the macrophage are comparable (19). There are many different factors involved with internalisation of *C. burnetii* within the host cell. Following uptake of the bacteria in a developing vacuole, the vacuole matures from an early endosome to a phagolysosome (19). As the vacuole matures through this process the vacuolar pH drops to become the acidic environment seen in the phagolysosome. *C. burnetii* requires the acidic environment for metabolic activation and production of proteins that allows vacuole expansion and inhibition of apoptosis(22). This change in pH triggers the translocation of bacterial proteins by a Dot/Icm type 4b secretion system (T4SS) (22). These proteins are required for development of a large parasitophorous vacuole (PV) which takes up the majority of the host cytoplasm and allows ongoing replication of *C. burnetii* (22). The T4SSs are large protein complexes spanning the cell envelope allowing translocation of proteins into the cytoplasm. They allow translocation of proteins across the bacterial cell envelope which are critical for cell infection and avoidance of host cell apoptosis (23). Once internalised the bacteria modulates the host cell response allowing bacterial replication, and fusion with autophagosomes. This fusion provides the bacteria with additional membrane for vacuole expansion and nutrients for pathogen growth (19). A further regulatory protein involved with *Coxiella* survival is the Outer membrane protein A (OmpA). OmpA is found in the cell wall of Gram-negative bacteria and has an important role in pathogenesis. In the case of *C. burnetii* it has been shown to induce cellular uptake (19,22,23). Mutations in this
protein significantly reduce C. burnetii internalisation, and replication within host cells (22).

1.3. Modes of transmission

Inhalation of contaminated aerosols is the main mode of Q fever transmission to humans (16). Animal and human experiments demonstrate that the incubation period is inversely related to the inhaled dose of C. burnetii (3) with as little as one organism being sufficient to infect a guinea pig and possibly a human (24). Farm animals are a major reservoir of Q fever and infection can be acquired from inhalation of contaminated aerosols from amniotic fluid, placenta or infected wool (16).

Laboratory acquired infection has been recognised since Dr Dyer became infected during the discovery of C. burnetii in the 1930s (25). Johnson and Kadull describe 50 cases of laboratory acquired infection from 1950 to 1956 amongst employees of the Army Biological Laboratories in Fort Detrick, USA (26). During many outbreaks staff not directly involved with animal work have been infected with Q fever thus demonstrating that Q fever can be transmitted via aerosol contamination (27).

Percutaneous transmission of Q fever has been demonstrated in needle stick injuries in the laboratory setting (28). There also exists a theoretical risk for transmission through blood transfusion from infected donors. Oei et al. estimated the risk of transmission from a blood transfusion during The Netherlands outbreak using computer modelling to be low (29).

Ingestion of contaminated milk can lead to infection (30,31). Heating raw milk to 71.7°C for 15 seconds is sufficient to kill the bacterium in raw milk (30,32). Pasteurisation of milk has been compulsory to prevent many different diseases since the 1940’s in the UK which has reduced the burden of disease from ingestion of contaminated milk (30).

C. burnetii was initially found in the wood tick, Dermacentor andersoni, near Nine Mile Creek in Montana, USA (28). The role of ticks in transmission of C. burnetii has been extensively reviewed. Duron et al. show that 7 types of hard and soft ticks are proven competent vectors in an experimental model (33).Ticks have been shown to shed up to 10⁻¹⁰ organisms per gram of faeces, and the organism has
also been found in the gut, salivary glands and ovaries (33). This highlights a potential risk for human acquisition of Q fever from inhalation of tick faeces, crushing a tick allowing direct entry of the organism or a tick bite. Ticks may contribute to transmission of *C. burnetii* between different animal species, but their role in human transmission is difficult to assess because infection from the aerosolised route is far more common and cannot be excluded as the source of infection even if the patient has been bitten by a tick.

*C. burnetii* has been isolated from the placentas of pregnant women (34,35), but there remains insufficient evidence to link Q fever to a poor maternofoetal outcome (36). Evaluation of 1174 pregnant women during the Netherlands outbreak did not find evidence of adverse foetal outcomes in women who had antibodies to *C. burnetii* during early pregnancy (37). A large Canadian study assessing adverse outcomes associated with Q fever found an association between seropositivity to phase I and phase II antibodies and premature delivery before 36 weeks, and an association between phase I antibodies and prior or current neonatal death (38).

Person to person transmission has been reported through direct contact with infected organs at autopsy (39), contact with infected patients whilst hospitalised (40), transmission through familial contact (41), and through sexual transmission (42). These are case reports and person to person transmission remains rare and there is no evidence to suggest infected patients should be isolated when hospitalised. Precautions should be used when handling infected tissues in the laboratory.

1.4. Q fever cases and outbreaks of note in the civilian population

Q fever is found worldwide with the exception of New Zealand (43). There have been many outbreaks reported throughout Europe since the 1980s (44). The largest recorded outbreak occurred in the Netherlands, affecting 4108 people from 2007 to 2011 (45). The outbreak was preceded by an increase in abortions amongst dairy goats and sheep. The proximity of affected patients to these abortions combined with the dry weather was thought to lead to the scale of this outbreak (46). A later study in the Netherlands showed that only 7.9% of cases are notified to the Public Health authorities in the Netherlands and therefore Van Loenhout *et al.* surmised that 52,000 people could have been affected by the outbreak from 2007-2011 which highlights the public health impact of Q fever (45).
French Guiana has the highest reported incidence of Q fever (47). The first case in this region was described in 1955 in a slaughterhouse worker with subsequent sporadic cases being reported until 1996 (48). Rates of Q fever increased over the following decade with a retrospective case-control study from 2004 - 2007 demonstrating a Q fever pneumonia prevalence of 24.4% in 131 community acquired pneumonia patients in Cayenne, French Guiana (49). In comparison to previous outbreaks the high prevalence of Q fever in Guiana was sustained and increasing, and many of the patients did not have the traditional risk factors associated with Q fever transmission, such as working or living with animals. In addition more patients were infected in the Cayenne, the capital city rather than the rural areas usually associated with Q fever (48). It has recently been shown that the likely reservoir for Q fever in this country is the three-toed sloth, which is an animal found specifically in South America living near the perimeter of Cayenne (47). The sloth had high concentrations of C. burnetii in its stool and the ticks parasitising it (47).

Q fever was first discovered in Great Britain in 1948 when Stoker found 3 out of 24 cases of sporadic atypical pneumonia were due to C. burnetii (50). There have been sporadic outbreaks in the UK since 1948 with the largest outbreak occurring in the West Midlands in 1987 (27,39,51-60). 147 people were infected in the West Midlands, epidemiological investigation found the outbreak to be related to nearby farms where outdoor lambing and calving was occurring at the time of strong southerly winds causing the windborne dispersal of infected birth products (61).

1.5. Military aspects of Q fever

1.5.1. Cases and outbreaks

Q fever was first identified as a problem for the military during World War II (WWII). Many thousands of Allied and German troops were affected by this agent. During the War these outbreaks were often identified as atypical pneumonia and only after 1945 were the outbreaks found to be due to C. burnetii (1). Infection within troops were widespread during WWII in the Mediterranean countries, the Balkans and Southern Europe, with the majority of cases occurring in troops in Italy. Dennig in 1948 recorded greater than a 1000 cases of atypical pneumonia amongst German troops in the Balkans (62) . The disease was called “Balkengrippe” but the aetiology
was not known, although Dennig noted that it was more common in troops that slept on hay or straw.

Robbins investigated 5 out of 8 outbreaks that occurred among Allied Troops in the Mediterranean region from the Winter 1944 to Spring 1945 (63). There were 613 cases reported from the 8 outbreaks. The first outbreak described was in the 339th Infantry where 53 cases occurred amongst 156 men (34%). During the outbreak they had been living in Italian farm houses and outbuildings in the Apennine mountains, Italy. The highest attack rate was seen amongst the troops living in the hay barns, outbuildings and apartment attics where the troops were in the closest proximity to animals including pigeons, cattle, horses, pigs, sheep and chickens. In April 1945, 269 soldiers (30%) of the 3rd Battalion, 362nd Infantry Regiment stationed in Italy were hospitalised with presumed atypical pneumonia which was later found to be Q fever (62). They had camped in tents around a farm, some using hay or straw for beds. All personnel had attended a training package in the attic of a nearby hay barn. The striking similarities between all the outbreaks described by Robbins were that most affected personnel were in close proximity to animals, and there is a strong association with dust particles either in attics or on hay and straw. Robbins postulated that the dust could be contaminated with animal excrement or insects which could be the mode of transmission.

More recently Anderson reported an outbreak of 19 cases of pneumonia in troops in Iraq in 2003 where subsequent serological investigation revealed that 8 patients had positive antibody titres for *C. burnetii* (64). This outbreak was not thought to be due to Q fever but led the US military to assess the extent of Q fever infections in the US military deployed in Iraq. Twenty-two patients, with available pre and post deployment serology, who had been diagnosed with pneumonia in Iraq were tested for Q fever. 5 patients (23%) had seroconverted to *C. burnetii* whilst on deployment. This led Anderson to complete a seroprevalence study in 2010 of 909 US servicemen deployed to Iraq. He found that 88 (10%) had seroconverted to Q fever during their deployment, and that working in a combat role was a risk factor for seroconversion (OR = 1.8, 95% CI: 1.1 -2.8, p < 0.05) (65).

Faix *et al.* reported a Q fever outbreak of 22 cases (58%) in a unit of 38 US Marines operating in Iraq in 2005 (66). This led to 64 lost duty days which in a small unit on operations has a considerable impact on unit effectiveness. An outbreak investigation revealed the Marines were living amongst local livestock including
goats, sheep and feral dogs. In addition, wind and dust were a constant in the environment upon which they were operating which means the Marines were exposed to all the conventional risk factors for Q fever.

From 2006 it was noted that British Military troops were being seen in the British Field Hospital in Helmand province, Afghanistan with UFI. This led to a fever study from May to October 2008 within the British Field Hospital (67). During this period 26 military personnel were admitted with an UFI. Twenty-three diagnoses were made, some patients had multiple pathogens diagnosed, but there were 6 diagnoses (26%) of acute Q fever. Newman et al. subsequently performed an anonymous seroprevalence study in UK troops deploying to Afghanistan and found that of 467 personnel 1.5% had seroconverted prior to deployment, and 1.7% seroconverted during their 6-month deployment (68).

The most recent published military outbreak occurred in early 2016, 50 soldiers working for the Kosovo Force (KFOR) became unwell, 1 died and 35 were diagnosed with pneumonia (69). Their camp in Prizren was co-located with a sheep farm, and the nearby civilian population also had increased numbers of patients (69).

1.5.2. *C. burnetii* as a bioterrorist and biowarfare agent

*C. burnetii* is highly infectious, very stable in the environment and can be aerosolised and travel many miles via wind borne spread. For these reasons Q fever has been designated a CDC Category B agent of potential use in Biowarfare and Bioterrorism (70,71). Q fever causes incapacitation without a high mortality rate which would lead to loss of manpower if used against troops of between 23-77% (70). This would significantly affect operational effectiveness. The difficulty with this bacterium is the ability to culture it, but if achieved large volumes could be produced for aerosolisation. The World Health Organisation (WHO) estimated that if *C. burnetii* was aerosolised in a city of approximately 5 million people, 125,000 would become ill and there would be 150 deaths (72). WHO predict the organism would spread by wind for more than 20km (72). The US, Soviet Union and Japanese Biowarfare research programmes all contained Q fever programmes before being dismantled (70,72). In the event of a deliberate release of *C. burnetii* the current recommendation is to provide a 5-7 day chemoprophylaxis course of doxycycline or tetracycline starting 8-12 days post exposure (70,73).
1.6. The clinical presentation of Q fever in humans

1.6.1. Acute Q fever (AQF)

Up to 60% of patients that seroconvert to *C. burnetii* will remain asymptomatic (14). Symptomatic disease typically presents with an acute UFI. The fever of up to 40°C is usually sudden onset and can be accompanied by a severe headache, retrobulbar pain, general malaise, drenching sweats, myalgia, arthralgia, anorexia, chest pain and acute weight loss (24,74,75). AQF can present to varying degrees with pneumonia or hepatitis. There appears to be a geographical, possibly strain related, variability to this presentation. Pneumonia is the commonest presentation in French Guiana, Spain, the Netherlands and Croatia (76). Hepatitis at presentation is more common in countries where the bacterium is endemic causing sporadic cases rather than outbreaks, such as France, Taiwan, Israel and Portugal (76). The hepatitis is rarely associated with jaundice. Liver biopsies often demonstrate a granulomatous hepatitis. Rarer complications of AQF include cardiac involvement (pericarditis, myocarditis, and acute endocarditis), neurological involvement (meningitis and meningoencephalitis), lymphadenitis, cholecystitis and rashes (10,76).

Laboratory abnormalities seen in AQF will vary depending on the presenting clinical picture but common abnormalities include: raised transaminases (15-85%); high alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGT) (25 – 75%); raised C-reactive protein (CRP) (up to 95%); raised erythrocyte sedimentation rate (ESR)(32-88%); thrombocytopenia (3-25%); raised creatinine (30-40%); hyponatraemia (50%) and anaemia (30%) (10,16,57,77). White cell count (WCC) is abnormal in 10-30% of cases (10,77).

Clinical data from previous outbreaks demonstrates an increased risk for AQF acquisition associated with gender, age and smoking (78-80). Men are more likely to acquire disease with a male to female risk ratio of 2.45 amongst French adults (79). This is postulated to be due to the protective effect of female sex hormones (81). In the Netherlands outbreak most commonly patients were aged between 40 – 60 years (80), and in the 1989 UK outbreak the mean age was 48 years (57). Smoking is increased in patients diagnosed with AQF compared to the general population (57,80,82). This has been postulated to be due to a greater exposure to
outdoor contaminated aerosols, from hand to mouth transmission or due to alterations in the structure and immune function of the respiratory tract (80). The environmental conditions that have been associated with Q fever acquisition when in close proximity to infected animals include; dry soil, little vegetation and high particle matter concentration in the air (83). These environmental conditions are commonly present for British Military soldiers working in Afghanistan if they are near infected ruminant animals.

1.6.2. Chronic Q fever (CQF)

Chronic Q fever occurs in 1-5% of patients following infection with Q fever (84,85). Only 38% of patients diagnosed with proven, probable or possible CQF recall an episode of AQF (86). The most common manifestations of CQF include endocarditis and vascular infections, other presentations include osteomyelitis, pericarditis and hepatitis (87). Predictors of CQF include previous valvular surgery or valvular abnormalities, the presence of vascular protheses and aneurysms, mild renal insufficiency, older age and non-haematological malignancy (86,88,89).

In Q fever endocarditis patients will often present with non-specific febrile symptoms and chills, sweats, weight loss, headaches, myalgia and hepatosplenomegaly(90). Common laboratory abnormalities seen in a French cohort with endocarditis included: raised Alanine Aminotransferase (ALT)(39%); raised ESR (75%), raised WCC (33%); thrombocytopenia (48%); raised creatinine (65%) and haematuria (70%) (90).

1.6.3. Q fever fatigue syndrome (QFS)

Post infection fatigue is seen following many different infectious disease such as Lyme borreliosis and infectious mononucleosis (91). Following the 1989 UK outbreak, 60% of patients complained of fatigue symptoms for 6-12 months following an acute infection (92). These symptoms will often resolve, but 10-30% will have persistent fatigue lasting for greater than a year and this has been reported for up to 10 years following AQF (78,93). This is termed Q fever fatigue syndrome (QFS). The Dutch consensus group who have treated the greatest number of patients with Q fever fatigue syndrome define QFS as “severe fatigue lasting for greater than 6 months, causing significant disabilities in daily functioning, not being caused by chronic Q fever infection, and the fatigue should have been
either absent before or have significantly increased since the acute Q fever infection” (93). In addition to fatigue patients report symptoms of myalgia/arthritis, neurocognitive difficulties, sleep disturbance, headache, blurred vision, night sweats, respiratory/chest problems and mood disturbance(93).

The cause of QFS to date is unknown but it is hypothesised that persistent non-viable cell components of C.burnetii could result in an altered cell immunity profile and ongoing production of proinflammatory cytokines(17). Other theories include possible host or genetic factors or biopsychological causes(93,94). Theories are debated and the literature is contradictory(93). The severity of acute illness is a predictor of QFS (17,78,91). Symptomatic AQF appears to be associated with a decrease in health status as assessed by symptoms of fatigue, quality of life and functional impairment following AQF (95). This supports the suggestion that QFS does not occur in patients with asymptomatic seroconversion to C. burnetii(96).

Retrospective analysis of a Q fever outbreak in Canada in 1999 found no association between initial symptoms or antibiotics during acute disease and the development of persistent symptoms (97). Kremers et al. found that increased levels of IL-6 and C-reactive protein (CRP) were predictors of severe disease but not QFS (98). Whilst Pentilla et al report an association between long term fatigue and an increase in IL-6 and IFN-γ and a decrease in IL-2 (99). Other factors with a possible association with fatigue include young age, female gender and smoking(100). Strauss et al. found that patients diagnosed with QFS have a higher burden of psychosocial characteristics than control patients without past evidence of Q fever. The controls were not matched for fatigue but a subgroup analysis was performed on control patients reporting fatigue symptoms. It is not clear whether the psychosocial characteristics are as a consequence of Q fever or a predictor of QFS development (94).

1.7. Q fever diagnostics

Serology using an indirect immunofluorescence assay (IFA) is the gold standard diagnostic test for Q fever. Diagnosis requires a two-week convalescent sample. In acute Q fever Phase II IgM and IgG antibodies along with Phase I IgM develop, and in chronic infection IgG and IgA to both Phase I and Phase II antigens are seen (101). With Q fever being endemic worldwide serology results need to be interpreted carefully to ensure that positive results are not due to a previous infection. Standardised cut off levels for positive results vary between laboratories.
(14), as do results (102), demonstrating an intra-operator and intra-laboratory variability in the IFA results.

PCR testing can aid the earlier detection of acute Q fever. With PCR positive results being found in the first 11 days compared to 7-35 days (median 17 days) after symptom onset with serology (103). Using the primers Com1 and IS1111a, 63% of Q fever patients were found to be positive with one or both primers and the sensitivity was highest before the development of Phase II IgM (103). To directly detect the bacteria using PCR the test ideally needs to be performed prior to antimicrobial therapy (101). Therefore, PCR has benefits in detecting an acute infection prior to the development of antibodies but will only be positive for up to 11 days, and maybe considerably less, and ideally needs to be performed prior to antimicrobial therapy.

Interferon gamma release assays (IGRA) have recently been shown to be more sensitive at detecting previous Q fever infection than serology (104). Sixty-eight percent more positive results were found with the Q-detect™, Innatoss IGRA in comparison to serology by IFA in a highly endemic region in the Netherlands (104). The platform had a predicted test sensitivity of 94% (104). Serum antibodies decline over time and therefore seroprevalence studies can underestimate the true exposure within a population, measuring prior exposure using cell mediated immunity by an IGRA could provide a more accurate assessment (105).

1.8. Treatment of Q fever

1.8.1. Treatment of acute Q fever

First line therapy for acute Q fever is doxycycline (100 mg twice daily for 10 to 14 days) (106). Tetracycline administration within the first three days of illness can reduce the duration of the fever by up to 50% (24). The median duration of fever in acute illness is 12.5 days and this is reduced to 1.7 days with doxycycline, 3 days with aureomycin and 8 days with penicillin (107,108). There is a suggestion that inadequate treatment in the acute phase can increase the chance of progression to chronic Q fever (89). Other treatment options include quinolones, rifampicin, co-trimoxazole, macrolides and β-lactams (24,74,106). During the Netherlands outbreak primary healthcare data showed that empirical administration of doxycycline, moxifloxacin and other newer fluoroquinolones showed a lower hospitalisation rate for patients compared with β-lactams or azithromycin (109). β-
lactams have previously been shown to have limited effect in the treatment of Q fever and should be avoided (108,110). Macrolides appear to have a variable effect \textit{in vitro} but have been shown to be beneficial in Q fever, particularly the newer macrolides azithromycin, clarithromycin and roxithromycin (24,74,110,111). Rifampicin has been shown to be highly effective \textit{in vitro} and in vivo experiments (24). It has been recommended to be used in conjunction with quinolones, doxycycline or trimethoprim to prevent the development of resistance with monotherapy (24,74,106). Prolonged “prophylactic” use of antibiotics is sometimes used in AQF patients who have a higher risk of progressing to CQF (89,112).

It is not standard practice to check for antibiotic resistance when treating Q fever, but resistance to doxycycline and the quinolone, pefloxacin have been reported (113,114). Different strains of \textit{C. burnetii} have different sensitivity profiles (111) and combined with emerging resistance highlights the requirement to assess alternative antibacterials and their efficacy against this bacterium.

Delord \textit{et al.} suggest that doxycycline as chemoprophylaxis for malaria prevention will prevent most rickettsial diseases in travelers, although this is not evidence based. To date there has been no published data to support the efficacy of antibiotic use in acute Q fever prevention (115).

1.8.2. Treatment of chronic Q fever

Chronic Q fever most commonly presents as endocarditis or vascular disease (87). Eradication of the bacteria is difficult in view of the bacteriostatic nature of the antibiotics available. With doxycycline monotherapy, viable \textit{C. burnetii} bacteria have still been isolated from an infected heart valve 4 years later (110). Prolonged combination therapies improved the outcome. Until 1999 the regimen of choice was 3 years of doxycycline combined with a quinolone (106). Other combinations included 3 years of rifampicin and a quinolone, but after 2 years of therapy viable bacteria could still be isolated from the cardiac valve (110). More recently the combination of doxycycline with hydroxychloroquine has been recommended which improved eradication, reduced relapses and allowed the treatment course to be shortened to 18 months (116-118). This combination is thought to be efficacious because hydroxychloroquine is an alkalinising agent raising the intracellular pH and restoring the bactericidal function of doxycycline (118). Doxycycline plasma drug levels need to be monitored to ensure the levels remain above the MIC (74). The tolerability of this regimen can be difficult due to side effects, and the search for
better tolerated combinations is ongoing (119). A recent study found a combination of tetracycline with a quinolone to be a safe alternative to tetracycline and hydroxychloroquine if this combination is not tolerated (119).

1.8.3. Treatment of Q fever fatigue syndrome

A consensus definition does not exist for Q fever fatigue syndrome (QFS) (93), therefore comparing outcomes for studies is difficult. A study looking at the effectiveness of long-term doxycycline treatment and cognitive behavioral therapy (CBT) on fatigue severity in patients with QFS found that long-term doxycycline did not improve outcome but CBT was effective at reducing fatigue severity (120). However, this effect was not maintained for greater than 1 year after CBT finished (121). The current recommendation is to provide a course of CBT tailored to QFS with booster sessions to maintain the benefit (121).

1.9. Vaccines

Q-vax is a whole cell vaccine against C. burnetii developed in Australia. It is a purified killed preparation of C. burnetii prepared from the Phase I Henzerling strain (96). The vaccine is highly antigenic and can cause hypersensitivity reactions in patients previously exposed to the pathogen (122). For this reason, the vaccine is not licensed in the UK. A meta-analysis looking at the efficacy of Q-vax concluded that whilst individual studies showed the vaccine to be highly effective they were only investigating a subselection of the population (123). Q-vax in Australia is used for high risk animal workers and the population are generally young. Also it is not clear how long immunity will last if there is not constant environmental re-exposure (123). During the Netherlands outbreak it was decided to use Q-vax in an attempt to reduce the disease burden, localised and systemic side effects were commonly seen following vaccine administration (78).

Research is ongoing to look for a vaccine that will provide immunity without the risk of hypersensitivity reactions (71). Live attenuated and sub-unit vaccines are under development. Targeting the T-cell mediated immune response to C. burnetii is hoped to reduce the side effects thought to be secondary to the LPS in Q-vax (124). However, there is currently no new vaccine near to being licensed for human use.
1.10. Pathotype specific virulence of *C. burnetii*

*C. burnetii* infection in humans leads to a wide spectrum of clinical disease from asymptomatic infection, acute self-resolving illness, to chronic disease and ‘Q fever fatigue syndrome’. Two theories have been hypothesised to account for this spectrum of disease (125). The first theory postulates that the development of acute or chronic disease is dependent entirely on the immune state of the host. The second theory postulates that the genetic heterogeneity of different strains of *C. burnetii* account for whether the host develops acute or chronic disease. Since the bacterium was first discovered in the 1930s many different strains have been identified and analysed for comparison (126-132).

The relationship of the host or the strain type of *C. burnetii* with the spectrum of human disease remains unclear. Early studies located the presence of different plasmids in the bacterium’s cytoplasm(133). Four different plasmids were found QpH1, QpRS, QpDG and QpDV linked to different strains (127). Nine-Mile strain, used in laboratories to model acute infection possessed a QpH1 plasmid. Priscilla which is used to model chronic infection has a QpRS plasmid, QpDG was found in feral rodents in Utah, and is infectious but avirulent. Finally, QpDV was found in French and Russian isolates. It was thought that these plasmids might account for virulence and pathogenicity but this association was not consistently demonstrated (127,128,134,135).

Phase I LPS is required for virulence and analysis of the LPS of different strains yielded 3 different groups. Group 1 contained the LPS found on the Nine-Mile strain and other acute Q fever cases. The LPS from groups 2 and 3 were found from chronic Q fever cases (20).

Hendrix *et al.* 1991, compared chromosomal DNA for 33 isolates which allowed the strains to be divided into 6 groups (131). These groups correlate with the grouping based on plasmids. Groups 1 to 3 contain the plasmid QpH1, group 4 contains QpRS, group 5 is plasmidless and group 6 contains QpDG (131). More recently, the whole genome of the *C. burnetii* isolate, Nine-Mile phase I (NMI) RSA493, was sequenced using the random shotgun method(136). Using microarray based whole genome comparisons Beare *et al.* compared the genomes of 23 different strains to the open reading frames (ORFs) of the NMI reference strain(137). The isolates contained up to 20 different genomic polymorphisms consisting of 1-18 ORFs each.
Nineteen of the isolates were in the genomic groups I to VI as described by Hendrix et al (131). 4 ungrouped isolates were analysed, Q321, Le Bruges, Dugway 5G61-63 and BDT-1, and were found phylogenetically either to be in genomic group I or to be different to the previous groups and therefore placed into new genomic groups VII and VIII (137).

Long et al. (138) used an intraperitoneal guinea pig model to compare the pathogenicity of different genomic groups. They found the group 1, containing Nine-Mile and Ohio strain caused the most acute and severe disease. Group 2 containing Henzerling RSA343 strain also displayed acute but less severe disease. Group 3, Idaho Goat strain, was nearly as virulent as group 1 but does not cause human disease. Whilst groups 4 and 5 produce less severe, persistent focalised infections. Group 6 containing Dugway strains are avirulent in the guinea pig model(138). Differences in strain virulence and host cell response have also been demonstrated in G. mellonella larvae and macrophages respectively (126,139).

1.11. Immune response to Q fever

Macrophages are an essential part of innate immunity. They undergo phenotypic polarisation between M1 and M2 type macrophages dependent on their environmental stressors (140). M1 macrophages are typically involved in killing and M2 macrophages in repairing. The M1 phenotype is stimulated by pro-inflammatory cytokines (IFN-γ, TNF-α or Toll-like receptor (TLR) ligands), and lead to the production of further inflammatory cytokines (IL-1β, TNF-α, IL-12 and IL-6), chemokines (CCL2, CCL5, CXCL8) and express the surface receptors CCR7, CD80, TLR-2 and TLR-4(141). This inflammatory cascade triggers the release of nitric oxide (NO) and reactive oxygen intermediates (ROI) leading to cell death(140). IL-4, IL-10 and IL-13 induce the M2 macrophage response which leads to the production of IL-10, IL-1 receptor antagonist (IL-1ra), TGF-β1, CCL16, CCL17, CCL18 and CCL24(141). They express CXCR1, mannose receptor (MR), CD14 and arginase-1 which increases the release of ornithine and polyamines, blocking the NO pathway thus leading to cell repair (140,141). Macrophages in the M2 state generally excrete anti-inflammatory cytokines, enhance scavenging of cellular debris and promote tissue remodelling and repair (142).
Macrophages and their precursor monocytes are targeted by *C. burnetii*. Monocytes displaying an M1 type response allow *C. burnetii* survival without replication, whereas macrophages with an M2 type response allow *C. burnetii* to slowly replicate (143). Benoit *et al.* demonstrated that monocytes stimulated with *C. burnetii* led to macrophage polarisation towards a unique M2 programme (141). They showed an upregulation of IL-10, IL-1ra, TGF-β1, MR, CCL18 and arginase-1 which are associated with an M2 profile (141). MR expression can modify TLR signalling and is postulated to contribute to *C. burnetii* survival in macrophages. Arginase-1 is associated with *C. burnetii* persistence and has been shown in Vanin-1−/− mice to be associated with increased bacterial burden and defective granuloma formation (141).

Dellacasagrande *et al.* (144) found that the bacterial viability of *C. burnetii* infected THP-1 macrophages were reduced when treated with IFN-γ. This was not replicated if the cells were pre-treated with IFN-γ. It appears that *C. burnetii* cell death by apoptosis and secondary necrosis were dependent on IFN-γ and TNF(144). IFN-γ works in many ways to promote *C. burnetii* eradication. It is known that phase II (avirulent) *C. burnetii* is internalised by monocytes and macrophages more efficiently that phase I (virulent form) (145). Following uptake both phases multiply in cells but only phase I can multiply in macrophages, as phase II are killed in macrophages (145). Phase I bacteria multiply in small vacuoles which fuse to form a phagolysosome, and they escape intracellular killing by inhibiting the final phagosome maturation step (145). IFN-γ restores this step allowing intracellular killing. IFN-γ also alkalinises the vacuole preventing bacterial replication, and increases infected cell apoptosis by stimulating the release of TNF-α (145). TNF-α enhances internalisation of the phase I bacteria and stimulates granuloma formation in acute infection (18).

### 1.11.1. The cytokine profile of acute Q fever

In acute Q fever, *C. burnetii* antigens stimulate IFN-γ production which promote bacterial clearance (146). Although, this does not lead to complete clearance because *C. burnetii* DNA can still be isolated from peripheral blood mononuclear cells (PBMCs) and bone marrow months to years later (125). TNF-α, IL-6, IL-10 and IL-12 production by PBMCs is significantly higher in patients with acute Q fever without valve lesions compared to healthy controls (*p* <0.05) (146). TNF and IL-10
levels were significantly higher in acute Q fever patients with hepatitis compared with fever alone ($p<0.05$) (146). This was also seen with IL-6 and IL-12 but with greater variation in the findings compared to TNF-α and IL-10. Acute Q fever patients with valvulopathy had higher levels of IL-10 production by unstimulated PBMCs compared to both acute Q fever patients without valvulopathy and the control group ($p<0.05$) (146). TNF-α, IL-6 and IL-12 levels were higher than the control group, but were not significantly raised compared to the non-valvulopathy group(146). Within the valvulopathy group TNF-α and IL-10 production were significantly raised during acute Q fever in those that went on to develop endocarditis compared to the valvulopathy group without endocarditis ($p<0.05$)(146). Honstettre et al. propose that TNF-α and IL-10 are used to monitor acute Q fever patients with valve abnormalities for chronic endocarditis (146).

1.11.2. The cytokine profile of chronic Q fever

Long term persistence of bacterial DNA after acute infection is associated with disease relapse when the patient’s cell mediated immunity is depressed from either medication, concurrent illness or pregnancy (146). Schoffelen et al. 2014 found that following the Netherlands outbreak, IL-2 was significantly reduced in patients with CQF compared to seropositive controls, and IFN-γ was significantly higher than seronegative but not seropositive controls (147). An IFN-γ/IL-2 ratio of > 11 had a sensitivity and specificity of 79% and 96% respectively in predicting CQF, if corroborated in further studies this may be of use for CQF diagnosis (147).

1.11.3. The cytokine profile of Q fever fatigue syndrome

Chronic immune stimulation from *C. burnetii* cell components rather than viable bacteria has been postulated as a cause of QFS (17,99). Patients with persistent QFS had significantly higher levels of IL-6 and IFN-γ and lower levels of IL-2 compared to seronegative controls(99,148). Penttila et al. showed that IL-6 levels were also significantly higher than patients with CQF and seropositive controls. The same study showed a correlation with symptom score and fatigue and the level of IL-6 (99). Raijmakers et al. found that IL-6 and IL-1β were significantly higher in QFS patients compared with the asymptomatic seropositive controls, but that the acute phase IL-6 was not a predictor of QFS development (149) This finding has also been seen in other studies (98,150). The IFN-γ/IL-2 ratio in QFS is
comparable to seropositive controls and significantly reduced compared to CQF patients (150). Vollmer-Conna et al., failed to find a significant difference in the cytokine profile of QFS patients compared to resolved AQF controls (151).

1.12. In vitro models of C. burnetii infection

1.12.1. Cell based assays

Prior to 2009 axenic growth of C. burnetii was not possible. For in vitro experimentation, propagation of the bacteria in cell culture or embryonated eggs (152,153) was required. Cell infection assays have been used to investigate the cell/bacteria interactions that allow internalisation, replication and immune evasion of C. burnetii (19,22,23).

Cell culture assays have been used to model in vitro efficacy of antibiotics against C. burnetii. Cell lines used include vero cells (154), THP-1 monocytes (155), HEL cells (13,156), or Buffalo green monkey cell culture (BGM) (152). L929 mouse fibroblast cells have been used for a chronic cell infection model to assess antibiotic efficacy (13,157). The cell line chosen for bacterial growth needs to be selected carefully because they have distinctive growth requirements and different C. burnetii strains grow optimally in particular cell lines (15). Growth of the bacteria in embryonated eggs or cell cultures is complicated, time consuming and is liable to infection from environmental organisms during the lengthy incubation process. The difficulty in performing these assays is enhanced because this bacterium needs to be handled as a Biosafety Level 3 (BSL-3) organism. These challenges have historically led to limited in vitro investigation of antimicrobial efficacy against C. burnetii (158). The use of shell vial techniques initially in 1990, whilst still using infected cells, allowed the more timely determination of antibiotic susceptibility compared to previous methods (156). For this technique HEL cells are used for the cell monolayer on a glass coverslip and 1ml of the clinical sample is centrifugated onto the cells. This allows better attachment and penetration of the bacterium inside the cells. The infected cells are then incubated and visualised using Gimenez staining or IFA (15).

Quantification of the activity of antimicrobials on C. burnetii is difficult in cell cultures, shell vial assays and embryonated eggs, and can lack standardisation and reproducibility (158). Methods to visualise and quantify bacterial activity include by
plaque assay (159), dye uptake assay (13), immunofluorescence assay (IFA) (13), Gimenez staining (160), RT-PCR (161), nuclear magnetic resonance spectroscopy (162), phase contrast microscopy (154) and flow cytometry (163).

**Table 1:** Summary from published literature of the methods and results from cell culture assays assessing the antibiotic efficacy of doxycycline, levofloxacin, ciprofloxacin, moxifloxacin and co-trimoxazole against NMI + NMII *C. burnetii.*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Strain</th>
<th>Cell line used for <em>C. burnetii</em> culture</th>
<th>Cell line used for shell vial assay</th>
<th>Method of quantification</th>
<th>MIC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline</td>
<td>NMII</td>
<td>P388D1</td>
<td>P388D1</td>
<td>RT-PCR</td>
<td>2</td>
<td>(161)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>NMII</td>
<td>MRC5</td>
<td>MRC5</td>
<td>IF</td>
<td>1</td>
<td>(161)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>NMI</td>
<td>Vero</td>
<td>Vero</td>
<td>Plaque assay and dye uptake assay</td>
<td>1</td>
<td>(160)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>NMI</td>
<td>L929</td>
<td>L929</td>
<td>Fluorescence</td>
<td>1</td>
<td>(157)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>NMI</td>
<td>HEL</td>
<td>HEL</td>
<td>IFA</td>
<td>&lt; 4</td>
<td>(156)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>NMI</td>
<td>Vero</td>
<td>Vero</td>
<td>IFA</td>
<td>1</td>
<td>(164)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>NMI</td>
<td>BGM</td>
<td>BGM</td>
<td>IFA</td>
<td>0.25-0.5</td>
<td>(165)</td>
</tr>
<tr>
<td>Levofloxacine</td>
<td>NMI</td>
<td>L929</td>
<td>HEL</td>
<td>IFA</td>
<td>0.5</td>
<td>(13)</td>
</tr>
<tr>
<td>Levofloxacine</td>
<td>NMII</td>
<td>P388D1</td>
<td>P388D1</td>
<td>RT-PCR</td>
<td>2</td>
<td>(161)</td>
</tr>
<tr>
<td>Levofloxacine</td>
<td>NMII</td>
<td>MRC5</td>
<td>MRC5</td>
<td>IF</td>
<td>1</td>
<td>(161)</td>
</tr>
<tr>
<td>Levofloxacine</td>
<td>NMI</td>
<td>BGM</td>
<td>BGM</td>
<td>IFA</td>
<td>0.5</td>
<td>(165)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>NMII</td>
<td>P388D1</td>
<td>P388D1</td>
<td>RT-PCR</td>
<td>2</td>
<td>(161)</td>
</tr>
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<tr>
<td>Ciprofloxacin</td>
<td>NMII</td>
<td>MRC5</td>
<td>MRC5</td>
<td>IF</td>
<td>4</td>
<td>(161)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>NMI</td>
<td>Vero</td>
<td>Vero</td>
<td>Plaque assay and dye uptake assay</td>
<td>4</td>
<td>(160)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>NMI</td>
<td>HEL</td>
<td>HEL</td>
<td>IFA</td>
<td>&lt; 1</td>
<td>(156)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>NMI</td>
<td>L929</td>
<td></td>
<td>EM after Gimenez staining</td>
<td>MIC₉₀ = 1.8</td>
<td>(166)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>NMI</td>
<td>Vero</td>
<td>Vero</td>
<td>IFA</td>
<td>4</td>
<td>(164)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>NMI</td>
<td>BGM</td>
<td>BGM</td>
<td>IFA</td>
<td>2-8</td>
<td>(165)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>NMI</td>
<td>J774</td>
<td></td>
<td>IFA</td>
<td>&gt; 1</td>
<td>(167)</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>NMI</td>
<td>L929</td>
<td>L929</td>
<td>Fluorescence</td>
<td>0.5</td>
<td>(157)</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>NMI</td>
<td>BGM</td>
<td>BGM</td>
<td>IFA</td>
<td>1-2</td>
<td>(165)</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>NMII</td>
<td>P388D1</td>
<td>P388D1</td>
<td>RT-PCR</td>
<td>8</td>
<td>(161)</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>NMII</td>
<td>MRC5</td>
<td>MRC5</td>
<td>IFA</td>
<td>8</td>
<td>(161)</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>NMI</td>
<td>HEL</td>
<td>HEL</td>
<td>IFA</td>
<td>&lt; 2</td>
<td>(156)</td>
</tr>
</tbody>
</table>

**Cell line Key:** P388D1 cells = mouse macrophages, MRC₅ cells = human lung fibroblast, Vero cells = African Green Monkey kidney fibroblasts, L929 cells = mouse fibroblast cells, HEL cells = human erythroleukemia cell line, BGM cells = Buffalo Green Monkey kidney cells, J774 cells = mouse macrophages.
1.12.2. Axenic growth of C. burnetii

In order to facilitate axenic growth of C. burnetii the intracellular requirements for growth had to be ascertained (168). Hackstadt et al. in 1981 discovered that the acidic pH created by the fusion of the phagosome and lysosome were essential for bacterial replication (169). This acidic pH enabled the bacteria to metabolise glucose and glutamate. Zuerner et al. 1983, achieved transient (less than 4 hours) of protein synthesis by C. burnetii in phosphate buffered solution supplemented with amino acids and glucose to a pH of 4.5, but no bacterial growth (170). Early studies looking at energy sources used by C. burnetii, stability of the ATP pool, membrane energisation and acid activation allowed Omsland et al. to build on these discoveries and develop a complex Coxiella medium (CCM) which supported metabolic activity, in the form of protein and ATP synthesis outside of the host cell for 24 hours (168,171). The composition of CCM was designed to replicate the environment inside the phagolysosome, and consisted of acidic citrate buffer, neopeptone, fetal bovine serum and RPMI medium (171). CCM supported metabolic activity of C. burnetii for the first time for 24 hours outside of the host cell, this was seen by active transition from SCV to LCV in CCM. Although, replication was not seen in CCM and work continued to find the additional nutritional requirements for bacterial growth (171).

Using transcriptomic microarray, genomic reconstruction and metabolite typing the metabolic requirements for growth were further identified and led to the addition of casamino acid as a source of amino acids and L-cysteine. This second generation media was called acidified cysteine citrate medium (ACCM) (168). ACCM enhanced protein synthesis but replication was again not seen (172). Genome analysis had shown that C. burnetii growth was possible under different oxygen concentrations. Bacterial growth was assessed under different oxygen concentrations and it was found that the media when incubated in a microaerophilic environment (2.5% O2) enabled 3 logs growth of C. burnetii over 7 days (173). Further refinements were made by adding methyl-β-cyclodextrin to the media (ACCM-2) which allowed 4-5 logs of growth over 7 days (173). ACCM and ACCM-2 solid agarose support growth and allow enumeration of the bacteria (Figure 1) (173). Singh et al. have also shown that an empirical medium based on Vero cell extract (VCEM) allows axenic growth of C. burnetii but the media was required to be changed every 48 hours, and the bacteria would not grow on solid agarose-VCEM (174)
Figure 1: *C. burnetii* colonies on an ACCM-2 plate after 7 days of incubation in a microaerophilic environment (M.G. Hartley, Dstl, Porton Down).

1.13. *In vivo* models of *C. burnetii* infection

1.13.1. *Galleria mellonella*

The Lepidopteran greater wax moth larvae (*Galleria mellonella*) is an insect whose larvae have been used previously in antimicrobial efficacy studies against a range of bacteria and fungi, including *Pseudomonas aeruginosa* (175), *Francisella tularensis* (176), *Listeria monocytogenes* (177) and *Burkholderia pseudomallei* (178). There are many advantages of using *G. mellonella* larvae which include; low rearing costs, they are an ethically acceptable animal model and the bacterial inoculum can be delivered by topical application, oral or injection routes (Figure 2). The large larval size of 2-3 cm means that the bacterial inoculum or antibiotics can be injected straight into the haemolymph through the proleg. The large haemolymph volume of approximately 20 μl can be drained and used for pharmacokinetic studies or bacterial quantification. The larvae can be reared at 37°C which represents the bacterial growth conditions in humans (176, 177, 178, 179). In addition using larvae for high containment work is cheaper, and the larvae are easier to manipulate in comparison to mammalian models (179).
Figure 2: The injection technique used for inoculating through the proleg into the haemolymph. (I. Norville,Dstl, Porton Down).

G. mellonella larvae have an innate immune system which shares many similarities with the mammalian innate immune system. The host response of these larvae include structural and passive barriers as well as cellular and humoral immunity mediated by haemocytes within the haemolymph (176,180,181). The haemocytes are capable of phagocytosing bacterial and fungal cells and function much like human phagocytes (181). Microbial killing by haemocytes is through production of superoxide, a reactive oxygen species (ROS), which is also the process used by human neutrophils to kill bacteria (181). Insects differ to mammalian models as they do not have an adaptive immunity (177).

G. mellonella are susceptible to lethal infection with C. burnetii, NMI and NMII strains (179). No significant difference was seen between the 2 strains in time to death. All larvae infected with either strain died by day 9 (216 hours) post inoculation. Treatment with 50 mg/kg of doxycycline 24 hours after inoculation with $10^6$ GE/ml significantly extended time to death, with a median time to death of 96 hours in the untreated group versus 180 hours in the treated group ($p <0.0001$). Larvae can be monitored for disease progression through macroscopic colour changes secondary to an increase in melanisation as a response to infection (Figure 3), and a reduction in movement.
**Figure 3:** The macroscopic colour changes seen in *G. mellonella* larvae with disease progression. Melanisation occurs with infection. The larvae on the right hand side is healthy, increasing melanisation from right to left demonstrates an increased reaction to the infection. The larvae on the left hand side is dead. The larvae are lifesize. (*I. Norville. Dstl, Porton Down*)

1.13.2. Mammalian models

Once preliminary *in vitro* and non-mammalian *in vivo* work has been completed a higher order animal model is required to further test antibiotic efficacy. This is particularly important when human efficacy trials are not feasible or ethical as seen in Q fever.

Early *C. burnetii* pathogenicity studies were completed in guinea pigs\(^2,15,182\). The first antibiotic efficacy study was performed in 1948 investigating the effectiveness of streptomycin in guinea pigs inoculated via intraperitoneal injection with *C. burnetii*\(^182\). Guinea pigs are the most susceptible animal to *C. burnetii* which in untreated animals leads to death within 8 days\(^182\). Although this is dependent on the size of the bacterial inoculum because no mortality was seen in other studies\(^2\). Following inoculation, clinical disease will present with fever, anorexia and inactivity, and splenic enlargement at postmortem\(^2,182\). Other animal models utilised to study *C. burnetii* include rats, which are only mildly susceptible to disease displaying no clinical signs but do demonstrate splenic enlargement after inoculation\(^2,5\). Pathogenesis studies looking at strain variation in the development of endocarditis have been carried out in rabbits\(^183\), although Derrick found rabbits to be insusceptible to the bacteria\(^2\). Fourteen out of forty-
seven strains of mice were found to be susceptible to C. burnetii inoculation via the intraperitoneal (IP) route (184). AJ mice were the most susceptible with a 100% of mice showing signs of ruffled fur and lethargy with an IP inoculum of $10^{6.5}$ phase 1 burnetii Nine-Mile strain (184). The mean duration of illness was 4 days. Lethality was dose dependent with 100% mortality with an inoculum of greater than $10^{7.7}$ (184). Widespread dissemination of bacteria was seen in blood, heart, liver, lung, spleen, kidney and brain with a peak concentration at 7 days(184). Other sensitive strains include Balb and l29 mice (184). Sensitive and resistant mice seroconverted to the bacteria with no difference seen in the serology titres between the groups(184). The genetic modification in AJ mice led to deficiencies in both their innate and adaptive immune system (10,185). Their innate immune system is deficient in Complement component 5 (C5), Nod-Like Receptor (NLrp) and Neuronal Apoptosis Inhibitory Protein, which make the mice more susceptible to infection and reduces apoptosis and the phagocytic response to the pathogen (185). The adaptive immune response in AJ mice is shifted towards a TH2 helper T cell predominant response to a pathogen (185). A TH1 response is important in clearance of intracellular bacteria. These genetic modifications increase the susceptibility of AJ mice to C. burnetii but alter the immune response when exposed to this bacterium.

Infection can also be investigated in the non-human primates. Cynomologus monkeys demonstrate a clinical infection similar to human Q fever with fever, anorexia, cough, depression, shortness of breath and radiological changes consistent with pneumonia (15,186). Macaque monkeys are also susceptible and have been utilised in pathogenesis studies (5).

Pathogenicity and virulence in animal studies of C. burnetii depends on the animal species used, the route of infection and the size of the inoculum (15). Early studies relied on inoculation via the intraperitoneal route because the bacteria could not be isolated, and therefore samples from animals or humans known to have been infected were inoculated to cause disease. Intraperitoneal infection has a shorter incubation period with broad dissemination of the bacteria to organ sites (15,184). Whilst infection via the intranasal route or aerosolisation leads to infection mostly involving the lung with a decrease in systemic spread compared to the IP route (15). Aerosolisation studies have been completed in mice (187,188), and monkeys (186), and this route is now the preferred method for pathogenesis studies as it represents the most common route for human infection.
Norville et al. described the first inhalational model of infection in AJ mice (188). Mice were exposed to $10^6$ GE/ml of *C. burnetii* and displayed clinical signs from day 5 to 9, and weight loss starting at day 5, peaking at day 7 to 8. During the 13 day experiment the lung and spleen weight increased over time. Bacterial burden in lungs, spleen, liver, testes and blood by RT-PCR were documented. In addition, all mice by the end of the study had antibodies to phase I and II *C. burnetii* antigens. The AJ mouse model is an acute, inhalational, non-lethal model of *C. burnetii* infection and has been selected to be the model used for the antibiotic efficacy studies in this thesis.

1.14. The aim of this research

*C. burnetii* is a pathogen of interest to the military. Sporadic cases and outbreaks have been recorded since the bacterium was first identified in the 1930’s. The bacterium is also of military relevance due to its categorisation as a CDC category B biowarfare agent. Cases of acute Q fever and Q fever fatigue syndrome have been diagnosed in service personnel returning from the British military campaign in Afghanistan (Op Herrick, 2002 – 2014) (67). To date, there is no licensed Q fever vaccine for use in the UK. It has been suggested that doxycycline could prevent rickettsial infections in travellers (115), but this has not been proven and no data exists on the use of chemoprophylaxis in prevention of Q fever. A small human trial in the 1950’s showed the benefit of antibiotics administered late in the incubation period or around symptom onset, but this benefit was not seen with treatment early in the incubation period (189).

*In vitro* and *in vivo* studies have demonstrated that a range of antibiotics are efficacious against *C. burnetii*. Standard treatment for Q fever is doxycycline (106) but this antibiotic is bacteriostatic in the acidic intracellular environment within with *C. burnetii* replicates. This is why doxycycline monotherapy leads to disease relapse in chronic Q fever (74). Doxycycline and pefloxacin resistance have been reported highlighting the importance of evaluating the efficacy of alternative antibiotics for treatment of acute and chronic Q fever (106,113).
The aims of this research project are:

- To evaluate the efficacy of a range of antibiotics using *in vitro* and *in vivo* models.
- To evaluate the efficacy of chemoprophylaxis for prevention or amelioration of acute disease in animals models.
- To characterise Q fever prevalence and outcome in a military cohort.
Chapter 2: Materials and Methods

2.1 Bacterial strains and cell lines

2.1.1. Bacterial strains

The bacterial strains used during this research degree are listed in Table 1.

Table 1: The bacterial strains used for the in vitro and in vivo studies.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase II C. burnetii [Nine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mile (NM II) (RSA439, clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>□CBU0678-CBU0698; truncated LPS</td>
<td>Williams et al. (1981)(190)</td>
</tr>
<tr>
<td>Phase I C. burnetii [Nine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mile (NM I) (RSA493)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wild type (WT)</td>
<td>Montana, tick, 1935</td>
</tr>
<tr>
<td>Escherichia coli (ATCC®</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25922 ™)</td>
<td></td>
<td>American Type Culture Collection (ATCC)</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>subsp. aureus (ATCC®</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25923 ™)</td>
<td></td>
<td>American Type Culture Collection (ATCC)</td>
</tr>
</tbody>
</table>

2.1.2. Growth of strains

*C. burnetii* NMII strain was cultured axenically in Acidified Citrate Cysteine Medium-2 (ACCM-2, Sunrise Science Products, California) in a 75 cm$^3$ tissue culture containing 20 ml of medium. The flasks were placed in a plastic box with vented lid (0.2 µm filter) and incubated statically in a Galaxy 170 R incubator (New Brunswick Scientific) at 37°C adjusted to 5% CO$_2$ and 2.5% O$_2$ for 7 days (168). Cultures of the NMII strain were grown as required and not frozen for storage.

*C. burnetii* NMI strain was grown in 250 ml Erlenmeyer flasks containing 100 ml of ACCM-2. It was incubated at 37°C, shaking at 75 r.p.m. for 6 days in a GENbox microaer generator (bioMérieux) to displace oxygen (188). The bacteria were harvested via centrifugation at 10,000 X g at 21°C for 20 minutes and the supernatant was removed to leave a pellet of bacteria which was re-suspended in PBS and stored at -80°C for future use.
Frozen stocks of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 kept at -80°C were defrosted and streaked onto Lysogeny Broth (LB) plates and cultured overnight in an aerobic atmosphere at 37°C.

### 2.1.3. Cell lines

THP-1 cells are a myelomonocytic leukaemia cell line derived from a 1 year old boy with acute monocytic leukaemia(191,192). The cells were sourced from ECCAC (PHE Porton). Cells were maintained in 75 cm³ flasks in a loose suspension at a cell density greater than 1 x 10⁵ in Roswell Park Memorial Institute (RPMI 1640) medium (Life Technologies, Gaithersburg, MD) plus 2 mM L-glutamine (or glutomax) and 10% foetal calf serum (Life Technologies, Gaithersburg, MD) in 5% CO₂ at 37°C.

### 2.2. Antibiotics

**Table 2:** The antibiotic formulations and the diluents used for the *in vivo* and *in vitro* studies.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Formulation</th>
<th>Source</th>
<th>Diluent</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline hyclate</td>
<td>Powder</td>
<td>Sigma Aldrich®, UK</td>
<td>DiH₂O</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Galleria Mouse Study 1&amp;2</td>
</tr>
<tr>
<td>Doxycycline hyclate</td>
<td>100mg capsules, split and powder mixed with diluent</td>
<td>Chanelle Medical Ltd, UK</td>
<td>DiH₂O</td>
<td>Mouse study 4</td>
</tr>
<tr>
<td>Doxycycline monohydrate</td>
<td>100mg tablets, Vibramycin-D</td>
<td>Pfizer Limited, Sandwich, UK</td>
<td>DiH₂O</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mouse study 3 &amp; 4</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Powder</td>
<td>Sigma Aldrich®, UK</td>
<td>DiH₂O + 2mL of 1 M sodium hydroxide</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Galleria</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Solution for infusion (2 mg/ml)</td>
<td>Hospira UK Ltd, Horizon, Maidenhead UK</td>
<td>Used neat</td>
<td>Mouse study 1&amp;2</td>
</tr>
<tr>
<td>Moxifloxacin hydrochloride</td>
<td>Powder, neat</td>
<td>Sigma Aldrich®, UK</td>
<td>DiH₂O</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Galleria</td>
</tr>
</tbody>
</table>
### 2.3. Bacterial enumeration

#### 2.3.1. Axenic media

ACCM-2 agarose plates without an overlay were incubated at 37°C with 5% CO₂ and 2.5% O₂ for 14 days. One hundred microlitres per plate of standard dilutions from the neat culture were plated out in duplicate. Bacterial counts were enumerated using the plates with colony counts of between 30 – 300 to reduce error.
2.3.2. Real time PCR

In all four mouse studies, DNA extraction was performed using a modified method from the Qiagen QIAamp DNA minikit/blood kit. One hundred microliters of sample (containing less than 40 mg of homogenised spleen or 80 mg of homogenised lung tissue or 100 μl of whole blood) were added to ATL buffer and proteinase K and heated in a rotating block at 56°C for between 15 minutes and 1 hour as per manufacturer’s instructions. Two hundred microliters of AL buffer and 200 μl of ethanol were added to the tissue preparation above, pipetted to mix and then added to a 1.5 ml microcentrifuge tube and centrifuged at 8000 rpm for 1 minute. The filtrate was removed from the column and 500 μl of AW1 buffer was then added to the column and centrifuged at 8000 rpm for 1 minute. The filtrate was again removed and 500 μl of AW2 buffer added to the column and centrifuged at 14000 rpm for 3 minutes. The filtrate was removed and 200 μl of AE buffer was added to the column, left to stand for 1 minute, and centrifuged at 8000 rpm for 1 minute into a clean collecting tube. The filtrate was pipetted into Starstedt tubes and either used immediately or placed in the fridge or frozen at -80°C for later use.

For mouse study 1 & 2, *C. burnetii* was enumerated using real time PCR (RT-PCR) at Public Health England (PHE) Porton using the methodology described previously by Norville *et al.,* 2014 (188). RT-PCR targeted the *sod* gene using the forward primer TCTTCAACAATGCAGCACAACAT and reverse primer TGAAGCCAATTGCAGGAGAA. The probe sequence was CATTTTATTGCGACTGAGCAGGCTTG, and the probe was covalently labelled at the 5’ end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3’ end with the quencher dye, black hole quencher 1 (BHQ-1). For each PCR in the first two studies, a control of linearized synthetic plasmid was quantitated after linearization and purification using a ND-2500 NanoDrop spectrophotometer. A standard curve of the synthetic plasmid was run in duplicate in the range of 1 x 10⁷ GE/ml to 1 x 10² GE/ml for each PCR. A plasmid concentration of 1 x 10¹ GE/ml in triplicate is used as the lower limit of check in each assay. The lower limit of detect for this assay is 2.5 x 10³ GE/ml for culture or 4.4 x 10¹ GE/mg spleen or 2.2 x 10¹ GE/mg lung.

For mouse study 3 & 4, *C.burnetii* was enumerated using RT-PCR at the Defence Science and Technology Laboratory (Dstl) Porton Down by targeting the *com1* gene (forward primer, CGACCGAAGCATAAAGTCAATG; reverse primer,
ATTTCATCTTGCTCGCTCTAACAAC; probe, TTATGCGCGGTTTCGACTACCATTTC). Standards were made by spiking naive tissue/blood with serial dilutions of viable bacteria and extracted the same way. The appropriate standard was run every time. As the data obtained by PCR was directly compared to viable counts we have used the term PCR-CFU.

For all studies a 2 µl template DNA, forward primer (900 nM), reverse primer (900 nM), probe (250 nM) and ABI Fast TaqMan mastermix were used for the real-time PCR. PCR cycles consisted of 3 mins at 95°C, 30s at 60°C, followed by 50 two-step cycles, with one cycle consisting of 15 s at 95°C and 30s at 60°C.

2.4. In vitro studies

2.4.1. Minimum inhibitory concentrations (MICs)

2.4.1.1. Broth dilution: Phase II C. burnetii (NMII)

The concentration of C. burnetii was determined using a spectrophotometer set to a wavelength of 590 nm. The culture was diluted using ACCM-2 to an optical density (OD) of 0.1 which has been shown to be equivalent to 10^8 CFU/ml (I. Norville, personal communication, Dstl). Antibiotics were prepared at stock concentrations of 10 mg/ml and were diluted to the required starting concentration in ACCM-2. Four millilitres of ACCM-2 were aliquoted into wells in a 6 well plate and the antibiotic solution was double diluted across the plate to the lowest dilution required. A positive control containing no antibiotics and a negative control well containing no bacteria were included.

The C. burnetii culture was further diluted to -1 (10^7 CFU/ml) and 100 µl used to inoculate each well. The culture was plated out from -1 to -6 on solid axenic media to calculate viable counts. The 6 well plates were incubated statically as previously described for 7 days. The bacterial growth was estimated using the spectrophotometer and the MIC defined as the lowest concentration of antibiotic for which no visible growth was seen after incubation. All experiments were completed in triplicate.
2.4.1.2. Broth dilution: Phase I *C. burnetii (NMI)*

The MIC was determined for Phase I *C. burnetii* using the same methodology as for Phase II *C. burnetii* described in 2.2.1.1 with the exception that the *C. burnetii* culture was prepared from a frozen stock stored at -80°C. The bacterial growth was estimated using the spectrophotometer and the MIC defined as the lowest concentration of antibiotic for which no visible growth was seen after incubation. All experiments were completed in triplicate.

2.4.1.3. Epilosometer tests (Etests®): Phase II *C. burnetii (NMII)*

A bacterial lawn of 100 µl of *C. burnetii* at $10^7$ GE ml$^{-1}$ was created on an ACCM-2 agarose plate. The plates were allowed to air dry for 15 minutes in the microbiological safety cabinet (MSC-1) prior to application of the Etest®. The plates were incubated for 14 days as previously described, and the results read by two independent observers. The MIC by this methodology was defined as the point where the zone of no growth bisected the Etest® strip.

2.4.2. Broth assay: Phase I *C. burnetii (NMI)*

*C. burnetii NMI* was prepared from frozen stock stored at -80°C. The broth dilution assay to assess antibiotic sensitivity was set up using the methodology from section 2.2.1.1. A range of doxycycline hyclate, doxycycline monohydrate, levofloxacin, ciprofloxacin and co-trimoxazole concentrations were selected above and below the MICs established for NMI. The cultures were incubated statically as previously described for 7 days. At 7 days serial dilutions of the cultures were plated out on ACCM-2 agar for enumeration. The inoculum at day 0 was also plated out to enable growth kinetics to be established. Significant inhibition of growth between each antibiotic concentration and growth in the absence of antibiotics was established by log transforming the data and comparing the growth using a one-way ANOVA with Dunnett’s multiple comparisons. The Clinical and Laboratory Standards Institute (CLSI) define the limit of bactericidal effect as a 2 log decrease following incubation with each antibiotic concentration when compared with the bacterial concentration at time 0h (193). This definition was used to compare the bactericidal effects of each antibiotic.
2.4.3. THP-1 cell assay: Phase I C. burnetii (NMI)

THP-1 myelomonocytes were seeded at 5 x 10^5 cells/ml into a 24 well plate in RPMI 1640 containing 10% FCS + 2 mM glutamine with 200 nM of phorbol 12-myristate 13 – acetate (PMA, Sigma-Aldrich) to stimulate differentiation into the macrophage like cells, and incubated at 37°C, 5% CO₂ for 72 hours (194). Following incubation, cells were assessed for viability using trypan blue staining. Briefly, trypsin was added to one well to remove the adhered cells from the well surface, followed by addition of trypan blue to allow cell viability to be calculated. The cell count and viability were read on an automated cell counter (Cellometer® Auto T4, Bioscience). Phase I C. burnetii stock was diluted to the required concentration in Leibovitz’s L15 medium (Life Technologies, Gaithersburg, MD) with 10% FCS (Life Technologies, Gaithersburg, MD) dependent on the initial cell count. Cells were infected with at an estimated multiplicity of infection (MOI) of 20:1 ratio (T = - 24h). Antibiotic stocks were diluted to the required concentrations in L15 plus 10% FCS. Twenty-four hours after cell infection (T = 0 h), the cells were washed twice with PBS prior to 1 ml of antibiotic in L-15 (doxycycline hyclate, doxycycline monohydrate, levofloxacin, ciprofloxacin and co-trimoxazole) being added to each well. Each antibiotic concentration was duplicated in separate wells. At T=0 h, two wells were lysed with diH₂O, the contents of the wells pooled, serially diluted and then 100 µl plated out in duplicate to allow enumeration of intracellular bacteria prior to the addition of antibiotic. Seventy-two hours later (T = 72 h), the wells were washed twice with PBS, prior to cell lysis with diH₂O as previously described. The contents of the wells with corresponding antibiotic concentrations were pooled prior to serial dilution. One hundred microlitres of each pooled well was plated out in duplicate for enumeration. Each experiment was completed in triplicate.

The inhibitory effect and bactericidal effect of each antibiotic in THP-1 cells were assessed using the same methodology as for the broth assay in section 2.4.2. to allow comparison of antibiotic efficacy in axenic media versus cell culture.

2.4.4. Antibiotic stability

The stability of doxycycline hyclate, ciprofloxacin, levofloxacin, moxifloxacin and co-trimoxazole in ACCM-2 at pH 4.75 was determined using the well diffusion methodology (195), with the exception that solutions of antibiotics at varying
concentrations were placed into holes in the agar rather than using antibiotic discs. In brief, 2 ml of bacteria at 1 x 10^8 CFU/ml (E. coli was used for ciprofloxacin, levofloxacin, moxifloxacin and co-trimoxazole and S. aureus for doxycycline hyclate and co-trimoxazole) was spread evenly across the surface of LB agar plates and tilted to ensure an even coverage, with excess culture removed. LB agar and Muller-Hinton plates were used for Co-trimoxazole because the antibiotic was found to be inactive on LB agar requiring Muller-Hinton to be used to ensure there was not an interaction between the media and antibiotic. Antibiotic standards were serially diluted in PBS to final concentrations of 32, 16, 8, 4, 2, 1 and 0.5 µg/ml. Two equally spaced 5 mm holes were bored into the agar. Fifty microlitres of each antibiotic standard was added to each hole. The plates were incubated overnight at 37°C. After 24 hours of incubation, the zones of inhibition were measured using calipers to produce the standard curve. All tests were performed in duplicate.

To assess for antibiotic stability in ACCM-2 doxycycline hyclate, ciprofloxacin, levofloxacin, moxifloxacin and co-trimoxazole were diluted to a concentration of 32 µg/ml using ACCM-2. The antibiotics were placed into wells and the zone of inhibition was read using calipers at 0, 24, 48, 72 and 144 hours post-inoculation. The results were compared to the standard curve to test for antibiotic stability in ACCM-2.

2.5. In vivo studies

2.5.1. Galleria mellonella efficacy study

An in vivo non-mammalian antibiotic efficacy study was performed in the wax moth larvae, Galleria mellonella. The aim was to assess the survival benefit of the antibiotics being studied (doxycycline hyclate, levofloxacin, ciprofloxacin, moxifloxacin and co-trimoxazole) when administered 24 hours before or 24 hours after C. burnetii challenge in comparison to untreated larvae given only PBS.

G. mellonella were sourced from Live Foods UK and maintained on wood chips at 14°C. Only healthy G. mellonella weighing between 0.19 – 0.26 g were selected for the study to control for larvae age. The model of infection was set up as described by Norville et al. 2014(179). Groups of 10 larvae were injected with 10 µl of 10^8 GE/ml phase II C. burnetii (NMII) into their uppermost right proleg. The G. mellonella were treated with each antibiotic being studied into their left uppermost
prolong either 24 hours pre or 24 hours post challenge with *C. burnetii*. A group of 10 *G. mellonella* were challenged with *C. burnetii* and administered with 10 µl of PBS 24 hours post challenge as the positive control group. Larvae were monitored for survival every 24 hours over 264 hours. Larvae were recorded as dead if they were immobile after gentle stimulation with a pipette tip. This study was completed in triplicate. Groups of 5 uninfected larvae were treated with either PBS, or the antibiotics being studied (doxycycline hyclate, ciprofloxacin, moxifloxacin, levofloxacin and co-trimoxazole) to control for any side effects of the treatment.

**Table 3:** Study plan: Groups of 10 *G. mellonella* were challenged with 10 µl of 10⁸ GE/ml *C. burnetii* and treated with doxycycline hyclate, ciprofloxacin, co-trimoxazole, levofloxacin, moxifloxacin or PBS either 24 hours pre or 24 hours post challenge. Their outcome was observed over 264 hours. This study was completed in triplicate.

<table>
<thead>
<tr>
<th>Group</th>
<th>Numbers</th>
<th>Challenge</th>
<th>Treatment</th>
<th>24h Pre-dose</th>
<th>24 h Post-dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10⁸ <em>C. burnetii</em></td>
<td>Doxycycline hyclate</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>10⁸ <em>C. burnetii</em></td>
<td>Doxycycline hyclate</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>10⁸ <em>C. burnetii</em></td>
<td>Ciprofloxacin</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>10⁸ <em>C. burnetii</em></td>
<td>Ciprofloxacin</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>10⁸ <em>C. burnetii</em></td>
<td>Co-trimoxazole</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>10⁸ <em>C. burnetii</em></td>
<td>Co-trimoxazole</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>10⁸ <em>C. burnetii</em></td>
<td>Levofloxacin</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>10⁸ <em>C. burnetii</em></td>
<td>Levofloxacin</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>10⁸ <em>C. burnetii</em></td>
<td>Moxifloxacin</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>10⁸ <em>C. burnetii</em></td>
<td>Moxifloxacin</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>10⁸ <em>C. burnetii</em></td>
<td>PBS</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

2.5.2. AJ mouse pharmacokinetic study

Groups of age matched A/Jola (AJ) mice (Harlen) were sourced for the pharmacokinetic (PK) studies. The studies were performed at PHE Porton. The aim of the study was to understand the PK profile of doxycycline hyclate, levofloxacin, ciprofloxacin and co-trimoxazole in AJ mice in order to inform the dose...
and frequency required for each antibiotic to best replicate the PK profile in commonly used human drug regimens.

2.5.2.1. Pharmacokinetic studies

Levofloxacin was administered to 30 mice via the intraperitoneal route at a dose of 100 mg/kg. Groups of 3 mice were humanely euthanised at 1 min, 10 min, 20 min, 30 min, 1 h, 1.5 h, 2 h, 4 h, 8 h and 12 h after dosing.

Co-trimoxazole was administered to 30 mice orally via pipette at a dose of 48 mg/kg. Groups of 3 mice were humanely euthanised at 30 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 4 h, 6 h, 8 h and 12 h after dosing.

The time points were selected having reviewed literature on the rate of absorption of each drug in mice. Blood samples were collected from all animals via a terminal cardiac puncture. All samples were heparinised and centrifuged to separate the plasma fraction from the whole blood. Samples were immediately frozen and stored at -80°C until further analysis. Three untreated mice were included with each group.

Raw data from previous pharmacokinetic studies of doxycycline hyclate and ciprofloxacin in AJ mice completed at PHE Porton, were used to model the dose required for the antibiotic efficacy studies.

2.5.2.2. Determination of antibiotic concentrations in the blood samples by liquid chromatography-mass spectrometry (LC-MS)

The plasma samples were mixed with an equal volume of internal standard solutions and 3 volumes of acetonitrile. After centrifugation, the supernatant was removed and reduced in volume to remove the acetonitrile using a centrifugal evaporator (Geneva Ltd., Ipswich, UK), and injected onto the LC-MS system consisting of an Agilent 1100 binary pump (Agilent Technologies UK Ltd., Wokingham, UK), CTC PAL injector (Presearch Ltd., Basingstoke, UK) using an ACE-3-C18HL 20- by 2.1 mm column (Hichrom, Theale, UK) and a gradient mobile phase.

The LC-MS system was controlled by Analyst Software (AB Sciex, UK). Calibration curves were developed for naïve mouse plasma using reference standards of
antibiotics, co-trimoxazole (Septrin 240 mg/kg, Aspen Pharma Trading Limited) and levofloxacin (Levofloxacin solution for infusion 5 mg/kg, Hospira, UK Ltd).

2.5.2.3. Pharmacokinetic calculations

The terminal half-life \( t_{1/2} \), rate of clearance (CL), maximum concentration \( C_{\text{max}} \), and area under the concentration curve (AUC) were established by compartmental pharmacokinetic analysis using WinNonlin Phoenix (v6.1; Pharsight Corp., USA) on mean concentration data.

2.5.3. Aerosol challenge of AJ mice

AJ mice were chosen for the in vivo mammalian model to assess efficacy of antibiotics against C. burnetii due to their high degree of sensitivity to this bacterium(184). The AJ mouse model of acute C. burnetii infection has been set up at Dstl Porton Down as an aerosol model because this is the most common mode of acquisition in human infection (188).

Groups of age-matched AJ mice (Harlen) were housed on a 12-h day-night light cycle, with food and water available ad libitum in an Advisory Committee on Dangerous Pathogens (ACDP) (UK) level 3 flexible-film isolator and allowed to acclimatize before challenge. All procedures were conducted under a project license approved by internal ethical review, and in accordance with both the UK Animal (Scientific Procedures) Act (1986) and the 1989 Codes of Practice for the Housing and Care of Animals used in Scientific Procedures.

The AJ mouse model for C. burnetii has been published by Norville et al(188). Mice were challenged with an aerosol generated from \( 1 \times 10^9 \) PCR-CFU of Phase I C. burnetii using the AeroMP-Henderson apparatus. The challenge aerosol was generated using a six-jet Collison nebulizer (BGI Waltham, MA) operating at 15 litres/min. The aerosol was mixed with conditioned air in the spray tube and delivered via a head-only exposure chamber. Samples of the aerosol were taken using an AGI-30 (Ace Glass Inc. USA) at 6 litres/min containing PBS and an aerodynamic particle sizer (TSI Instruments, Ltd., Bucks, UK); these processes were controlled and monitored using the AeroMP management platform (Biaera Technologies, LLC, Frederick, MD). A back titration of the aerosol samples taken at the time of challenge was performed using RT-PCR. Direct bacterial enumeration
was used to calculate the retained dose using a derived respiratory minute volume of 19.9 ml estimated using the average weight of animals (196). An aerosol dose that is presented to the mouse in the exposure is calculated and then the retained dose in the mouse lungs is calculated by multiplying the presented dose by 0.4 which is the estimated retained dose in the lungs extrapolated from aerosol work with *Bacillus* spores from the 1950's (197). The calculation for the dose of *C. burnetii* retained by the mice is shown below:

**Aerosol Concentration** (cfu/L of air) 
\[= \text{Impinger count (cfu/mL)} \times \text{Impinger volume (mL)} \times \frac{\text{Impinger flow rate (L/minute)} \times \text{Impinger sampling duration (minutes)}}{1000}\]

**Presented dose (cfu)** 
\[= \text{Aerosol concentration (cfu/L of air)} \times \text{Minute respiratory volume (L)} \times \text{challenge duration}\]

**Retained dose (cfu)** 
\[= 0.4 \times \text{presented dose}\]

Impinger flow rate = 12 L/min  
Impinger volume = 10 ml  
Impinger sampling duration = 1 minute  
Minute respiratory volume = 0.0199 L  
Challenge duration = 10 minutes

2.5.4. AJ mouse antibiotic efficacy study 1

AJ mouse efficacy study 1 was designed to assess the effectiveness of treatment with doxycycline hyclate 105 mg/kg once daily and twice daily orally or ciprofloxacin 22 mg/kg twice daily via intraperitoneal injection administered either 24 hours pre or 24 hours post challenge (Table 4). The treatment duration was for 7 days post challenge, which meant the mice received a total of 9 days of antibiotics if initiated pre challenge or 7 days if initiated post challenge. A group of mice were challenged with *C. burnetii* but given only PBS instead of antibiotics as a positive control group. There were 10 mice in each group.
Table 4: Study plan for A/J mouse antibiotic efficacy study one. Seven groups of 10 mice were challenged by aerosol challenge with a mean retained dose of $6.4 \times 10^5$ PCR-CFU of Phase 1 \textit{C. burnetii} and given antibiotic treatment of doxycycline hyclate or ciprofloxacin, either 24 hours pre or 24 hours post challenge or the control group of PBS. \textit{(od = once daily, bd = twice daily, IP = intraperitoneal)}

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment regimen</th>
<th>Antibiotic dose</th>
<th>Route of administration</th>
<th>Length of treatment (days)</th>
<th>No of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Doxycycline hyclate一次 daily 24h PRE CHALLENGE</td>
<td>105 mg/kg od</td>
<td>Oral</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Doxycycline hyclate once daily 24h POST CHALLENGE</td>
<td>105 mg/kg od</td>
<td>Oral</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Doxycycline hyclate twice daily 24h PRE CHALLENGE</td>
<td>105 mg/kg bd</td>
<td>Oral</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Doxycycline hyclate twice daily 24h POST CHALLENGE</td>
<td>105 mg/kg bd</td>
<td>Oral</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Ciprofloxacin twice daily 24h PRE CHALLENGE</td>
<td>22mg/kg bd</td>
<td>IP</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>Ciprofloxacin twice daily 24h POST CHALLENGE</td>
<td>22 mg/kg bd</td>
<td>IP</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>PBS (control) 24h PRE CHALLENGE</td>
<td>50 µl od</td>
<td>Oral</td>
<td>9</td>
<td>10</td>
</tr>
</tbody>
</table>

The mice were observed daily and clinical signs of piloerection, arched back, dehydration, eyes shut, wasp-waisted appearance, immobility and death were recorded, and a corresponding score given to each mouse (Table 5). Weights were recorded once daily at the same time each day +/- 1 hour, and in accordance with PHE Porton’s Animal Home Office Licence, the mice were deemed to have reached their humane end point and were culled if they lost greater than 20% of their baseline weight over two consecutive time-points. All remaining mice were culled at day 14 post challenge and lungs and spleen were aseptically removed and weighed. The right lung (maximum 80 mg) and remaining spleen (maximum 40 mg) were homogenized to extract DNA using a Precellys24 homogeniser. Bacteria were enumerated using RT-PCR. The remaining tissue was transferred toDstl Porton Down where it was plated out on ACCM-2 agar for viable counts.
Table 5: At PHE Porton, AJ mice were observed daily, and a clinical score given to their clinical appearance in order to monitor their disease progression in conjunction with weight loss. The clinical score is summative as an aggregate of the clinical signs seen. The clinical observation score is specific to the Home Office Animal Licence that is held at PHE Porton.

<table>
<thead>
<tr>
<th>Description</th>
<th>Clinical Observation Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>0</td>
</tr>
<tr>
<td>Ruffled fur</td>
<td>1</td>
</tr>
<tr>
<td>Dehydration</td>
<td>1</td>
</tr>
<tr>
<td>Arched back</td>
<td>2</td>
</tr>
<tr>
<td>Eyes shut</td>
<td>2</td>
</tr>
<tr>
<td>Wasp wasted</td>
<td>2</td>
</tr>
<tr>
<td>Immobile</td>
<td>9</td>
</tr>
<tr>
<td>Dead</td>
<td>10</td>
</tr>
</tbody>
</table>

2.5.5. AJ mouse antibiotic efficacy study 2

AJ mouse efficacy study 2 was designed to investigate the efficacy of doxycycline hyclate 105 mg/kg twice daily orally, ciprofloxacin 22 mg/kg twice daily intraperitoneally, levofloxacin 40 mg/kg twice daily intraperitoneally or co-trimoxazole 48 mg/kg twice daily orally following an aerosol challenge with a mean retained dose of $4 \times 10^6$ PCR-CFU of *C. burnetii*. Antibiotics were commenced either 24 hours pre or 24 hours post challenge (Table 6) and continued for 9 days if commenced pre challenge or 7 days if commenced post challenge. A control group of 10 mice received only PBS as therapy.
Table 6: Study plan for AJ mouse antibiotic efficacy study 2. Nine groups of 10 mice were challenged with an aerosol generated from $1 \times 10^9$ PCR/CFU of Phase 1 C. burnetii and given antibiotic treatment of doxycycline hyclate, ciprofloxacin, levofloxacin or co-trimoxazole either 24 hours pre or 24 hours post challenge or the control group of 25 µl PBS twice daily.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment regimen</th>
<th>Antibiotic dose</th>
<th>Route of administration</th>
<th>Length of treatment (days)</th>
<th>No of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Levofloxacin once daily 24h PRE CHALLENGE</td>
<td>40mg/kg bd</td>
<td>IP</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Levofloxacin once daily 24h POST CHALLENGE</td>
<td>40mg/kg bd</td>
<td>IP</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Co-trimoxazole once daily 24h PRE CHALLENGE</td>
<td>48mg/kg bd</td>
<td>Oral</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Co-trimoxazole once daily 24h POST CHALLENGE</td>
<td>48mg/kg bd</td>
<td>Oral</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Ciprofloxacin twice daily 24h PRE CHALLENGE</td>
<td>22mg/kg bd</td>
<td>IP</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>Ciprofloxacin twice daily 24 hrs POST CHALLENGE</td>
<td>22 mg/kg bd</td>
<td>IP</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>Doxycycline hyclate twice daily 24h PRE CHALLENGE</td>
<td>105 mg/kg bd</td>
<td>Oral</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>Doxycycline hyclate twice daily 24h POST CHALLENGE</td>
<td>105 mg/kg bd</td>
<td>Oral</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>PBS (control) 24h PRE CHALLENGE</td>
<td>50 µl bd</td>
<td>Oral</td>
<td>9</td>
<td>10</td>
</tr>
</tbody>
</table>

The mice were observed daily and clinical signs of piloerection, arched back, dehydration, eyes shut, wasp-waisted appearance, immobile and death were recorded and a corresponding summative score of all clinical signs given to each mouse (Table 4). Weights were recorded once daily, and as in study 1, the mice were deemed to have reached their humane end point and were culled if they lost greater than 20% of their baseline weight over two consecutive time-points. All remaining mice were culled at day 14 post challenge and lungs and spleen were aseptically removed. The organs were processed as described for study 1.
2.5.6. AJ mouse antibiotic efficacy study 3

The aim of AJ mouse efficacy study 3 was to investigate the effectiveness of doxycycline commenced at 1 day pre or 5 days post an aerosol challenge with a mean retained dose of \(6.7 \times 10^4\) PCR-CFU of \(C.\ burnetii\). Doxycycline monohydrate was used instead of doxycycline hyclate to see if it was better tolerated in AJ mice. The mice were treated with doxycycline monohydrate for a total of 7 or 14 days post challenge. Therefore, when antibiotics were started 24 hours prior to \(C.\ burnetii\) challenge the mice received 9 or 16 days of treatment. If antibiotics were commenced 5 days post challenge they received 7 or 14 days of treatment (Table 8).

**Table 7:** At Dstl Porton Down AJ mice were observed daily and a score given to their clinical appearance in order to monitor their disease progression in conjunction with weight loss. The clinical score was summative and if a mouse reached a score of 6 this was considered to be the humane end point. The clinical observation score is specific to the Home Office Animal Licence that is held at Dstl Porton Down.

<table>
<thead>
<tr>
<th>Description</th>
<th>Clinical Observation Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>0</td>
</tr>
<tr>
<td>Slightly ruffled fur</td>
<td>1</td>
</tr>
<tr>
<td>Ruffled fur</td>
<td>2</td>
</tr>
<tr>
<td>Hunched</td>
<td>1</td>
</tr>
<tr>
<td>Very hunched</td>
<td>2</td>
</tr>
<tr>
<td>Eyes shut</td>
<td>2</td>
</tr>
<tr>
<td>Limited immobility</td>
<td>1</td>
</tr>
<tr>
<td>Immobile</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 8: Study plan for AJ mouse antibiotic efficacy study 3. One hundred and five mice were challenged via an aerosol challenge with a mean retained dose of \(6.7 \times 10^4\) PCR-CFU of Phase I *C. burnetii* and given doxycycline monohydrate or water (DiH\(_2\)O) either 24 hours pre or 5 days post challenge. Two groups of mice were treated with either antibiotic or water to assess the immunological effects of doxycycline alone without *C. burnetii* challenge.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment Regimen</th>
<th>Antibiotic dose</th>
<th>Length of Treatment (days)</th>
<th>Day euthanised</th>
<th>No of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Doxycycline monohydrate twice daily 24h PRE CHALLENGE</td>
<td>105 mg/kg</td>
<td>9</td>
<td>8 + 35</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Distilled water twice daily 24h PRE CHALLENGE</td>
<td>50 µl</td>
<td>9</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Doxycycline monohydrate twice daily 24h PRE CHALLENGE</td>
<td>105 mg/kg</td>
<td>16</td>
<td>15 + 35</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Distilled water twice daily 24h PRE CHALLENGE</td>
<td>50 µl</td>
<td>16</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Doxycycline monohydrate twice daily 5 days POST CHALLENGE</td>
<td>105 mg/kg</td>
<td>7</td>
<td>12 + 35</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>Distilled water twice daily 5 days POST CHALLENGE</td>
<td>50 µl</td>
<td>7</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>Doxycycline monohydrate twice daily 5 days POST CHALLENGE</td>
<td>105 mg/kg</td>
<td>14</td>
<td>19 + 35</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>Distilled water twice daily 5 days POST CHALLENGE</td>
<td>50 µl</td>
<td>14</td>
<td>19</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>Distilled water twice daily 5 days POST CHALLENGE</td>
<td>50 µl</td>
<td>14</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>Doxycycline monohydrate twice daily INITIATED DAY OF CHALLENGE</td>
<td>105 mg/kg</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>Distilled water twice daily INITIATED DAY OF CHALLENGE</td>
<td>50 µl</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>Doxycycline monohydrate twice daily INITIATED DAY OF CHALLENGE</td>
<td>105 mg/kg</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>Distilled water twice daily INITIATED DAY OF CHALLENGE</td>
<td>50 µl</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

*Not challenged with *C. burnetii*
The mice were observed twice daily and clinical signs of ruffled fur, hunched appearance, eyes shut, limited mobility and immobility were recorded with a corresponding score given to each mouse (Table 7). This study was completed at Dstl Porton Down where the humane end point was reached if the clinical signs score equated to a score of 6 or more. Weights were recorded daily. In groups 1, 3, 5 and 7, there were 5 mice terminally anaesthetised at completion of the antibiotic treatment and the remaining 10 mice in each group were terminally anaesthetised at completion of the study (day 35) (Table 8). Group 9 contained 5 mice treated with 14 days of water commenced at day 5 post challenge and culled at completion of study (day 35). They provided the positive control group for the treatment groups culled at the end of the study. All remaining groups were culled at completion of therapy. Blood was removed by cardiac puncture along with any urine produced by the mouse and the brain, lungs, spleen, a portion of adipose tissue, testes, and femur were aseptically dissected. All tissues were weighed. One of the lungs and approximately half of the spleen were placed in 10% neutral buffered formalin (NBF) and sent to the pathology department at PHE Porton for processing to paraffin, sectioning and staining with haematoxylin and eosin (H&E). The brain, remaining lung and spleen, and adipose tissue were homogenised through a 40 µm sieve (Falcon®) using a 10ml syringe plunger into 1ml of PBS. The homogenate was stored in 2ml Starstedt tubes at -80ºC for future processing. The testes were placed in the 40 µm sieve above 1ml of PBS and gently dissected using blunt scissors to allow the spermatozoa to pass into the PBS. One hundred microliters of this solution was placed in a Starstedt and were inactivated with a final concentration 4% PFA for 40 hours at 4ºC. The remaining testes were homogenised through the 40 µm sieve (Falcon®) into the PBS and the homogenate stored at -80ºC as above. The muscle was stripped off the femur to allow the bone to be visualised. The bone was cut at the hip and knee joints allowing visualisation of the bone marrow. One ml of PBS was injected through the bone marrow using a 2 mm needle and the marrow was collected in a sieve and homogenised into the remaining PBS. This homogenate was stored at -80ºC for future analysis. Two x 100 µl of blood from the cardiac puncture was frozen as whole blood for PCR and viable counts, the remainder was spun down in a microcentrifuge and frozen as plasma. Two groups of 5 mice were treated with antibiotic or water for 5 days without exposure to C. burnetii to investigate the immunological effects of doxycycline monohydrate alone.
2.5.6.1. Spermatozoa imaging

200 µl of permfix buffer (BD) was added to sperm samples and incubated for 20 mins at room temperature. Two microlitres of monoclonal anti-cox antibody (BBI™Detection) in 100 µl permfix buffer was added to each sample and incubated for 1hr at room temp. Samples were washed in 1 ml PBS, spun at 200 RPM. Supernatant was removed and a primary antibody, anti C. 48burnetiiid-LPS antibody and secondary antibody anti-mouse IgG conjugated to FITC (Sigma) was added at 1:200 dilution. Images were reviewed on the confocal microscope (Carl Zeiss, LSM 710).

2.5.6.2. Cytokine analysis

To investigate the cytokine response to C. burnetii in the different treatment groups 26 different cytokines were analysed using a 26-plex ProcartaPlex™ multiplex immunoassay kit (ThermoFisher EpXR260-26088-901). The 26 cytokines being tested for included; IFN-gamma, TNF-alpha, IL-1 beta, IL-10, IL12p70, IL-13, IL-17A, IL-18, IL-2, IL-22, IL-23, IL-4, IL-5, IL-6, IL-9, Eotaxin, GM-CSF, Gro-alpha/KC, IP-10, MCP-1, MCP-3, MIP-1alpha, MIP1-beta, MIP-2 and RANTES. The tests were carried out in accordance with manufacturer’s instructions. In summary; a serial dilution of the antigen standards was prepared, the magnetic capture beads were added to the 96 well round bottomed plate, mouse serum which had previously been stored at -80°C or antigen standards were added to Universal Assay Buffer and added to the plate, a plate sealer was applied along with a black microplate lid and shaken at 500 rpm at room temperature for 60 minutes. Plates were washed 3 times and Detection Antibody Mixture and plate seals applied, and again incubated at room temperature for 30 minutes at 500 rpm. R-Phycoerythrin (SAPE) was added to each well, plate seals applied and incubated at room temperature for 30 minutes at 500 rpm. The plates were washed 3 times and then C. burnetii was fixed using 4% paraformaldehyde which was added to each well for 40 hours at 4 °C which differs from the manufacturer’s instructions. Reading buffer was added to each well, plate sealers applied and incubated at room temperature for 5 minutes at 500 rpm. The plates were then run on the Luminex™ machine and analysed using the Luminex™ software. Luminex™ uses magnetic beads to detect and quantify multiple protein targets simultaneously. The mixture of beads have differing fluorescence profiles allowing the cytokines to be individually interrogated.
2.5.6.3. Serology

Serology titres were quantified using an Enzyme Linked Immunosorbent Assay (ELISA) on all mice culled at the end of the study (day 35). Coxevac (inactive veterinary Coxella burnetii vaccine, European Medicines Agency) was used for the antigen to coat the plate. The vaccine was used as a 1:2 dilution in Carbonate coating buffer solution (Sigma). The 96 well plate was coated with 100 µl of antigen solution which was approximately 1 x 10^8 bacteria/ml. Plates were incubated overnight in the fridge at 4°C. One hundred microlitres of skimmed milk (2% skimmed milk powder (Sigma) in PBS with 0.5% Tween (Sigma)) was added as the blocking agent to prevent non-specific binding of the antibodies and the plates incubated at 37°C for one hour, then washed 3 times with 200µl of PBS tween. One hundred microlitres of sera were added to the wells at a 1:100 dilution whilst the negative control sera was added at a 1:50 dilution. 1:5 dilutions of the sera were carried out to allow quantification of the serological response from a 1:100 to a 1:19531250 dilution. The plates were incubated at 37°C for 2 hours, then washed 3 times before the secondary antibody, mouse anti IgG horseradish peroxidase (HRP) (Sigma) was added. The mouse HRP was diluted in skimmed milk to a 1:2000 dilution and 100 µl added to each well. The plates were incubated at 37°C for an hour before being washed 3 times and 100 µl of 3,3’,5,5’-Tetramethylbenzidine (TMB) ELISA substrate (Sigma) added to each well to detect the mouse HRP. TMB and HRP react to produce a blue by-product. Once the colour change has progressed a stop solution (0.16M sulfuric acid) (Sigma) was added which changes the solution to a yellow colour and stabilises any further colour change. The intensity of the colour change was read on a plate reader at an absorbance maximum of 450 nm. Each mouse sera was completed in duplicate. Standards of non-exposed mice to C. burnetii were carried out in duplicate on each plate. The mean of these values + 2 standard deviations was recorded. An experimental serum was considered positive if greater than the mean + 2SD of the negative sera. The results were recorded as the highest titre that the sera remained positive for.

2.5.6.4. Splenocyte stimulation

Splenic tissue was minced gently using a pair of blunt ended scissors aseptically. The tissue was then stroked through a 40 µm sieve into 1 ml of sterile PBS.
Splenocytes were stimulated with either concanavalin A (ConA, Sigma-Aldrich) at a concentration of 5 µg/ml, Phase 2 and Phase 1 *C. burnetii* at a concentration of 1 x 10^6 bacteria/ml, or media alone as the negative control. Twenty-five microliters per well of the splenocytes was added to 175µl of stimulants in L15 with 10% FCS and incubated at 37°C for 24 hours. One hundred microliters of the supernatant was removed and frozen at -80°C for future processing.

The IFN-γ production of the stimulated and unstimulated splenocytes was measured by ELISA (MABTEC, 3321-1H-6). The test was carried out within manufacturer’s instructions. Pre-coated plates with capture antibody were used. The samples and standard dilutions were added to the wells and captured by the bound antibodies. A biotinylated detection antibody was added to the wells to enable detection of the captured protein. Streptavidin conjugated with HRP was added to the wells to bind the biotinylated antibody and a colorimetric substrate added to the wells to form a coloured solution. The absorbance was measured on an ELISA reader to quantify the IFN-γ response by the splenocytes.

2.5.7. AJ mouse antibiotic efficacy study 4

The aim of AJ mouse efficacy study 4 was to investigate the effectiveness of doxycycline hyclate versus doxycycline monohydrate commenced at different timepoints from point of *C. burnetii* challenge. Groups of 6 mice were challenged by an aerosol challenge with a mean retained dose of 2.9 x 10^4 PCR-CFU of *C. burnetii*. Treatment was with doxycycline monohydrate 105 mg/kg twice daily orally or doxycycline hyclate 105 mg/kg twice daily orally for a total of 14 days post challenge starting 1 day prior to challenge or 2, 5 of 10 days post challenge. The 1 day prior to challenge group represented the use of antibiotics as prophylaxis, 2 days post challenge represented treatment early in the incubation phase, day 5 post challenge represented treatment initiated late in the incubation phase or at time of symptom onset, and day 10 post challenge represented treatment initiated late following recovery from acute infection. Mice with treatment initiated pre challenge received a total of 16 days of antibiotics, whereas the groups receiving antibiotics post challenge received a total of 14 days.

The mice were observed twice daily and clinical signs of ruffled fur, hunched appearance, eyes shut, limited mobility and immobility were recorded with a
corresponding score given to each mouse (Table 7). This study was completed at Dstl Porton Down where the humane end point was reached if the clinical signs score equated to a score of 6 or more. Weights were recorded daily. All mice were culled 14 days after completion of antibiotic therapy. A water control group received 16 days of water 50 µl twice daily initiated 1 day pre challenge were culled at day 37 as a positive control for all of the antibiotic treatment groups. A group of 6 mice were treated with water 50 µl twice daily for 16 days but without C. burnetii challenge to investigate the effects of daily manipulation with oral therapy on mice without bacterial challenge (Table 9).
Table 9: Study plan for AJ mouse antibiotic efficacy study 4. Fifty-four mice were challenged via an aerosol challenge with a mean retained dose of $2.9 \times 10^4$ PCR-CFU of Phase 1 *C. burnetii* and given doxycycline monohydrate, doxycycline hyclate or water (DiH$_2$O) either 24 hours prior to challenge, or 2, 5 or 10 days post challenge. Mice received 16 days therapy if treatment was initiated pre challenge and 14 days of therapy if treatment was initiated post challenge.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment Regimen</th>
<th>Antibiotic dose</th>
<th>Length of Treatment (days)</th>
<th>Day euthanised</th>
<th>No of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Doxycycline monohydrate twice daily initiated 24h PRE CHALLENGE</td>
<td>105 mg/kg</td>
<td>16</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Doxycycline hyclate twice daily initiated 24h PRE CHALLENGE</td>
<td>105 mg/kg</td>
<td>16</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Distilled water twice daily initiated 24h PRE CHALLENGE</td>
<td>50 µl</td>
<td>16</td>
<td>37</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Doxycycline monohydrate twice daily initiated 2 days POST CHALLENGE</td>
<td>105 mg/kg</td>
<td>14</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>Doxycycline hyclate twice daily initiated 2 days POST CHALLENGE</td>
<td>105 mg/kg</td>
<td>14</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>Doxycycline monohydrate twice daily initiated 5 days POST CHALLENGE</td>
<td>105 mg/kg</td>
<td>14</td>
<td>32</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>Doxycycline hyclate twice daily initiated 5 days POST CHALLENGE</td>
<td>105 mg/kg</td>
<td>14</td>
<td>32</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>Doxycycline monohydrate twice daily initiated 10 days POST CHALLENGE</td>
<td>105 mg/kg</td>
<td>14</td>
<td>37</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>Doxycycline hyclate twice daily initiated 10 days POST CHALLENGE</td>
<td>105 mg/kg</td>
<td>14</td>
<td>37</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>Distilled water twice daily No exposure to <em>C. burnetii</em></td>
<td>50 µl</td>
<td>16</td>
<td>37</td>
<td>6</td>
</tr>
</tbody>
</table>

Mice were terminally anaesthetised 14 days after completion of antibiotics or at the end of the study (day 37) in the water control groups and blood removed by cardiac
puncture. Lungs and spleen were aseptically dissected, weighed and processed as per study 3 for bacteriology and splenocyte stimulation. The splenocyte stimulation assay was prepared as per section 2.3.6.4. in Study 3, but concentrations of $1 \times 10^6$ bacteria/ml, $1 \times 10^7$ bacteria/ml and $1 \times 10^8$ bacteria/ml were used for stimulation of Phase 1 *C. burnetii*.

2.6. Statistical analysis of *in vivo* studies

All statistical analyses were performed using Graphpad Prism v7. Kaplan-Meier survival curves for the *G. mellonella* data were analysed using a Log-rank (Mantel-Cox) test. For mouse study 1, 2, 3 and 4, weight loss data was compared to controls using a two-way repeated measures ANOVA and Tukey's multiple comparisons test. Organ weight was analysed as a percentage of the total body weight. In studies 1 and 2, organ bacterial burden (RT-PCR) data was transformed by $\log_{10}$ and then analysed by a two-way repeated measures ANOVA.

Due to the number of results that did not reach the limit of detection (LOD) for bacterial burden by viable counts and RT-PCR in study 3 the data could not be analysed using a parametric test. Therefore, values that did not reach the LOD were entered as 1 and the non-parametric rank tests, Kruskall-Wallis was performed with Dunn's multiple comparisons to assess for statistically significant differences. Kruskall-Wallis with Dunn's multiple comparisons were also used to analyse the cytokine results. Statistical significance was indicated as follows, *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$.

2.7. Cohort Study design

2.7.1. Population

A retrospective cohort study of the 86 military patients seen at the UK Role 4 Military Infectious Disease & Tropical Medicine clinic at Birmingham Heartlands Hospital following an episode of acute Q fever from 2007 to 2015 was performed.

2.7.2. Variables selected for data collection

Having reviewed previous outbreak reports and Q fever review articles, it was decided to capture a breadth of epidemiological, clinical and laboratory variables for future
analysis in order to investigate whether there were any predictors of development from acute Q fever (AQF) to Q fever fatigue syndrome (QFS).

The clinical outcomes of chronic Q fever (CQF) and QFS were recorded using data from both hospital and primary healthcare records. These outcomes are defined in section 2.8.2.1 The predictive variables selected for collection included; gender, age, rank, military trade choice, date of symptom onset, hospital length of stay, smoking history, observations when first seen by a healthcare professional, symptoms as first reported by the patient, serial bloods during the acute illness and serological titres over the course of the 2 year follow up in clinic. Notes were reviewed for antibiotic prescriptions and number of days after symptom onset that the antibiotics were commenced. For full list of predictive variables collected see Appendix A.

2.7.2.1. Clinical outcome definition

The endpoint of interest in this study is the clinical outcome for the patient following acute Q fever. For the purposes of the study there are 4 outcomes;

1. Resolved AQF with no long term sequelae
2. QFS
3. CQF
4. CQF & QFS

The main outcome of interest in this study is QFS. The definition of QFS has been adapted from the Dutch QFS guidelines (93). In our military Q fever cohort study QFS was defined as: severe fatigue causing significant disability in daily life for more than 12 months, with a temporal relationship with acute Q fever and not caused by co-morbidities. As stated by the Dutch, fatigue should be absent before acute Q fever or should have significantly increased since the infection (93).

A diagnosis of CQF and QFS for the military cohort in accordance with the chosen definitions for both of these outcomes was recorded using data from the military notes. Only cases classified as proven CQF using the Dutch Consensus Guidelines in 2012 were recorded as a case of CQF for the military cohort. Cases of QFS were defined as above, based on a modified Dutch criteria.
The results from the data collection for the military cohort were used to record the number of cases of proven, probable and possible CQF in accordance with the Dutch Consensus Guidelines published in 2012 (Table 10) (85) and the French guidelines for CQF endocarditis and CQF vascular infection published in 2012 (Table 11 & 12) (198).

**Table 10:** The Dutch consensus guideline on diagnosis of CQF. Patients were categorised as either proven, probable, possible or no CQF (85).

<table>
<thead>
<tr>
<th><strong>Proven chronic Q fever</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Positive <em>C. burnetii</em> PCR in blood or tissue OR</td>
</tr>
<tr>
<td>2. IFA ≥ 1:1024 for <em>C. burnetii</em> phase I IgG AND</td>
</tr>
<tr>
<td>- Definitive endocarditis according to the modified Duke criteria</td>
</tr>
<tr>
<td>- OR</td>
</tr>
<tr>
<td>- proven large vessel or prosthetic infection by imaging studies (<em>18</em>FDG-PET, CT, MRI or AUS)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Probable chronic Q fever</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>IFA ≥ 1:1024 for <em>C. burnetii</em> phase I IgG AND one or more of the following criteria:</td>
</tr>
<tr>
<td>- Valvulopathy not meeting the major criteria of the modified Duke criteria</td>
</tr>
<tr>
<td>- Known aneurysm and/or vascular or cardiac valve prosthesis without signs of infection by means of TEE/TTE, <em>18</em>FDG-PET, CT, MRI or abdominal Doppler ultrasound</td>
</tr>
<tr>
<td>- Suspected osteomyelitis or hepatitis as manifestation of chronic Q fever</td>
</tr>
<tr>
<td>- Pregnancy</td>
</tr>
<tr>
<td>- Symptoms and signs of chronic infection. Such as fever, weight loss and night sweats, hepatosplenomegaly, persistent raised ESR and CRP</td>
</tr>
<tr>
<td>- Granulomatous tissue inflammation, proven by histological examination</td>
</tr>
<tr>
<td>- Immunocompromised state</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Possible chronic Q fever</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>IFA ≥ 1:1024 for <em>C. burnetii</em> phase I IgG without manifestation meeting the criteria for proven probable chronic Q fever</td>
</tr>
</tbody>
</table>
Table 11: The French definition of CQF endocarditis. IE = Infectious Endocarditis(198)

<table>
<thead>
<tr>
<th>A. Definite criterion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive culture, PCR, or immunohistochemistry of a cardiac valve.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Major criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbiology: positive culture or PCR of the blood or an emboli or serology with IgG1 antibodies ≥6400</td>
</tr>
<tr>
<td>Evidence of endocardial involvement:</td>
</tr>
<tr>
<td>Echocardiogram positive for IE: oscillating intra-cardiac mass on valve or supporting structures, in the path of regurgitant jets, or on implanted material in the absence of an alternative anatomic explanation; or abscess; or new partial dehiscence of prosthetic valve; or new valvular regurgitation (worsening or changing of pre-existing murmur not sufficient).</td>
</tr>
<tr>
<td>Pet-scan showing a specific valve fixation and mycotic aneurysm.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. Minor criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predisposing heart condition (known or found on echocardiography)</td>
</tr>
<tr>
<td>Fever, temperature &gt; 38 °c.</td>
</tr>
<tr>
<td>Vascular phenomena, major arterial emboli, septic pulmonary infarcts, mycotic aneurysm (seen at Pet-scan), intracranial haemorrhage, conjunctival haemorrhages, and Janeway's lesions.</td>
</tr>
<tr>
<td>Immunologic phenomena: glomerulonephritis, Osler’s nodes, Roth’s spots, or rheumatoid factor.</td>
</tr>
<tr>
<td>Serological evidence: PI IgG antibodies ≥800 &lt;6400</td>
</tr>
</tbody>
</table>

**Diagnosis definite**
1. 1A criterion
2. 2B criteria
3. 1B criterion, and 3C criteria (including 1 microbiology evidence, and cardiac predisposition)

**Diagnosis possible**
1. 1B criterion, 2C criteria (including 1 microbiology evidence, and cardiac predisposition).
2. 3C criteria (including positive serology, and cardiac predisposition).
Table 12: The French criteria for diagnosis of CQF vascular infection (198)

<table>
<thead>
<tr>
<th>A. Definite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive culture, PCR or immunohistochemistry of arterial samples (prosthesis or aneurysm) or a periarterial abscess or a spondylodiscitis linked to the aorta.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Major criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbiology: Positive culture, PCR or the blood or emboli, or serology with IgG antibodies ≥ 6400.</td>
</tr>
<tr>
<td>Evidence of vascular involvement:</td>
</tr>
<tr>
<td>CT-scan: aneurysm or vascular prosthesis + periarterial abscess, fistula or spondylodiscitis.</td>
</tr>
<tr>
<td>Pet-scan specific fixation on an aneurysm or vascular prosthesis.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. Minor criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serological IgG ≥ 800 &lt; 6400</td>
</tr>
<tr>
<td>Fever, temperature ≥ 38 ºc</td>
</tr>
<tr>
<td>Emboli</td>
</tr>
<tr>
<td>Underlying vascular predisposition (aneurysm or vascular prosthesis)</td>
</tr>
</tbody>
</table>

**Diagnosis definite**

1. 1A criterion
2. 2B criteria
3. 1B criterion and 2C criteria (including microbiology and vascular predisposition)

**Diagnosis possible**

Vascular predisposition, serological evidence and fever or emboli

2.7.3. Diagnostic laboratory testing

Blood for confirmation and monitoring of Q fever was sent as serum or whole blood to the Rare & Imported Pathogens Laboratory (RIPL) at PHE Porton where it was stored at -80°C for future processing. The whole blood was processed for PCR if requested. Serum was tested initially for *C. burnetii* by ELISA for PII IgM and IgG (Vирion Serion, *Coxiella burnetii*, PII IgM ELISA item number ESR 1312G, PII IgG ELISA item number ESR 1312M), and if positive or equivocal, confirmatory testing by immunofluorescence assay (IFA) for PII IgM, PII IgG, PI IgA and PI IgG was performed (Vircell microbiologists, *Coxiella burnetii* IFA, PI + PII IgG, IgM, IgG/M/A, reference number PCOBU I+II).
2.7.4. Data collection

Clinical notes were accessed from the Military Role 1 (Primary Health Care records whilst in the UK and when deployed overseas), Military Role 3 (Deployed Military Field Hospital at Camp Bastion, Afghanistan) and the Military Role 4 (Birmingham Heartlands Hospital, UK or The Princess Mary’s Hospital, RAF Akrotiri, Cyprus). Notes from the Role 3 in Afghanistan and the Role 4 in Cyprus were accessed from a digitised system held at MOD Shoeburyness, Essex. Patient notes were also reviewed from other NHS trusts where available if the patient had accessed alternative healthcare sites.

The independent variables selected for data collection were chosen following a literature review of the clinical presentation and possible predictors of AQF/CQF and QFS cases. The variables selected are listed in Appendix A.

2.7.5. Ethical considerations

Approval was gained to access patient notes for the purposes of this study from the Military Caldicott Guardian, Brigadier Williamson in 2016. The study was approved through the Defence Science and Technology Scientific Advisory Committee in 2016. It was decided at this meeting that the study did not require formal ethical approval since it was a retrospective epidemiological study that under MODREC guidance would be regarded as clinical audit. All patient identifiable data has been removed from the database and been replaced with a unique code in accordance with Caldicott guidelines.

2.7.6. Statistical analysis

Data was stored for analysis on a database using SPSS v24 (IBM). Descriptive analysis of the cohort data was performed, and graphs produced on SPSS v24. To investigate the presence of a clinical predictor for progression from acute disease to QFS the dataset was initially analysed using univariate binomial logistic regression with the variables of interest. Any variables approaching significance (p <0.1) were entered into a multivariate binomial logistic regression model to control for confounding factors. Only patients with a data entry for each variable entered into the multivariate model were analysed.
GraphPad Prism 7.0a was used to analyse the serology titres. The graphical presentation of the serology titres is from GraphPad Prism 7.0a. To test for the hypothesis that a difference existed between the serology titres in patients with acute resolved disease versus QFS the mean titres of between 1 and 12 months were compared via unpaired t-test if the data was parametric or Mann-Whitney U test if the data was non-parametric. The period of 1 to 12 months was selected because it usually takes 2-4 weeks for the serology to become positive and the definition of QFS is fatigue lasting longer than 12 months. All data was initially non-parametric in distribution. In an attempt to transform the data into a parametric distribution the log transform of the data and the square root were taken. Finally, the cube root of the data was taken, and this transformed the P2 serology into parametric data for analysis. The P1 serology remained non-parametric throughout.
Chapter 3: *In vitro* evaluation of antibiotic efficacy against *C. burnetii*

3.1. Introduction

In order to reduce the use of animals through *in vivo* investigation into antibiotic efficacy against *C. burnetii* it was important to complete as much preliminary antibiotic efficacy work against *C. burnetii* in an *in vitro* or *in silico* (199, 200) model as possible. The antibiotics selected for the *in vitro* studies included doxycycline, ciprofloxacin, levofloxacin, moxifloxacin and co-trimoxazole. These antibiotics were selected because doxycycline is first line treatment for Q fever (106) and the fluoroquinolones (ciprofloxacin, levofloxacin and moxifloxacin) have been shown by a retrospective analysis during the Netherlands outbreak to lower the rate of hospitalisation compared with patients receiving a beta-lactam or azithromycin (109). In addition, ciprofloxacin and doxycycline are readily available to the military on deployment for anthrax and plague treatment. Levofloxacin is frequently used for patients presenting with atypical pneumonias and is now licensed for treatment of inhalational anthrax (201). Therefore, due to the military experience and access to quinolones when deployed, utilisation of these drugs as prophylaxis against Q fever would be a possibility if proven to be beneficial. Co-trimoxazole was selected as this is the recommended treatment for pregnant patients.

Prior to 2009 assessing the *in vitro* efficacy of antibiotics against *C. burnetii* required propagation of the bacteria in embryonated eggs (152, 153), or in cell based cultures (13, 154-156). Following the development of an axenic (host cell-free) culture method in 2009 the growth of this intracellular bacteria became easier to perform (168). It has taken 3 decades to develop an axenic media capable of sustaining the metabolic function and growth of *C. burnetii*. ACCM-2 is capable of supporting 4-5 logs of growth over 7 days (168), and ACCM-2 solid agarose supports growth and allows enumeration of the bacteria (168). Using ACCM-2 media the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were carried out against a range of antibiotics using a broth dilution method (202).

*C. burnetii* is an intracellular organism, preferentially infecting mononuclear phagocytes (203). In order to understand how the intracellular environment in
combination with the antibiotic may affect the survival of C. burnetii it was decided to assess antibiotic efficacy against C. burnetii in a THP-1 cell assay. To date work on C. burnetii within the THP-1 model has been investigating the mode of infection and survival within the intracellular milieu (155,204,205). No antibiotic efficacy work has been completed within this human myelomonocytic cell line for C. burnetii.

3.2. Determination of Minimum Inhibitory Concentrations (MICs) using the broth dilution method and Epsilometer tests (E-tests®), and the Minimum Bactericidal Concentrations (MBCs)

In order to determine the antibiotic efficacy against C. burnetii NMII and NMI, the MICs were established using axenic media for a range of antibiotics (doxycycline hyclate, doxycycline monohydrate, ciprofloxacin, levofloxacin, moxifloxacin and co-trimoxazole) using broth dilution and E-test® methods. The MBCs were established via broth dilution in axenic media. All results were repeated in triplicate and 2 independent observers reviewed the E-tests® (Table 1).

Table 1: Summary of the MICs for doxycycline hyclate, doxycycline monohydrate, levofloxacin, moxifloxacin, ciprofloxacin and co-trimoxazole against phase II C. burnetii (NMII) and phase I C. burnetii (NMI). The MICs and E-tests® were performed in triplicate. (NP = not performed)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC NMII (µg/ml) (broth dilution)</th>
<th>E-tests® NMII (µg/ml)</th>
<th>MIC NMI (µg/ml) (broth dilution)</th>
<th>MBC NMI (µg/ml) (broth dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline hyclate</td>
<td>0.04 - 0.16</td>
<td>0.16 – 0.23</td>
<td>0.01 – 0.04</td>
<td>8</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1- 2</td>
<td>0.064 - 1.5</td>
<td>1 - 4</td>
<td>16</td>
</tr>
<tr>
<td>Doxycycline monohydrate</td>
<td>NP</td>
<td>NP</td>
<td>0.03 - 0.04</td>
<td>16</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.5 - 4</td>
<td>0.064 - 4</td>
<td>0.5 - 1</td>
<td>&gt;16</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>1 - 4</td>
<td>0.19 - 1</td>
<td>NP</td>
<td>&gt;16</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>16 - 32</td>
<td>32</td>
<td>8- 32</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>
3.2.1. Determination of the MICs for phase II C. burnetii (NMII) via broth dilution

The results for the NMII MICs using the broth dilution method have been published in the International Journal of Antimicrobial Agents (206).

The antibiotic efficacy of doxycycline hyclate, ciprofloxacin, levofloxacin, moxifloxacin and co-trimoxazole was assessed. Doxycycline hyclate exhibited the lowest MIC (0.04 – 0.16 µg/ml) and co-trimoxazole the highest MIC (16-32 µg/ml). The MICs for levofloxacin ranged from 0.5 - 4 µg/ml, 1-2 µg/ml for ciprofloxacin and 1 – 4 µg/ml for moxifloxacin (Table 1).

3.2.2. Determination of the MICs for phase II C. burnetii (NMII) via E-tests®

The results for the NMII MICs using the E-tests® method have been published in the International Journal of Antimicrobial Agents (206).

The antibiotic efficacy of doxycycline hyclate, ciprofloxacin, levofloxacin, moxifloxacin and co-trimoxazole were assessed. The results show good, distinct zones of inhibition (Figure 1). The results shown in Table 1 correlate well with the broth dilution method.

![Figure 1: Representative image of E-tests for C. burnetii NMII. Red arrow indicates MIC. E-tests were performed in triplicate and read by two independent individuals. A – Doxycycline (0.032 µg/ml) B – Ciprofloxacin (0.25 µg/ml) C – Levofloxacin (0.064 µg/ml) D – Moxifloxacin (0.125 µg/ml) E – Co-trimoxazole (32 µg/ml).](image)

3.2.3. Determination of the MICs and MBCs for phase I C. burnetii (NMI)

The antibiotic efficacy of doxycycline hyclate, doxycycline monohydrate, levofloxacin, ciprofloxacin and co-trimoxazole against C. burnetii NMI were assessed by means of the same methodology as used for NMII for comparison.
3.2.3.1. Determination of the MICs and MBCs for phase I C. burnetii (NMI) via broth dilution method and culture

Doxycycline hyclate and doxycycline monohydrate exhibited the lowest MIC against NMI (0.01 - 0.04 µg/ml and 0.03 - 0.04 µg/ml respectively), and co-trimoxazole the highest MIC (8 - 32 µg/ml). The MIC for levofloxacin was 0.5 - 1 µg/ml and for ciprofloxacin was 1 - 4 µg/ml (Table 1). These results are consistent with the MICs established against NMII. The broth was plated out at the neat concentration and the MBCs, as defined by no visible bacterial growth when plated out after 7 days were 8 µg/ml for doxycycline hyclate and 16 µg/ml for doxycycline monohydrate, the remaining antibiotics had an MBC greater than the highest concentration tested for the MICs (levofloxacin > 16 µg/ml, ciprofloxacin > 8 µg/ml and co-trimoxazole > 128 µg/ml) (Table 1).

3.3. Assessment of growth inhibition and bactericidal effects of the antibiotics under investigation against phase I C. burnetii (NMI)

The growth inhibition of C. burnetii in the presence of a range of concentrations of doxycycline monohydrate, doxycycline hyclate, levofloxacin, ciprofloxacin and co-trimoxazole was assessed. Viable bacteria were enumerated at day 0 and day 7 to assess the growth kinetics in the presence of the antibiotics under investigation.

A significant inhibition of bacterial growth compared to the bacterial count after 7 days in the presence of no antibiotics (0 µg/ml) was assessed using a One-Way ANOVA with Dunnett’s multiple comparisons. The concentrations of antibiotics that significantly inhibit growth are shown in the legend of the graphs in Figure 2. Antibiotic concentrations below the blue dashed line significantly inhibit bacterial growth compared to the bacterial count at 7 days in the absence of antibiotics. The blue line is not the minimum concentration of antibiotic required to achieve inhibition it reflects the minimum concentration tested in these studies. Doxycycline hyclate and monohydrate both had significant inhibitory effects at concentrations of > 0.01 µg/ml (Figure 2a, b), ciprofloxacin at concentrations > 0.5 µg/ml (Figure 2c), levofloxacin at concentrations of > 0.5 µg/ml (Figure 2d), and co-trimoxazole at concentrations > 1 µg/ml (Figure 2e).

The definition of limit of bactericidal effect has been taken from the Clinical and Laboratory Standards Institute (CLSI) as -2 log from the bacterial concentration at
time 0 hours(193), this is shown on the graphs in Figure 2 as a red dashed line. Concentrations below the red dashed line are considered to be bactericidal using this definition. In broth after 7 days, doxycycline hyclate had the lowest bactericidal concentration of > 0.04 µg/ml (Figure 2a). Doxycycline monohydrate was bactericidal at concentrations > 1 µg/ml (Figure 2b), ciprofloxacin at > 2 µg/ml (Figure 2c), levofloxacin at > 2 µg/ml (Figure 2d), and co-trimoxazole at > 32 µg/ml (Figure 2e).
a. Doxycycline hyclate broth assay

- 16 µg/ml (p < 0.001)
- 8 µg/ml (p < 0.001)
- 4 µg/ml (p < 0.001)
- 2 µg/ml (p < 0.001)
- 1 µg/ml (p < 0.001)
- 0.5 µg/ml (p < 0.001)
- 0.25 µg/ml (p < 0.001)
- 0.16 µg/ml (p < 0.001)
- 0.08 µg/ml (p < 0.001)
- 0.04 µg/ml (p < 0.001)
- 0.02 µg/ml (p < 0.001)
- 0.01 µg/ml (p < 0.001)
- 0 µg/ml

b. Doxycycline monohydrate broth assay

- 16 µg/ml (p < 0.001)
- 8 µg/ml (p < 0.001)
- 4 µg/ml (p < 0.001)
- 2 µg/ml (p < 0.001)
- 1 µg/ml (p < 0.001)
- 0.5 µg/ml (p < 0.001)
- 0.25 µg/ml (p < 0.001)
- 0.16 µg/ml (p < 0.001)
- 0.08 µg/ml (p < 0.001)
- 0.04 µg/ml (p < 0.001)
- 0.02 µg/ml (p < 0.001)
- 0.01 µg/ml (p < 0.001)
- 0 µg/ml
Ciprofloxacin broth assay

- 0 µg/ml
- 0.25 µg/ml
- 0.5 µg/ml (p < 0.05)
- 1 µg/ml (p < 0.001)
- 2 µg/ml (p < 0.001)
- 4µg/ml (p < 0.001)
- 8 µg/ml (p < 0.001)

Levofloxacin broth assay

- 0.08 µg/ml
- 0.16 µg/ml
- 0.04 µg/ml
- 0.01 µg/ml
- 0.02 µg/ml
- 0.01 µg/ml
- 0 µg/ml
- 0.5 µg/ml (p < 0.01)
- 1 µg/ml (p < 0.05)
- 2 µg/ml (p < 0.001)
- 4 µg/ml (p < 0.001)
- 8 µg/ml (p < 0.001)
- 16 µg/ml (p < 0.001)
**Figure 2:** Growth of phase I *C. burnetii* (NMI) in ACCM-2 broth in the presence the antibiotics under investigation at differing concentrations. (a) Doxycycline hyclate. (b) Doxycycline monohydrate. (c) Co-trimoxazole. (d) Ciprofloxacin. (e) Levofloxacin. The figures shown represent the mean +/- SEM for 2 experimental repeats. The blue dashed line represents the antibiotic concentration below which a significant inhibition to growth was demonstrated compared to the bacterial count after 7 days in the presence of no antibiotics (0 µg/ml). Any antimicrobial concentrations below the blue line had a significant inhibitory effect as determined by a One-Way ANOVA with Dunnett’s test for multiple comparisons of the log transformed data. The significance values are shown in the graph legends. The red dashed line shows the limit of bactericidal effect (-2 log from the bacterial concentration at time 0h in accordance to Clinical and Laboratory Standards Institute (CLSI)). Antimicrobial concentrations shown below the red dashed line are bactericidal.
3.4. Antibiotic stability: Well Diffusion Assay

ACCM-2 is acidic (pH 4.75) this is to replicate the environment within the intracellular phagolysosome where C. burnetii replicates. The stability of the antibiotics in this acidic media were evaluated using a well diffusion assay against Escherichia coli (levofloxacin, moxifloxacin, ciprofloxacin and co-trimoxazole) or Staphylococcus aureus (doxycycline hyclate and co-trimoxazole).

The zones of inhibition around each well were measured using callipers for each antibiotic at concentrations ranging 0.5 – 32 µg/ml, extended up to 128 µg/ml for co-trimoxazole after 24 hours of incubation (Figure 3).

**Figure 3:** Well diffusion standards for doxycycline hyclate against Staphylococcus aureus ATCC 25923 at increasing concentrations. The zones of clearance were measured 24 hours after incubation at 37°C (a) 0.5 µg/ml (b) 1 µg/ml (c) 2 µg/ml (d) 4 µg/ml (e) 8 µg/ml (f) 16 µg/ml (g) 32 µg/ml

The zones of inhibition for each antibiotic concentration were graphed in order to make a standard curve for the ACCM-2 stability assay (Figure 4).
Figure 4: The standard curves of zones of inhibition for (a) Doxycycline hyclate (b) Moxifloxacin (c) Levofloxacin (d) Ciprofloxacin and (e) Co-trimoxazole against either *Escherichia coli* or *Staphylococcus aureus* using antibiotic concentrations of 32, 16, 8, 4, 2, 1 and 0.5 µg/ml diluted in PBS. Co-trimoxazole antibiotic concentrations were extended up to 128 µg/ml. The experiments were completed in duplicate and the mean with standard error is represented in these graphs. The zone of clearance on the standard curve when the concentration of antibiotic is 32 µg/ml is indicated on the graph and correlates with the zone of clearance seen in the stability results seen in Figure 4.
In order to ascertain the stability of the antibiotics in ACCM-2, each antibiotic was diluted in ACCM-2 to 32 μg/ml, added to the well, and incubated for 144 hours. Seven days (144 hours) was selected because this is the length of time required for 4-5 logs growth of C. burnetii(168), and therefore the length of time the antibiotics are required to remain stable in ACCM-2 for efficacy studies. The zone of clearance was measured at 24 hour increments to assess the duration of stability in ACCM-2 (Figure 5.). The zone of clearance on these test plates were compared with the zone of clearance at 32 μg/ml on the standard curve for each antibiotic. All antibiotics except co-trimoxazole were active for 7 days in ACCM-2 at 37°C (Figure 6).

**Figure 5:** Well diffusion test plates. Doxycycline hyclate 32 μg/ml in ACCM-2 was tested against Staphylococcus aureus ATCC 25923 at 24 hour time increments and the zone of clearance measured at 24 hours and read against the standard curve to assess the stability of the antibiotic in ACCM-2 over the course of 144 hours. Time course (a) 0 hours (b) 24 hours (c) 48 hours (d) 72 hours (e) 144 hours. Doxycycline hyclate was stable for 144 hours in ACCM-2.
**Figure 6**: Doxycycline hyclate, moxifloxacin, levofloxacin and ciprofloxacin remain stable up to 144 hours in ACCM-2 at 37°C against either *Escherichia coli* or *Staphylococcus aureus* growth on LB agar plates or Mueller Hinton for co-trimoxazole. Co-trimoxazole shows no zone of clearance at any time point against these bacteria.

### 3.5. THP-1 Cell Infection Assay: Phase I *C. burnetii* (NMI)

The THP-1 cell assay is an established cell infection model for phase I *C. burnetii* (data from M.G.Hartley. Dstl Porton Down). *C. burnetii* NMI was incubated with THP-1 cells for 24 hours then intracellular growth monitored for 144 hours. Cells were lysed every 24 hours, and numbers of intracellular bacteria enumerated by viable counts on ACCM-2 agar plates. In the absence of antibiotics, *C. burnetii* replicates intracellularly within THP-1 cells, with a 2.5 log in growth over 144 hours. In establishing the model at Dstl it has been shown that doxycycline hyclate at concentrations of 2 and 8 µg/ml prevent intracellular growth of *C. burnetii* over 144 hours (Figure 7).
Figure 7: THP-1 intracellular infection assay using phase I C. burnetii. The experiment was completed in duplicate and the mean represented with standard error in this graph. Doxycycline at 2 and 8 µg/ml prevent intracellular growth of C. burnetii in this model (M.G.Hartley, Dstl).

THP-1 cells infected with C. burnetii exposed to 8 µg/ml of doxycycline hyclate or non-treated controls were stained with DAPI (Deoxyribonucleic acid stain) and anti C. burnetii LPS-FITC stain (green). The slides were then fixed with 4% paraformaldehyde for 40 hours at 4°C before staining and visualising under the confocal microscope (Figure 8). The representative images in Figure 8 show that doxycycline hyclate at 8 µg/ml reduces the intracellular bacterial burden whilst cell wall integrity is maintained (data from M.G.Hartley, Dstl).
Figure 8. THP-1 cell assay investigating the effects of doxycycline hyclate on the intracellular bacterial burden of *C. burnetii*. Cells were infected with a MOI of 200:1 and treated with doxycycline hyclate 8 μg/ml for 72 hours. The slides were then fixed with 4% paraformaldehyde for 40 hours at 4°C before staining and visualising under the confocal microscope. (a) THP-1 cells treated with 8 μg/ml of doxycycline hyclate, the image is at x100 magnification. (b) THP-1 cells treated with 8 μg/ml doxycycline hyclate, the image is at x40 magnification. (c) THP-1 cells were untreated, the image is at x100 magnification. (d) THP-1 cells were untreated, the image is at x40 magnification. (Blue = DAPI, DNA stain, green = mouse anti *C. burnetii* LPS monoclonal FITC stain) (M.G.Hartley,Dstl).

The growth of intracellular *C. burnetii* in the presence of the antibiotics under investigation at differing concentrations was evaluated over a 72-hour period. The range of antibiotic concentrations was selected from the MIC via broth dilution results (Table 1). A statistically significant inhibition of bacterial growth was
assessed comparing the bacterial growth at 72 hours for each antibiotic concentration in relation to the bacterial growth at 72 hours in the absence of antibiotics using a One-Way ANOVA with Dunnett’s multiple comparisons. When assessing the bactericidal activity of antimicrobial agents the Clinical and Laboratory Standards Institute (CLSI) define a bactericidal effect as a greater than -2 log drop from the bacterial concentration at time 0 hours in the presence of no antibiotics (0 µg/ml) (193). This CLSI definition is for assessing the bactericidal effect in broth medium, but the definition has been applied to the THP-1 cell assay to allow comparison between the efficacy of antibiotics in broth (Figure 2) and in the intracellular environment (Figure 9).

Doxycycline hyclate at concentrations > 0.2 µg/ml significantly inhibited growth in comparison to growth in the absence of antibiotics (Figure 9a. p < 0.01). Doxycycline monohydrate significantly prevented growth at concentrations > 0.01 µg/ml (Figure 9b. p < 0.05). Neither doxycycline preparation displayed bactericidal effects up to 2 µg/ml, and this was shown up to 8 µg/ml for doxycycline hyclate as demonstrated in the original data by M.G. Hartley (Figure 7).

Co-trimoxazole at concentrations of > 2 µg/ml demonstrated significant inhibitory effects of bacterial growth (Figure 9c, p < 0.05), but did not show a bactericidal effect at the concentrations tested (< 128 µg/ml, Figure 9c).

Ciprofloxacin demonstrated a significant inhibition of bacterial growth in the THP-1 assay at concentrations > 0.5 µg/ml (Figure 9d. p < 0.01). A bactericidal effect was seen at > 2 µg/ml (Figure 9d). Levofloxacin displayed a significant inhibition of intracellular bacterial growth at concentrations > 0.16 µg/ml (fig 9e. p = 0.001). In addition, concentrations of > 2 µg/ml demonstrated a bactericidal effect (Figure 9e). All experiments were completed in triplicate, with the exception of co-trimoxazole which was completed in duplicate, and the mean and standard error of the mean (SEM) are shown in Figure 9.
a. Doxycycline hyclate THP-1 cell assay

- 0 µg/ml
- 0.01 µg/ml (p <0.001)
- 0.02 µg/ml (p <0.01)
- 0.1 µg/ml (p <0.01)
- 0.2 µg/ml (p <0.05)
- 2 µg/ml (p <0.05)

b. Doxycycline monohydrate THP-1 cell assay

- 0 µg/ml
- 0.2 µg/ml (p <0.05)
- 2 µg/ml (p <0.05)
- 0.02 µg/ml (p <0.01)
- 0.1 µg/ml (p <0.01)
- 0.01 µg/ml (p <0.001)
Ciprofloxacin THP-1 cell assay

Levofloxacin THP-1 cell assay
Figure 9: Intracellular growth of *C. burnetii* in a THP-1 cell assay in the presence of a range of antibiotics at differing concentrations for 72 hours. The multiplicity of infection (MOI) for bacteria to cell count was 20:1 throughout the replicates. The experiments were completed in triplicate, with the exception of co-trimoxazole which was completed in duplicate, and a mean +/- SEM is represented in this figure. (a) Doxycycline hyclate (0 - 2 μg/ml). (b) Doxycycline monohydrate (0 - 2μg/ml). (c) Co-trimoxazole (0 - 128 μg/ml). (d) Ciprofloxacin (0 - 4μg/ml). (e) Levofloxacin (0 - 16μg/ml). The blue dashed line represents the antibiotic concentration below which a significant inhibition to growth was demonstrated. A statistically significant inhibition of bacterial growth was assessed comparing the bacterial growth at 72 hours for each antibiotic concentration in relation to the bacterial growth at 72 hours in the absence of antibiotics using a One-Way ANOVA with Dunnett’s multiple comparisons. The red dashed line shows the limit of bactericidal effect (the definition is adapted from the bactericidal definition in broth medium of a -2 log from the bacterial count at 0 hours in accordance with Clinical and Laboratory Standards Institute guidelines (CLSI)). Antimicrobial concentrations shown below the red dashed line are bactericidal.
3.6. Discussion

The antibiotic efficacy of doxycycline, levofloxacin, ciprofloxacin and co-trimoxazole against *C. burnetii* NMI and NMII, and the efficacy of moxifloxacin against NMII were assessed in this study. Doxycycline for 2 weeks is the standard treatment for *C. burnetii* in humans (106). However, resistance has started to emerge against this antibiotic (106,113). It is therefore important to assess alternative antibiotic options for patients where doxycycline is contraindicated, cannot be tolerated or who have phenotypic resistance. NMII was initially investigated because it can be used in class 2 Microbiological Safety Cabinets (MSC) and allowed training to be carried out on the bacteria at a lower safety level than NMI which requires a class 3 MSC. In addition, due to the increased ease of working with NMII antibiotic efficacy studies have been carried out using this less virulent form of the bacteria (161). Carrying out *in vitro* efficacy studies against both phases of *C. burnetii* allows a direct comparison between previous published data and the virulent form of the bacteria (NMI) which will be used for future *in vivo* models.

Antibiotic efficacy was assessed in ACCM-2 broth and within a THP-1 cell culture for comparison. It was important to investigate the efficacy in both environments because broth assays are easier and quicker to perform than cell based assays, and they are more reproducible because it is easier to replicate the broth environment than the intracellular environment. Investigations in broth also reduces the variables being tested, allowing a direct assessment of the antimicrobial effect. Whereas in the intracellular environment some of the efficacy seen will be dependent on the ease of uptake of the bacteria and the antibiotic, and their localisation within the cell. The cell also independently exerts a bactericidal effect, with release of reactive oxygen intermediates (ROIs), nitric oxide (NO) and cytokines (207). Therefore, understanding the ability of the antibiotic to inhibit growth of *C. burnetii* independently to the cell is of interest. Which allows the combined effect of antibiotic efficacy, intracellular uptake and localisation of drug and bacteria, and the cellular bactericidal effects to be investigated in cell based assays.

This research has used axenic media for the first time to establish the MICs for first and second line antibiotics (doxycycline hyclate, doxycycline monohydrate, ciprofloxacin, levofloxacin, moxifloxacin and co-trimoxazole) against *C. burnetii* using the conventional methods of broth dilution and antimicrobial gradient method.
(E-tests®). The MIC results for both doxycycline preparations and the fluoroquinolones determined using axenic media in this study were comparable to those derived from shell vial assays and cell culture methods(13,156,157,160,161,164-167,208,209). Furthermore, there was a good correlation between the NMII MICs shown by broth dilution and by E-tests®. Comparable results were also seen for the MICs by broth dilution for NMI and NMII (Table 1). NMII, as previously discussed, is easier to use due to the lower containment level required and as shown can be effectively used to assess antibiotic efficacy in broth.

The intracellular assay chosen for this in vitro work was the human THP-1 myelomonocytic cell line. The THP-1 model of C. burnetii infection is well established(155) but to date had not been used to assess antibiotic efficacy against C. burnetii. It is known that phase I C. burnetii are poorly internalised by macrophages in comparison to phase II bacteria(203). In addition, phase I organisms can survive within the macrophage whereas phase II organisms are eliminated(203). Therefore, only NMI was used for cell infection. The THP-1 model of infection supports 1-2 log growth of NMI over 72 hours (Figure 9). Many different cell lines support bacterial growth and have been used to investigate the mechanism of C. burnetii infection and treatment efficacy. The THP-1 human monocytic leukaemia cell line following PMA stimulation differentiates into the human macrophage which is the primary target of C. burnetii. These macrophages resemble native monocyte derived macrophages keeping the functional aspects of a macrophage such as their role in host defence and as a secretary cell(194). The THP-1 cell model allows the efficacy of the antibiotic alongside the human macrophage immune functions to be investigated which suggests that the results from the THP-1 experiments in this chapter are a good in vitro approximation of human infection.

Doxycycline is in the class of tetracyclines and inhibits bacterial protein synthesis by binding to the 30s ribosomal subunit. Doxycycline hyclate and monohydrate have the lowest MICs of the antibiotics tested in this study (0.01 – 0.04 and 0.03 – 0.04 respectively against NMI). Both doxycycline preparations had enhanced efficacy in broth compared to the THP-1 assay. Using the CLSI definition of bactericidal effect as -2 log drop in counts from time 0h, both preparations had a low bactericidal concentration in broth (Doxycycline hyclate, \( \geq 0.04 \mu g/ml \), and doxycycline monohydrate, \( \geq 0.16 \mu g/ml \)). In the intracellular model doxycycline hyclate was not
bactericidal up to 8 µg/ml and doxycycline monohydrate showed no bactericidal effects up to the highest concentration tested (2 µg/ml). Doxycycline is bacteriostatic against *C. burnetii* and is only bactericidal when combined with hydroxychloroquine in chronic infection(106). Hydroxychloroquine increases the pH in the lysosome which is the postulated mechanism for allowing doxycycline to exert a bactericidal effect(106). The disparity in results seen for this antibiotic between broth and THP-1 demonstrate the negative impact that the acidic intracellular environment has on antibiotic efficacy.

Fluoroquinolones are bactericidal drugs that target 2 enzymes, DNA gyrase and topoisomerase IV which leads to inhibition of DNA replication(210). Mutations in these 2 targets can lead to resistance. In this study ciprofloxacin, levofloxacin and moxifloxacin had comparable MICs against NMII (1-2 µg/ml, 0.5 – 4 µg/ml and 1- 4 µg/ml respectively), therefore due to the experience and access the military have with levofloxacin and ciprofloxacin it was decided to continue *in vitro* studies with NMI on ciprofloxacin and levofloxacin. *C. burnetii* has shown some resistance to ciprofloxacin due to a *gyrA* gene mutation(113) and therefore assessment of a range of antibiotics in this class would be useful in guiding future antibiotic treatment choices. In this study ciprofloxacin and levofloxacin show a bactericidal effect against NMI in broth and the THP-1 cell assay at concentrations > 2 µg/ml. Quinolones accumulate within neutrophils and macrophages(211) and therefore have access to the intracellular location of *C. burnetii*. Levofloxacin and ciprofloxacin have previously been shown to have comparable inhibitory effects against NMI *C. burnetii*(157). No bactericidal effect was seen with levofloxacin up to 4 µg/ml in a shell vial assay(13), but ciprofloxacin was bactericidal in persistently infected L929 fibroblast cells at concentrations > 5 µg/ml(166). It is also worth noting that the *C. burnetii* strain appears to affect the efficacy of this class of antibiotics, with NM being the most susceptible of the strains(166).

When interpreting the results of previous intracellular assays, it appears that the cell type chosen to investigate the efficacy of fluoroquinolones may affect the outcome. Ciprofloxacin shows low accumulation in J774 mouse macrophage cells whereas moxifloxacin shows high intracellular accumulation(212). The difference is due to the presence of the ciprofloxacin efflux transporter, Mrp4(212). No active efflux of fluoroquinolones has been seen in THP-1 cells(212). Intracellular uptake and antibiotic efficacy do not appear to be related in fluoroquinolones(212-214). The levels of ciprofloxacin, moxifloxacin and levofloxacin in human alveolar
macrophages are higher than in serum(212,215). The lungs, in general, are the primary site of infection and therefore accumulation in the alveolar macrophages would be one explanation for the efficacy of quinolones in treatment of Q fever. *In vitro* work completed in this study investigating the efficacy of ciprofloxacin and levofloxacin show similar results, therefore further *in vivo* work will be carried out to ascertain any differences between the two antibiotics.

Co-trimoxazole is a bacteriostatic drug used in the treatment of *C. burnetii* infection during pregnancy(216) and as an alternative if doxycycline is contraindicated(106). Co-trimoxazole is a combination of trimethoprim and sulphamethoxazole which are both folic acid antagonists and block DNA synthesis reducing bacterial replication. No resistance to co-trimoxazole has been reported to date(15). The published MIC for co-trimoxazole against *C. burnetii* determined via RT-PCR (8 μg/ml) (161) and the MIC determined via axenic media in this study (16 – 32 μg/ml, Table 1) suggests that *C. burnetii* has a low susceptibility to this antibiotic. In this study the MIC for co-trimoxazole by broth dilution is difficult to interpret because by well diffusion assay it was shown to be unstable in ACCM-2 over 7 days in contrast to the other antibiotics under investigation. Comparing bacterial growth by culture in broth and in the THP-1 assay shows that all concentrations tested (> 1 μg/ml in broth over 7 days, > 2 μg/ml in THP-1 cells over 72 hours) had a significantly inhibitory effect. Using the CLSI definition of bactericidal, co-trimoxazole was bactericidal at > 32 μg/ml in broth but up to 128 μg/ml was not bactericidal within the intracellular assay. These experiments investigating the efficacy of co-trimoxazole against *C. burnetii* are interesting and demonstrate that despite a high MIC in broth the effectiveness of this antibiotic is on a gradient and there is significant inhibition at lower concentrations. Comparing growth in broth and THP-1 cells it appears that co-trimoxazole may be more effective in the intracellular environment. The apparent clinical efficacy of co-trimoxazole within the intracellular environment could be accounted for by good penetration and activity within the acidic phagolysosome. This finding is consistent with the shell vial assay using P388D1 cells where the MIC for NM *C. burnetii* was found to be 8 μg/ml by immunofluorescence and PCR(161). A study looking at the intracellular versus extracellular efficacy of co-trimoxazole against *Listeria monocytogenes*, another intracellular bacteria, found that there was no correlation between the MICs in extracellular culture versus intracellular growth of bacteria(217). The same study found that the efficacy of co-trimoxazole varied depending on the culture medium used(217). The reduced efficacy of co-trimoxazole in the ACCM-2 broth is likely due to inhibition from the
components of the broth, but this cannot be tested because no other culture medium currently exists that will allow the growth of *C. burnetii* in an antibiotic containing media for 7 days. VCEM media (174) which will allow axenic growth needs to be changed every 48 hours and therefore is not appropriate for this assay. All treatment regimens for acute Q fever (AQF) are bacteriostatic and it is postulated that the acidic intracellular environment may decrease the antibiotic efficacy of these drugs. Although in the case of co-trimoxazole it is known to work well in the acidic intracellular environment against *Brucella* (218).

Clinical experience with co-trimoxazole treatment during pregnancy yields conflicting results. Since 1996 Raoult et al. (219) have recommended the use of co-trimoxazole throughout pregnancy to reduce poor obstetric outcome and the development of chronic Q fever (CQF) in the mother. In 2007 a large obstetric study of 53 patients found that treatment with at least 5 weeks of co-trimoxazole during pregnancy significantly reduced obstetric complications (*p* = 0.009) and protected against maternal CQF (*p* = 0.001) (216). The benefits of co-trimoxazole in pregnancy have been replicated in further studies (220-222). Yet a large clustered randomised control trial looking at the benefits of screening and treating patients infected with Q fever during the large outbreak in the Netherlands did not find an association between treatment and a reduction in obstetric complications (223). This finding was replicated in a screening programme during 2 outbreaks in Germany (36). The difference in outcome for treatment of Q fever during pregnancy has been proposed to be due to strain variation (220).

The results from these *in vitro* efficacy studies provide an insight into the efficacy of a range of antibiotics of interest in the treatment of Q fever. The limitations of this study are related to the limitations of each model chosen. No model can fully replicate the host response seen in human Q fever and each of the selected models aims to reproduce the environment required for the bacterium and provided by the host. The limitations of the THP-1 cell line are that they are not a primary cell line taken directly from human tissue. There are many benefits to using a secondary continuous cell line including ease and robustness of use and reproducibility between experiments. Primary cell lines are not modified and therefore experimental results will have a greater approximation to the human *in vivo* response. Future work will enhance the range of antibiotic concentrations tested in the THP-1 assay. The concentrations selected for doxycycline will be increased up to 16 µg/ml to allow a direct comparison of the effect seen in broth and to
investigate any bactericidal activity at higher concentrations. Similarly, the levofloxacin concentrations in the THP-1 assay will be expanded down to 0.01 µg/ml to allow a direct comparison to the broth results and also a comparison between doxycycline and levofloxacin efficacy. The range of co-trimoxazole concentrations will be expanded in the THP-1 assay to find the limitation of significant inhibition and also ascertain if higher concentrations would have a bactericidal effect. Further work will be carried out investigating the intracellular uptake of each antibiotic in the absence of bacterial infection, alongside the influx and efflux rate of each antibiotic to understand the intracellular mechanism of action related to its antibacterial efficacy. The cytokine response of the uninfected THP-1 cell in response to each antibiotic will be investigated to understand the immunomodulatory effects of antibiotics. Quinolones performed well against *C. burnetii* in the broth and intracellular environment exerting some bactericidal effects, this will be further investigated using newer antibiotics in this class such as finafloxacin. Finally, a difference in antibiotic efficacy has been shown against different strains of *C. burnetii* (111). Strain variation with the antibiotics selected in this study in the THP-1 model and broth will be further assessed.

In summary, the main findings from the *in vitro* work completed assessing the efficacy of doxycycline monohydrate, doxycycline hyclate, levofloxacin, ciprofloxacin, moxifloxacin and co-trimoxazole include:

- The MICs established via broth dilution are comparable using NMI and NMII. This means that the less virulent strain can be used for basic *in vitro* antibiotic efficacy work in broth. The comparability of the 2 phases of *C. burnetii* has not been investigated in the THP-1 cell assay.

- The THP-1 myelomonocytic cell line was used for the first time to assess antibiotic efficacy against *C. burnetii*. It has been shown to be a useful and reproducible model within which to assess antibiotics. The benefit of the THP-1 cell line is that it is a human macrophage therefore more closely representing human infection than other cell lines previously used.

- Doxycycline monohydrate and hyclate appear to have comparable efficacy against *C. burnetii* in the *in vitro* investigations. Both preparations have the lowest MIC out of the antibiotics investigated, but no bactericidal effect is seen in the THP-1 cell assay in contrast to the broth experiments which is consistent with the lack of bactericidal effect in human infection.
• The fluoroquinolones, ciprofloxacin and levofloxacin, have comparable efficacy to each other against *C. burnetii* in the broth and intracellular environment. Both antibiotics have been shown to have bactericidal effect at concentrations greater than or equal to 2 μg/ml using the CLSI definition.

• Co-trimoxazole appears to have superior efficacy in the intracellular environment, which may account for its apparent clinical efficacy despite high MICs in ACCM-2 broth.

• The efficacy of doxycycline, levofloxacin, ciprofloxacin and co-trimoxazole *in vitro* has been shown. Levofloxacin and ciprofloxacin look promising as alternatives to doxycycline, especially with regards to their apparent bactericidal effect.
Chapter 4: *In vivo evaluation of antibiotic efficacy against C. burnetii*

4.1 Introduction

Acute Q fever (AQF) in humans leads to Q fever fatigue syndrome (QFS) in approximately 10-30% of patients (78,93). Within the British military acute infection whilst on deployment in Afghanistan between 2007 to 2015 has left some patients with long term debilitating fatigue (greater than 1 year) and the inability to continue working within the military setting. The military medical service looking after these patients have questioned whether providing antibiotics as chemoprophylaxis prior to deployment to Afghanistan would have prevented acquisition of the infection or reduced the severity of the acute and long-term symptoms. Doxycycline is the standard antibiotic of choice for treatment of AQF (106) and is already an established drug for long-term chemoprophylaxis against malaria (224). Other antibiotics are also known to be efficacious against AQF (106) and so a range of antibiotics known to be effective against Q fever and available to the military in the deployed setting have been chosen. The reason for selecting the antibiotics used in these *in vivo* efficacy studies have been discussed in Chapter 3.

The search for new antimicrobials or repurposing old antibiotics for novel indications requires antibiotics to be tested in a mammalian system prior to human trials. The ethical considerations, cost and limitations placed on mammalian models has led to the increased use of insect models for the primary screening of antibiotics amongst other pathogenesis studies (225). Norville et al. demonstrated that the greater wax moth larvae (*Galleria mellonella*) are susceptible to lethal infection with *C. burnetii*, NMI and NMII strains, and treatment with 50 mg/kg of doxycycline 24 hours after inoculation significantly extended the time to death (179). This study demonstrates the utility of *G. mellonella* as a non-mammalian model for assessing antibiotic efficacy against *C. burnetii*.

Once preliminary *in vitro* and non-mammalian *in vivo* work has been completed a higher order animal model is required to further test antibiotic efficacy. This is particularly important when human efficacy trials are not feasible or ethical as seen in Q fever. The last human study to be completed was with Seventh Day Adventist conscientious objectors in the USA during the 1950s’ (189), and due to the possible chronic sequelae further human studies would now be considered unethical. Norville et al. described the first inhalational model of infection with *C. burnetii* in AJ
mice(188). The AJ mouse model is an acute, inhalational, non-lethal model of C. burnetii infection and hence a good model of AQF in humans. The G. mellonella model and the AJ mouse model have been used to assess the benefit of antibiotics provided as chemoprophylaxis versus treatment for Q fever in this chapter.

4.2. *Galleria mellonella* antibiotic efficacy study

The efficacy of doxycycline hyclate (50 mg/kg), ciprofloxacin (50 mg/kg), levofloxacin (50 mg/kg), moxifloxacin (50 mg/kg) or co-trimoxazole (50 mg/kg) given pre and post challenge with *C. burnetii* were determined. *G. Mellonella* were injected with antibiotics either 24 hours pre or post challenge with 10 μl of 1 x 10⁸ GE/ml NMII *C. burnetii* and monitored for 264 hrs. One group of larvae were unchallenged and given only PBS 24 hours post challenge as a negative control group. The median time to death for larvae challenged with *C. burnetii* and given only PBS 24 hours post challenge was 180 hrs. Levofloxacin pre challenge significantly extended median time to death to 264 hrs (Figure 1a; p < 0.025) and levofloxacin or doxycycline hyclate post challenge significantly extended the median time to death both to 264 hrs (Figure 1b; p < 0.0001, p = 0.0003 respectively). Doxycycline hyclate, ciprofloxacin, moxifloxacin or co-trimoxazole pre challenge did not confer survival benefit with median times to death of 192, 194, 168, 168 (Figure 1c). Whilst ciprofloxacin, moxifloxacin or co-trimoxazole post challenge did not confer survival benefit with median times to death of 168, 192 and 144 hrs respectively (Figure 1d). No deaths were observed in the unchallenged larvae treated with antibiotic alone (data not included).
Figure 1. Survival of G. mellonella following challenge with 10 µl of 1 x 10^8 GE ml^-1 C. burnetii NMII. The results are the means of three replicates, each with 10 larvae. (a) Treatment with levofloxacin (50 mg/kg) 24 hrs pre challenge. (b) Treatment with doxycycline hyclate (50 mg/kg) or levofloxacin (50 mg/kg) 24 hrs post challenge. (c) Treatment with doxycycline hyclate (50 mg/kg), ciprofloxacin (50 mg/kg), moxifloxacin (50 mg/kg), or co-trimoxazole (50 mg/kg) 24 hours pre challenge. (d) Treatment with ciprofloxacin (50 mg/kg), moxifloxacin (50 mg/kg), or co-trimoxazole (50 mg/kg) 24 hours post challenge. Treatment groups that are significantly different to the larvae that received C. burnetii challenge but were treated with only PBS by Mantel Cox log rank test have the significance values indicated in the key.
A pharmacokinetic study was completed in AJ mice in order to inform the antibiotic dose and regime required for each antibiotic in the murine efficacy studies. Levofloxacin (100 mg/kg) and co-trimoxazole (48 mg/kg) were administered to naïve AJ mice via the intraperitoneal or oral route, respectively. The animals were euthanised at set time points over a 24-hour period and plasma tested for drug concentration post mortem (Figure 2, Table 1). Data from previous PK studies completed at Dstl for doxycycline hyclate administered orally and ciprofloxacin via the intraperitoneal route on AJ mice were re-analysed (Figure 2, Table 1).

To understand the antibiotic profile against a specific bacteria the MICs for a range of bacterial strains, alongside pharmacokinetic and pharmacodynamic data, and
clinical studies showing efficacy of drug eradication need to be taken into account\(^{(226)}\). For the purposes of the future mouse efficacy studies; the MIC data from the previous \textit{in vitro} studies (Chapter 3), the PK data in AJ mice (Figure 2, Table 1), and the published pharmacokinetic parameters determined in humans for each antibiotic (Table 2) were used to calculate the murine drug doses required to model concentrations of antibiotics used to treat Q fever in humans (Figure 3, Table 3). All the antibiotics reached steady state within 24 hours, therefore pre exposure antibiotics can be administered 24 hours prior to \textit{C. burnetii} exposure.

**Figure 2:** The pharmacokinetic profile of (a) levofloxacin (b) co-trimoxazole (c) ciprofloxacin and (d) doxycycline hyclate. Three mice were euthanised at each time point for plasma samples to assess the concentration of levofloxacin and co-trimoxazole. The graphs show a mean of the results with standard errors represented. The concentrations of the component drugs of co-trimoxazole (sulphamethoxazole and trimethoprim) were sampled separately at each time point. Doxycycline hyclate and ciprofloxacin data has been re-analysed from previous pharmacokinetic studies completed by Defence Science and Technology Laboratory (Dstl), Porton Down.
**Table 1:** Pharmacokinetic parameters in AJ mice for levofloxacin, co-trimoxazole (sulphamethoxazole and trimethoprim), doxycycline hyclate and ciprofloxacin

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Route</th>
<th>Plasma terminal half-life (h)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;0-t&lt;/sub&gt; (µg.h/ml)</th>
<th>Clearance (g/h/kg or Litre/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levofloxacin</td>
<td>IP</td>
<td>2.39</td>
<td>62</td>
<td>0.167</td>
<td>48.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>Oral</td>
<td>1.93</td>
<td>48</td>
<td>1</td>
<td>123.5</td>
<td>0.324</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>Oral</td>
<td>1.56</td>
<td>0.241</td>
<td>1</td>
<td>0.427</td>
<td>18.7</td>
</tr>
<tr>
<td>Doxycycline hyclate</td>
<td>Oral</td>
<td>3.5</td>
<td>3.4</td>
<td>2</td>
<td>21.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>IP</td>
<td>1.6</td>
<td>29</td>
<td>0.017</td>
<td>15.6</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**Table 2:** Summary of the target pharmacokinetic parameters in humans from published literature for ciprofloxacin, levofloxacin, doxycycline and co-trimoxazole.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Human regimen</th>
<th>Pharmacokinetic target in humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>500mg od</td>
<td>AUC = 11.6 mg/L/h(227)</td>
</tr>
<tr>
<td></td>
<td>500mg bd</td>
<td>AUC = 23.2 mg/L/h</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>500mg od</td>
<td>AUC = 45.6 mg/L/h(228)</td>
</tr>
<tr>
<td>Doxycycline hyclate</td>
<td>100mg od</td>
<td>AUC = 37-40 mg/L/h(229)</td>
</tr>
<tr>
<td></td>
<td>100mg bd</td>
<td>AUC = 40 – 123 mg/L/h</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>960mg od</td>
<td>T &gt; MIC for ⅔ of the time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIC for co-trimoxazole combination = 2-8 µg/mL(156,161)</td>
</tr>
</tbody>
</table>
**Figure 3:** Pharmacokinetic (PK) calculations to ascertain the required murine dose of antibiotic (AJ mice) to replicate the human PK metric observed in humans in doses used to treat Q fever.

**Table 3:** Summary of the antibiotic dose and route selected for the AJ mouse based on *in vitro* sensitivities and pharmacokinetic data. The murine doses have been calculated to model the human dose of antibiotics used to treat Q fever. (IP = intraperitoneal, od = once daily, bd = twice daily)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Human dose</th>
<th>Route</th>
<th>AJ mouse dose</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline hyclate</td>
<td>100mg od</td>
<td>Oral</td>
<td>105mg/kg od</td>
<td>Oral</td>
</tr>
<tr>
<td>Doxycycline hyclate</td>
<td>100mg bd</td>
<td>Oral</td>
<td>105mg/kg bd</td>
<td>Oral</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>500mg od</td>
<td>Oral</td>
<td>40mg/kg bd</td>
<td>IP</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>500mg bd</td>
<td>Oral</td>
<td>22mg/kg bd</td>
<td>IP</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>960mg bd</td>
<td>Oral</td>
<td>48mg/kg bd</td>
<td>Oral</td>
</tr>
</tbody>
</table>

Two AJ mouse antibiotic efficacy studies were completed. The antibiotics regimens used are summarised in Table 3. Doxycycline hyclate 105 mg/kg was administered orally once daily versus twice daily. These regimens represent the human PK profile seen with 100 mg daily of doxycycline used as malaria prophylaxis and the 100 mg twice daily PK profile seen with treatment doses of doxycycline used in
acute Q fever. Ciprofloxacin 22 mg/kg twice daily and levofloxacin 40 mg/kg twice daily were administered via the intraperitoneal (IP) route because previous studies at Dstl, Porton Down have shown that quinolones via the IP route as opposed to the oral route provide a PK profile with a greater correlation to the human PK profile of these drugs taken orally(188). Co-trimoxazole was provided at 48 mg/kg twice daily which provides a human PK profile equivalent to 480 mg twice daily which is the dose used for prophylaxis of Pneumocystis Carinii in HIV positive patients(230).

4.4. AJ mouse antibiotic efficacy study 1

Doxycycline is the standard treatment for Q fever, and both the efficacy of oral doxycycline hyclate (50 mg/kg twice daily) and the lack of effectiveness of oral ciprofloxacin (50 mg/kg twice daily) against C. burnetii in the AJ mouse model have been previously published(188).

In this study AJ mice were challenged with a mean retained dose of 6.4 x 10^5 copies of C. burnetii via the aerosol route. The efficacy of oral doxycycline hyclate (105 mg/kg once daily and twice daily) and intraperitoneal ciprofloxacin (22 mg/kg twice daily) commenced either 24 hours pre or 24 hours post challenge were assessed. Treatment in the pre challenge group continued for 9 days, and in the post challenge group for 7 days. A control group of mice were administered 50 μl of PBS daily from 24 hours pre challenge to 7 days post challenge. All groups stopped therapy 7 days post challenge (Figure 4).

The doses of doxycycline and ciprofloxacin differ from those completed in the previous antibiotic efficacy study by Norville et al(188) following re-evaluation of the pharmacokinetic data in AJ mice. Doxycycline hyclate 105 mg/kg once daily provides a PK profile equivalent to 100mg daily in humans, and the 105 mg/kg twice daily provides a PK profile equivalent to 100mg twice daily in humans. One hundred milligrams daily is the dose of doxycycline used for malaria prophylaxis and twice daily is the treatment dose for Q fever in humans. These 2 doses are compared in this study for the effectiveness in preventing and treating disease in this model. Ciprofloxacin 22 mg/kg twice daily IP provided a PK profile in AJ mice equivalent to 500mg twice daily in humans, which is the treatment dose for uncomplicated infections in humans.
**AJ mouse antibiotic efficacy study plan** 1. **AJ mice** were challenged with a mean retained dose of $6.4 \times 10^5$ copies of *C. burnetii* and treated with doxycycline hyclate 105 mg/kg once daily or twice daily orally or ciprofloxacin 22 mg/kg twice daily IP commenced either 1 day pre or 1 day post challenge. Pre challenge treatment continued for 9 days and post challenge treatment for 7 days. A control group of mice were administered 50 µl of PBS daily from 1 day pre challenge to 7 days post challenge. Mice were weighed daily from -1 to +14 days. At study completion (+14 days) mice were euthanised for lung and spleen weight and bacterial burden. **Key**: -1 days – pre challenge antibiotics and PBS commenced. 0 days - aerosol mouse challenge. +1 days – post challenge antibiotics commenced. +7 days – pre and post challenge antibiotics and PBS stopped. +14 days – end of study. IP = intraperitoneally. The dashed line indicates the time period where mice are being observed and no longer receiving antibiotics or water.

### 4.4.1. Percentage body weight loss from baseline and clinical signs

Mice in the control group treated with PBS reached peak weight loss by day 9 (14%) (Figure 5), and 90% exhibited clinical signs of ruffled fur at least once from days 7 to 11 post challenge. No mice were euthanised due to reaching the humane end point in the PBS group.

Doxycycline hyclate administered 105 mg/kg once daily, commenced pre and post challenge significantly reduced weight loss (Figure 5a; $p <0.05$), with a peak weight loss seen during the treatment phase in the pre challenge group at day 6 (5%) and at day 6 (1%) in the post challenge group. Both groups showed weight loss once the treatment had finished, starting at day 12. In the once daily pre challenge group, up to 40% showed signs of ruffled fur from day 3-12 and up to 20%
developed signs of an arched back from day 6-7. The signs were milder in the once daily post challenge group with ruffled fur being seen in up to 30% from day 6-9 and 10% showed an arched back at day 7. These findings were consistent with the increased number of mice euthanised due to reaching the humane end point of > 20% weight loss in the once daily pre exposure treatment arm (Table 4).

In the doxycycline 105 mg/kg twice daily treatment groups there was no significant reduction in weight compared to the PBS treated arm for both groups. The pre challenge group reached maximal weight loss at day 10 (13%), and the post exposure group reached maximal weight loss at day 6 (10%). Although these figures do not accurately represent the weight loss because 7 mice were euthanised due to > 20% weight loss in the twice daily pre challenge arm and 1 in the twice daily post challenge arm (Table 4). The weight loss figures (Figure 5 a & b) included the data of the mice that reached the humane end point, up until the point where they were euthanised. The clinical signs were the most marked out of all doxycycline treatment groups in the twice daily pre challenge group, with up to 90% of mice demonstrating ruffled fur and an arched back from days 3 – 10, and 1 mouse (10%) was found to be immobile at day 8 and was humanely euthanised. This mouse is counted in the number of mice that reached the humane end point. Clinical signs were also marked in the post challenge group, with 80% exhibiting ruffled fur at least once from day 5 to 9 and up to 30% displaying and arched back from days 5 to 8.

Both pre challenge treatment arms display weight loss within 24 hours from doxycycline initiation. The weight loss commences prior to that seen in the PBS treated control arm and is therefore suggestive of a drug side effect.

Ciprofloxacin 22 mg/kg twice daily did not confer any weight loss benefit compared to the PBS treated arm (Figure 5c). The pre challenge group reached maximal weight loss of 13% from baseline at day 10, and the post challenge group reached maximal weight loss of 10% from baseline by day 9 (Figure 5c). The clinical signs are consistent with the weight loss. In the pre challenge group up to 80% of mice displayed ruffled fur from day 6 to 9 and 40% displayed an arched back at day 8. In the post challenge group up to 80% displayed ruffled fur from day 5 to 10.
Days post challenge

Mean % change in bodyweight

Doxycycline 24h
pre challenge od
Doxycycline 24h
post challenge od
PBS

Days post challenge
Figure 5: Efficacy of oral doxycycline 105 mg/kg once daily and twice daily, or ciprofloxacin 22 mg/kg once daily commenced 24h pre or 24h post challenge (n=10) versus PBS treated control mice challenged with a mean retained dose of $6.4 \times 10^5$ of *C. burnetii*. Antibiotics were administered from days -1 to +7 in the pre challenge arm and +1 to +8 in the post challenge arm. Mean percentage change in body weight of mice infected with $1.6 \times 10^6$ GE/ml of *C. burnetii* and treated with (a) Doxycycline hyclate 105 mg/kg once daily (b) Doxycycline hyclate 105 mg/kg twice daily (c) Ciprofloxacin 22 mg/kg twice daily are displayed. Values that are significantly different ($p < 0.05$) to the PBS control are marked with an *, the colour corresponds to the group. The coloured arrows represent the time points where mice were euthanised due to reaching the humane end point, the colour corresponds to the group and the corresponding number identifies how many mice were euthanised at each point.

Table 4: The number of mice euthanised for reaching the humane end point of weight loss of ≥ 20% weight loss from baseline. In this study mice were only euthanised for reaching their humane end point in the doxycycline hyclate treatment groups.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Number of mice euthanised for weight loss ≥ 20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline hyclate pre exposure once daily</td>
<td>4</td>
</tr>
<tr>
<td>Doxycycline hyclate post exposure once daily</td>
<td>1</td>
</tr>
<tr>
<td>Doxycycline hyclate pre exposure twice daily</td>
<td>7</td>
</tr>
<tr>
<td>Doxycycline hyclate post exposure twice daily</td>
<td>1</td>
</tr>
</tbody>
</table>
4.4.2. Organ weight as a percentage of body weight and bacterial colonisation of lungs and spleen

At day 14 post challenge, mice were euthanised, lungs and spleens aseptically removed, weighed and bacterial load determined using RT-PCR. The spleen weight is significantly lower in the doxycycline twice daily pre and post treatment groups and the ciprofloxacin pre and post treatment groups compared to the PBS treated control mice ($p < 0.01$) (Figure 6a). Using PCR as a marker of bacterial colonisation, the doxycycline twice daily post exposure group and both ciprofloxacin groups had significantly lower PCR counts compared to the PBS treated control mice ($p < 0.01$) (Figure 6b). The doxycycline once daily pre exposure group had a significantly higher count than the PBS treated mice at day 14 ($p < 0.05$) (Figure 6b). At day 14 the PBS treated control group have controlled the disease and their weight was approaching baseline, whilst the doxycycline once daily treatment groups started to lose weight at day 12, and appeared to reach peak weight loss at day 13 but the study ended at day 14 (Figure 5a). Therefore, a relapse in disease may account for this difference in bacterial colonisation.
Figure 6: (a) Weight of lung and spleen as a percentage of total body weight at day 14 post challenge and treated with doxycycline 105 mg/kg once daily (od) or twice daily (bd), or ciprofloxacin 22 mg/kg twice daily (bd) for 7 days starting either 24 hours pre or 24 hours post challenge. (b) Bacterial colonisation of lung and spleen as determined by RT-PCR at day 14 post challenge and treated with doxycycline 105 mg/kg once daily (od) or twice daily (bd), or ciprofloxacin 22 mg/kg twice daily (bd) for 7 days starting either 24 hours pre or 24 hours post challenge. Values are means +/- standard error of the mean (SEM). Values that are significantly different from the value of the PBS control are indicated by asterisks as follows: * p < 0.01; *** p < 0.0001
4.5. AJ mouse antibiotic efficacy study 2

Mouse study 2 was completed in order to broaden the spectrum of antibiotics assessed as pre and post challenge treatment for *C. burnetii*. In this study, 100 mice were exposed via the aerosol route to a mean retained dose of $4 \times 10^6$ copies of *C. burnetii* and treated with doxycycline 105 mg/kg twice daily orally, ciprofloxacin 22 mg/kg twice daily intraperitoneally, levofloxacin 40 mg/kg twice daily intraperitoneally or co-trimoxazole 48 mg/kg twice daily orally commenced either 24 hours pre or 24 hours post challenge. Treatment in the pre challenge group continued for 9 days, and in the post challenge group for 7 days. A control group of mice were administered 50 $\mu$l of PBS daily from 24 hours pre challenge to 7 days post challenge. All groups stopped therapy 7 days’ post challenge (Figure 7).

![Figure 7: AJ mouse antibiotic efficacy study plan 2. AJ mice were challenged with a mean retained dose of $4 \times 10^6$ copies of *C. burnetii* and treated with doxycycline hyclate 105 mg/kg twice daily orally, ciprofloxacin 22 mg/kg twice daily IP, levofloxacin 40 mg/kg twice daily IP or co-trimoxazole 48 mg/kg orally commenced either 1 day pre or 1 day post challenge. Pre challenge treatment continued for 9 days and post challenge treatment for 7 days. A control group of mice were administered 50 $\mu$l of PBS daily from 1 day pre challenge to 7 days post challenge. Mice were weighed daily from -1 to +14 days. At study completion (+14 days) mice were euthanised for lung and spleen weight and bacterial burden. **Key:** -1 days – pre challenge antibiotics and PBS commenced. 0 days - aerosol mouse challenge. +1 days – post challenge antibiotics commenced. +7 days – pre and post challenge antibiotics completed, and +14 days – end of study. IP = intraperitoneally. The dashed line indicates the time period where mice are being observed and no longer receiving antibiotics or water.](image-url)
4.5.1. Percentage body weight loss from baseline and clinical signs

Mice treated with PBS reached peak weight loss by day 10 (18%) and up to 90% exhibited ruffled fur from days 6 to 9 and up to 40% displayed an arched back at least once from days 7 to 8 post challenge.

The doxycycline 105 mg/kg twice daily pre challenge group start to show clinical signs at day 1 post challenge to *C. burnetii*, with up to 50% displayed ruffled fur from day 1 to 10 and 10% an arched back from day 2 to 3. Whilst the doxycycline 105 mg/kg twice daily post exposure group start to show signs at day 5 post challenge, with up to 50% displaying ruffled fur from day 5 to 10. Both groups were clinically well from day 10 but showed weight loss towards the end of the experiment at day 12 for the pre challenge group and day 14 for the post challenge group (Figure 8a). Doxycycline 105 mg/kg twice daily pre and post exposure both provide significant weight loss protection compared to the PBS treated control group (*p* <0.05) (Figure 8a).

As seen in the study 1 there was weight loss seen in the doxycycline treatment groups that commenced prior to the PBS treated control group and appeared to be a drug side effect (Figure 8a). This is supported by the early onset of symptoms in the doxycycline pre exposure group. Four mice in the pre exposure group and 3 in the post exposure group were euthanised due to reaching their humane end point (Table 5).

The levofloxacin 40 mg/kg twice daily pre and post exposure group show no clinical signs during the experiment. This correlates with the significant weight loss protection seen in both levofloxacin pre and post exposure groups (*p* <0.001) when compared to the PBS treated control group (Figure 8b). The levofloxacin pre exposure group started to lose weight at the end of the experiment suggestive of a relapse in disease (Figure 8b).

The co-trimoxazole 48 mg/kg twice daily and ciprofloxacin 22 mg/kg twice daily pre and post challenge groups conferred no weight loss benefit over the PBS control treated group. Both groups started to lose weight at day 4-5 reaching a maximum between 9-10 returning to baseline by day 14 which is consistent with the PBS treated control group (Figure 8c).
The co-trimoxazole 48 mg/kg twice daily pre challenge group showed ruffled fur in up to 100% of mice from day 5 to 10, 80% displayed an arched back from day 7 to 8 and 50% were wasp waisted at day 6. The post challenge group in up to 100% of mice showed ruffled fur from day 6 to 10 and 10% an arched back at day 8. Five mice in the pre exposure group were euthanised due to reaching their humane end point (Table 5). The ciprofloxacin 22 mg/kg twice daily pre challenge group displayed ruffled fur in up to 100% of mice from day 5 to 8. The post challenge group displayed ruffled fur in up to 50% of mice from day 7 to 9 and an arched back in 30% at day 9.
Figure 8: Percentage weight loss from baseline for mice challenged with a mean retained dose of $4 \times 10^6$ of *C. burnetii* and treated with either doxycycline 105 mg/kg orally twice daily, levofloxacin 40 mg/kg twice daily intraperitoneally, ciprofloxacin 22 mg/kg twice daily intraperitoneally or co-trimoxazole 48 mg/kg twice daily for 7 days starting 24 hours pre or post challenge to *C. burnetii* compared to a PBS treated control group. (a) Doxycycline 105 mg/kg twice daily commenced pre and post exposure (b) Levofloxacin 40 mg/kg twice daily commenced pre and post exposure. (c) Ciprofloxacin 22 mg/kg twice daily and co-trimoxazole 48 mg/kg twice daily commenced pre and post exposure. Values that are significantly different from the value of the PBS treated control group are indicated by asterisks as follows * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. The coloured arrows represent the timepoints where mice were euthanised due to reaching the human end point, the colour corresponds to the group and the corresponding number identifies how many mice were euthanised at each point.
Table 5: The number of mice euthanised for reaching the humane end point of weight loss of ≥ 20% weight loss from baseline. In this study mice were euthanised for reaching their human end point in the doxycycline hyclate, co-trimoxazole and PBS treatment groups.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Number of mice euthanised for weight loss ≥ 20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline hyclate twice daily pre challenge</td>
<td>4</td>
</tr>
<tr>
<td>Doxycycline hyclate twice daily post challenge</td>
<td>3</td>
</tr>
<tr>
<td>Co-trimoxazole pre challenge</td>
<td>5</td>
</tr>
<tr>
<td>PBS</td>
<td>1</td>
</tr>
</tbody>
</table>

4.5.2. Organ weight as a percentage of body weight and bacterial colonisation of lungs and spleen

At day 14 post challenge, mice were euthanised, lungs and spleens aseptically removed, weighed and bacterial load determined using RT-PCR. Splenic weight gain in the doxycycline twice daily post exposure treatment group was significantly less than the PBS control group suggesting less bacterial burden ($p <0.05$) (Figure 9a). There was no significant difference between the doxycycline treatment groups and the PBS control groups and bacterial burden as determined by RT-PCR (Figure 9b).

There was no significant difference in organ weight and bacterial burden as determined by RT-PCR between the levofloxacin treatment groups and the PBS control groups (Figure 9).

The bacterial load as determined by RT-PCR was significantly lower in the spleen of the ciprofloxacin 22 mg/kg pre challenge group ($p <0.001$), and significantly lower in the spleen and lungs in the ciprofloxacin 22 mg/kg post challenge group ($p <0.001$, $P <0.05$ respectively, Figure 9b), yet there was no difference in organ weight between the groups (Figure 9a). There was no significant difference in organ weight and bacterial burden as determined by RT-PCR between the co-trimoxazole treatment groups and the PBS control groups (Figure 9).
Figure 9: (a) Weight of lung and spleen in mice challenged with a mean retained dose of 4 x10^6 GE of C. burnetii and treated with doxycycline hyclate 105 mg/kg twice daily (bd), levofloxacin 40 mg/kg twice daily (bd), ciprofloxacin 22 mg/kg twice daily (bd) or co-
trimoxazole 48 mg/kg for 7 days starting either 24 hours pre or 24 hours post exposure. The weight of lung and spleen for unchallenged, untreated AJ mice is shown for comparison. (b)
The bacterial colonisation of lung and spleen as determined by RT-PCR at day 14 post challenge from mice challenged with a mean retained dose of 4 x 10^6 GE of C. burnetii and treated with doxycycline hyclate 105 mg/kg twice daily (bd), levofloxacin 40 mg/kg twice daily (bd), ciprofloxacin 22 mg/kg twice daily (bd) or co-trimoxazole 48 mg/kg twice daily (bd) for 7 days starting either 24 hours pre or 24 hours post exposure. Values are means +/- standard error of the mean (SEM). Values that are significantly different from the value of the PBS treated control group are indicated by asterisks as follows * p < 0.05; ** p < 0.01; *** p < 0.0001.

4.6. Discussion

In the G. mellonella studies, levofloxacin 50 mg/kg given 24 hours’ pre-challenge, and doxycycline hyclate 50 mg/kg or levofloxacin 50 mg/kg given 24 hours post challenge were shown to provide a significant survival benefit (p < 0.05). Levofloxacin given post challenge conferred a greater survival benefit than pre challenge. All the antibiotics evaluated in the larvae model were shown to have activity against C. burnetii in the in vitro assays (Chapter 3) but this was not replicated in the larvae. Previous pre and post challenge antimicrobial efficacy studies have been completed in G. mellonella larvae for P. aeruginosa(175) and B. thailandensis and B. pseudomallei(180) with good effect. Although in these studies the antibiotic doses and timing of antibiotic administration were different to the above studies. The pharmacokinetics and pharmacodynamics of the antibiotics in G. mellonella may account in part for the lack of efficacy of some of the antibiotics in these C. burnetii studies. The pharmacokinetics of levofloxacin(175), doxycycline(175) and ciprofloxacin(175) in G. mellonella larvae have been described previously (Table 6).

Table 6: Summary of the published pharmacokinetic parameters of levofloxacin, doxycycline and ciprofloxacin in G. mellonella larvae.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Dose</th>
<th>Cmax (µg/ml)</th>
<th>AUC (µgh/ml)</th>
<th>T1/2 (h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levofloxacin</td>
<td>10 mg/kg</td>
<td>32.5</td>
<td>86.6</td>
<td>2.25</td>
<td>(175)</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>5 mg/kg</td>
<td>12.7</td>
<td>37.9</td>
<td>2.4</td>
<td>(175)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>50 mg/kg</td>
<td>80.5</td>
<td>299.6</td>
<td>17</td>
<td>(180)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>5 mg/kg</td>
<td>18</td>
<td>17.6</td>
<td>2.4</td>
<td>(180)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>20 mg/kg</td>
<td>26</td>
<td>422.1</td>
<td>15.7</td>
<td>(180)</td>
</tr>
</tbody>
</table>
A drug is considered to be eliminated from the system after 4-5 half lives (233). Therefore, administering the antibiotics 24 hours prior to the challenge in this study may have been too early leaving little or no antibiotic to provide a possible survival benefit. This is the not the case for doxycycline at 50 mg/kg where the $t_{1/2}$ is 17 hours and therefore the drug would not be eliminated for 68-85 hours. No data exists for the other drugs at 50 mg/kg and therefore the level in the haemolymph prior to challenge is not known.

Ciprofloxacin and doxycycline administered 1 hour prior to challenge, or 2 or 6 hours post challenge provided significant larval protection from the lethal infection of *B. pseudomallei* (180). For *P. aeruginosa* (175), *B. pseudomallei* (180) and *C. burnetii* (179) the lethal bacterial dose in *G. mellonella* larvae is dose dependent. At the doses selected in these studies the majority of larval death occurred within 24 hours for *P. aeruginosa* and *B. pseudomallei*, in contrast to *C. burnetii* with lethality for all larvae occurring by 216 hours. The time to lethality will influence the ideal scheduling of pre and post challenge antibiotics. Further pharmacokinetic research in the larvae to find optimum timing for pre and post challenge antibiotics is required for *C. burnetii* to inform future efficacy studies.

In the AJ mouse model, doxycycline once daily initiated pre challenge as prophylaxis and post challenge as treatment significantly reduced weight loss compared to the PBS control at the peak of disease, but both groups appeared to relapse by the end of the study with weight loss. The same was seen with levofloxacin pre exposure which significantly reduced weight loss at the peak of disease compared to the PBS control but again the mice start to lose weight by the end of the study. In both experiments the groups treated with ciprofloxacin pre and post challenge had no weight loss reduction compared to the PBS control. Yet in both groups significantly lower bacterial burden in the spleen as assessed by RT-PCR was seen. The data from study 1 and 2 suggests that treating the disease too soon and controlling signs and symptoms may not be beneficial in eradication of this intracellular bacterium in the longer term. The doxycycline and levofloxacin results from studies 1 and 2 in AJ mice also are suggestive that treatment initiated early in the incubation period may extend the incubation period rather than prevent disease. Treatment initiation later in the incubation period was not performed in these studies for comparison.
Splenomegaly in AJ mice following infection with *C. burnetii* appears to be proportional to the infecting dose(3). It is a useful marker of immune response and the pathogenesis of splenic enlargement is thought to be due to extensive proliferation of the immunoglobulin producing B cells(3). Doxycycline and ciprofloxacin twice daily initiated pre and post challenge in study 1 led to significantly less enlargement at post mortem than the spleens in the PBS treated group. This was only seen for the doxycycline twice daily post challenge group in study 2. This difference in splenic size could be due to a reduction in immune stimulation in the spleens of the treated mice. *C. burnetii* requires a cell mediated immune response to clear the infection(234). Cutler et al. (14) postulated that cytokine dysregulation and the subsequent impairment of the bactericidal responses may enable persistence of bacteria in chronic cases. Some antibiotics cause downregulation of the immune response to bacteria which can be useful in certain circumstances where there is a hyperinflammatory state such as systemic inflammatory response syndrome (SIRS) (235).

As an explanation for the results shown in study 1 and 2, it is hypothesised that antibiotic immunomodulation could be disadvantageous when used as prevention, or early in the treatment of intracellular bacteria such as *C. burnetii*. Tetracyclines and fluoroquinolones regulate the expression of Toll Like Receptors (TLRs) and cytokines which manifests as a reduction in proinflammatory cytokines such as IL-1, IL-6 and TNF-α(236,237). The effect of fluoroquinolones on the immune system appears to vary for drugs within the class and is also dose dependent. For example, in intracellular assays ciprofloxacin has been shown to both increase and decrease production of IL1, IL6 and TNF-α depending on the dose(237). Whereas levofloxacin produces a dose related decrease in IL6 and IL8 and decreases IFN-γ, TNF-α and increases IL10(237,238). Data from a mouse model showed that ciprofloxacin administered prior to an LPS challenge lowered IL6 and TNF-α production 1 hour after LPS administration and TNF-α remained lower at 4 hours(237). In an intracellular study of *Chlamydia pneumoniae*, doxycycline but not ciprofloxacin reduced production of IFN-γ(231). Doxycycline has been shown to reduce the proinflammatory cytokines, TNF-α, IL1, IL6 and IL8(236,239,240) and the levels of reactive oxygen intermediates (ROIs), and LPS induced nitric oxide (NO) production(236). *C. burnetii* inhibits the ROI response of polymorphonuclear leukocytes (PMNs) (241) which allows the bacteria to evade cell death, the further reduction by doxycycline may aid bacterial evasion. NO reversibly inhibits
replication of *C. burnetii* and formation of the parasitophorous vacuole\(^{(242)}\), so inhibition of NO by doxycycline may allow the bacteria to replicate. The possible relapse seen in disease at the end of Study 1 and 2 for doxycycline and levofloxacin could be because of immune downregulation seen with these antibiotics. The literature discussed above demonstrates a difference in the immune modulation between levofloxacin and ciprofloxacin. If ciprofloxacin had a lesser effect on immune suppression this may account for why the mice treated with ciprofloxacin lost weight in line with the PBS control mice and had lower spleen weights and splenic bacterial burden.

Co-trimoxazole appeared to perform better against *C. burnetii* in the intracellular assay rather than in axenic media (Chapter 3). Yet the results from study 2 show weight loss and clinical signs consistent with the PBS control group for co-trimoxazole and no lowering of splenic weight or bacterial burden compared with the control group. Co-trimoxazole did not appear to reduce the burden of disease in this model. The immunomodulatory effects of co-trimoxazole are not well documented. Limited studies have been performed looking at the anti-inflammatory benefits of co-trimoxazole in conditions such as rheumatoid arthritis, pulmonary fibrosis and granulomatosis with polyangiitis\(^{(232,243,244)}\). The results suggest that co-trimoxazole may reduce pro-inflammatory cytokine production and the release of ROIs from neutrophils and macrophages\(^{(243,244)}\). The *in vitro* and *in vivo* studies investigating the efficacy of co-trimoxazole against *C. burnetii* suggest that it has limited efficacy in ACCM-2 broth, *G. mellonella* and AJ mice. No survival benefit was seen with co-trimoxazole in *G. mellonella*, and in AJ mice no reduction in weight, organ weight or bacterial burden was seen. There are limitations to all *in vitro* and *in vivo* models. Observational human studies of the benefits of co-trimoxazole in pregnancy have shown conflicting results and the efficacy of co-trimoxazole in Q fever remains unclear\(^{(36,219,223,245)}\).

Further work needs to be carried out assessing the pharmacokinetic profile of antibiotics in *G. mellonella* larvae in order to allow further antibiotic efficacy studies to be carried out. In the AJ mouse model further work is required to assess the mechanism of antibiotic action against *C. burnetii*, to assess the immunomodulatory effects of the antibiotics being studied and to ascertain the most advantageous time to initiate treatment in order to support the innate and adaptive immune response in clearing the infection. A limitation to these *in vivo* studies is the weight loss seen as a side effect of doxycycline hyclate which led to the humane euthanisation of many
mice, particularly in the pre challenge groups. The drug associated weight loss confounds the analysis of weight loss prevention and the reduction in numbers of mice by the end of the study for organ weight and bacterial burden has reduced the power of finding a drug effect. Therefore, future work will focus on an alternative, better tolerated formulation of doxycycline. The other limitation is the length of the study. At day 14 when the study ended mice in the doxycycline and levofloxacin groups were starting to lose weight but due to the study timeline the outcome in these groups could not be further assessed. Future work will extend the study timescale to further evaluate disease progression and clearance.

In summary;

- Levofloxacin and doxycycline as treatment, and levofloxacin as prophylaxis provided a survival benefit in the *G. mellonella* model.
- Levofloxacin and doxycycline reduced weight loss in the AJ mouse model. Although the weight loss data with doxycycline hyclate was difficult to interpret due to the drug induced weight loss seen particularly in the high dose pre exposure groups.
- Doxycycline hyclate once daily and twice daily, both pre and post challenge regimens and levofloxacin pre challenge display weight loss at the end of study suggestive of a disease relapse.
- Ciprofloxacin pre and post challenge reduced bacterial burden in the spleen. A reduction in bacterial burden was not seen in the levofloxacin and doxycycline hyclate treatment groups.
- Antibiotic associated immune suppression may account for the disease relapse and failure to produce a reduction in bacterial burden in the levofloxacin and doxycycline treatment groups when administered pre challenge or early in the incubation period.
- Ciprofloxacin does not appear to have the same class effect as levofloxacin on weight loss, clinical signs and bacterial burden. It is postulated that the immunomodulatory effect of ciprofloxacin is different to levofloxacin and therefore the immune system with ciprofloxacin has been able to be primed to aid clearance of the infection.
- Co-trimoxazole does not appear to be efficacious against *C. burnetii* in these *in vivo* models.
Chapter 5: *In vivo* evaluation of alternative doxycycline regimens in treatment of Q fever

### 5.1. Introduction

Doxycycline is the recommended antibiotic for use in acute Q fever\(^{106}\). In chapter 4 the results of AJ mouse antibiotic efficacy studies 1 and 2 were presented assessing the efficacy of doxycycline, levofloxacin, ciprofloxacin and co-trimoxazole against *C. burnetii*. The results showed that doxycycline and levofloxacin administered pre and post challenge reduced mouse body weight loss compared to the PBS control group but no decrease in bacterial burden as assessed by RT-PCR was seen in the lungs or spleen at the end of the study. In contrast the ciprofloxacin pre and post challenge treated groups displayed no reduction in weight loss but did have a reduced bacterial burden and spleen weight at completion of the study. The doxycycline hyclate treatment groups were difficult to analyse due to drug associated weight loss requiring humane end point culls. This reduced the power of the study and ability to assess the effectiveness of doxycycline prophylaxis and treatment. As doxycycline is the standard therapy, the decision was made to focus on doxycycline for the next 2 studies. The main questions that arose from AJ mouse studies 1 and 2 were regarding the optimum timing of antibiotic initiation, finding a better tolerated doxycycline preparation in order to reduce drug associated weight loss, and assessing the immunomodulatory effects of pre and post challenge treatment and the consequent impact on disease burden. In addition, it was decided to extend the duration of the study to assess for disease relapse.

In study 3 tissue samples were taken from a greater range of sites than previous studies to assess the differences in bacterial dissemination and clearance with the different treatment regimens. At post mortem samples were taken for bacteriology from the lungs, spleen, brain, testes, bone marrow, adipose and blood. The lungs and spleen were taken for comparison to the previous studies. These sites are important because the mice are challenged with *C. burnetii* by the aerosolised route and splenic colonisation is a sign of dissemination once the mouse becomes bacteraemic. The brain was chosen because in human disease severe headaches, often with a retrobulbar distribution are a common feature of acute Q fever, likely as a consequence of the febrile illness and possibly dehydration. Yet neurological complications such as meningitis and meningoencephalitis are well
documented(246). Brain biopsies are not performed as part of the diagnostic work up and therefore understanding if the bacteria spreads to the brain in this model was of interest. Testes were assessed to gain further information on the sexual transmissibility of the bacteria. C. burnetii DNA is commonly found in bone marrow biopsies at all stages of the disease(247,248). It has been postulated that the bone marrow may be a sanctuary site for recrudescence of disease(248), along with the adipose tissue(249). DNA detection in the blood is a useful diagnostic tool. Culture cannot be performed in routine diagnostic laboratories, therefore understanding the benefits of PCR and how it compares to culture results in the mouse model will be beneficial when investigating human cases.

5.2. AJ mouse antibiotic efficacy study 3

For efficacy study 3 doxycycline monohydrate was selected instead of doxycycline hyclate due to its enhanced tolerability in humans(250). A tolerability study in AJ mice was performed at Dstl, Porton Down and found doxycycline monohydrate at 50 mg/kg or 100 mg/kg twice daily led to a less than 5% weight loss over 7 days (K.Hamblin, Dstl, data not included).

Mouse study 3 was completed using doxycycline monohydrate 105 mg/kg twice daily as prophylaxis starting at 1 day prior to C. burnetii challenge or as treatment starting at 5 days’ post challenge. The dose of 105 mg/kg twice daily is equivalent to the treatment dose in humans of doxycycline 100 mg twice daily. This regimen was selected because only one dosing regimen could be investigated in this study in view of the number of other variables being examined. The AJ mouse tolerability study (Hamblin, Dstl) demonstrated limited side effects with the 100 mg/kg twice daily regimen. A known effective dosing regimen was required to be able to assess the other variables of treatment timing, and length of treatment on outcome. The efficacy of a prophylaxis dose of 100 mg daily against C. burnetii is not known in humans. Day 5 post challenge was selected as a consequence of the previous two studies because it is at this point that the mice start to lose weight (Figure 8, Chapter 4) and display clinical signs. This time point is a better representation of when patients would receive treatment following C. burnetii exposure because it is at this point that they would feel unwell and present to the medical services.

Doxycycline monohydrate or distilled water (diH2O) as a control were continued for 7 or 14 days following the challenge. Therefore, the pre challenge group received a total of 9 or 16 days of treatment and the post challenge group a total of 7 or 14
days of treatment (Figure 1). The antibiotics were extended to 14 days for this study because the standard treatment for human infection is 2 weeks of doxycycline (106). The study was continued for 35 days to assess for disease relapse.

Four further groups of mice were added to this study to assess the immunomodulatory effects of antibiotics. One group commenced doxycycline monohydrate on the day of challenge (day 0) and continued for 5 days alongside a water treated control group. The final 2 groups were not challenged with C. burnetii but were given either 5 days of doxycycline monohydrate or water. The aim of these groups was to assess the immunomodulatory effect of the antibiotics in the presence and absence of C. burnetii.

AJ mice were challenged via the aerosol route with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of C. burnetii. Each doxycycline pre and post challenge treatment group consisted of 15 mice. Five from each group were euthanised 24 hours after completion of therapy, along with a water treated control group for interim analysis. The further 10 mice from each group were euthanised at completion of the study (day 35) to look for clinical and microbiological signs of treatment failure or relapse as seen for doxycycline hyclate in studies 1 and 2. Five mice from the control group treated with water from day 5 to day 19 were also euthanised at day 35 for comparison with the treatment groups (Figure 1). The 4 groups investigating the immunomodulatory effects of antibiotics consisted of 5 mice per group and were all euthanised at day 5 after completion of therapy.
Figure 1: Study plan for AJ mouse efficacy study 3. Mice were exposed to an aerosol challenge with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii*. The antibiotic treatment regimens were doxycycline monohydrate 105 mg/kg twice daily orally for 5 days commenced on the day of challenge, or doxycycline monohydrate 105 mg/kg twice daily orally for 9 or 16 days commenced 1 day prior to challenge or for 7 or 14 days commenced 5 days post challenge. The dashed line indicates the time period where mice are being observed and no longer receiving antibiotics or water.

Key: -1 – pre challenge antibiotics commenced. 0 – mice exposed to aerosol challenge. +5 – 5 day treatment group completed antibiotics; post challenge antibiotics commenced. +8 – 9 day pre challenge treatment completed. +12 – 7 days post challenge treatment completed. +15 – 16 days pre challenge treatment completed. +19 – 14 days post challenge antibiotics completed. Group 1 – Doxycycline monohydrate or water commenced on the day of challenge and continued for 5 days. Each group contained 5 mice and were euthanised at completion of treatment (day 5). Group 2 – Doxycycline monohydrate or water commenced 1 day prior to challenge for 9 days. Five mice were euthanised in treatment group and water group at completion of therapy (day 8), 10 mice euthanised in treatment group at end of study (day 35). Group 3 - Doxycycline monohydrate or water commenced 1 day prior to challenge for 16 days. Five mice euthanised in treatment group and water group at completion of therapy (day 15), 10 mice euthanised in treatment group at end of study (day 35). Group 4 - Doxycycline monohydrate or water commenced 5 days post challenge and continued for 7 days. Five mice euthanised in treatment group and
water group at completion of therapy (day 12), 10 mice euthanised in treatment group at end of study (day 35). **Group 5** - Doxycycline monohydrate or water commenced 5 days post challenge and continued for 14 days. Five mice euthanised in treatment group and water group at completion of therapy (day 19), 10 mice euthanised in treatment group at end of study (day 35). **Group 6** – Water (DiH$_2$O) was initiated at day 5 and continued for 14 days. All 5 mice in this group were euthanised at completion of the study (day 35) for comparison with the treatment groups.

Mice were euthanised at 6 time points, day 5, 8, 12, 15, 19 and 35 post challenge. These time points were selected because they correspond to the completion of antibiotic therapy (day 8 and 15 for pre challenge groups, and day 12 and 19 for post challenge groups) or the end of the study. The water treated groups provide the positive control for comparison to the treated groups so that antibiotic efficacy can be assessed. The water treated control mice which were euthanised at day 5 provided an assessment of the bacterial burden at the point where mice start to lose weight. At post mortem the lungs, spleen, brain and testes were weighed and along with bone marrow, adipose and blood processed for bacterial colonisation. Bacteria were quantified by culture and RT-PCR for study 3 to gain more information about the disease process and to assess for clearance of viable bacteria. The results from the control groups of mice treated with water only will be presented first to enable an increased understanding of the disease in the AJ mouse model, followed by the results from the doxycycline monohydrate efficacy study.

### 5.3. AJ mouse pathogenesis study

The number of scheduled interim post mortems, the lengthened study duration, and broad range of tissue sites taken for microbiological analysis allowed an in depth look at the pathogenesis of *C. burnetii* in this model. Thirty mice were exposed to mean retained dose of 6.7 x 10$^4$ PCR-CFU of *C. burnetii* via the aerosol route. All mice were provided with 50 µl of distilled water (diH$_2$O) via the oral route twice daily for varying lengths of time in accordance with the antibiotic efficacy study 3 (Figure 1). Five mice were euthanised at interim time points (day 5, 8, 12, 15, 19 and 35) and lung, spleen, testes, adipose and brain weighed and processed for bacterial quantification by culture and RT-PCR. Blood, bone marrow and urine were also taken for bacterial quantification. Seminal fluid was isolated to evaluate the mechanism of sexual transmission of *C. burnetii* in the AJ mouse model.
5.3.1. Percentage body weight loss from baseline during the study for the water treated control mice

A total of 30 mice were included in the water treated control groups. The groups were combined for weight loss analysis. The mice started to lose weight at day 5 post challenge (Figure 2). Reaching maximal weight loss of 15% from baseline at day 8 before returning to baseline weight at day 11 (Figure 2). From day 11 the mice continued to gain weight until completion of the study at day 35 (Figure 2). Clinical signs correspond to the weight loss profile with signs of ruffling in up to 100% of mice from day 5 to 12. One mouse became immobile at day 7 from the group treated with 16 days of water commenced 1 day prior to challenge and was originally scheduled to be euthanised at day 15. This mouse had lost 18.1% of his body weight from baseline and had displayed ruffled fur in the preceding 2 days, and along with immobility had reached a humane end point and was euthanised.

![Graph showing weight loss in AJ mice challenged with an aerosol challenge of a mean retained dose of 6.7 x 10^4 PCR-CFU of C. burnetii and treated with 50 μl of water twice daily orally. Five mice were euthanised at each time point marked. Data shown is the mean percentage weight loss from baseline +/- standard error of the mean (SEM). The red arrow represents the time point where 1 mouse was euthanised due to reaching a humane end point.]

**Figure 2:** Weight loss in AJ mice challenged with an aerosol challenge of a mean retained dose of 6.7 x 10^4 PCR-CFU of *C. burnetii* and treated with 50 μl of water twice daily orally. Five mice were euthanised at each time point marked. Data shown is the mean percentage weight loss from baseline +/- standard error of the mean (SEM). The red arrow represents the time point where 1 mouse was euthanised due to reaching a humane end point.
5.3.2. Organ weight as a percentage of body weight for water treated control mice

Organ weights for lung, spleen and testicles were measured at autopsy and compared for the water treated groups at different time points against a baseline weight for each organ in a group of 5 male AJ mice not exposed to *C. burnetii*. Adipose and brain were weighed at autopsy to enable a weight based standardisation of bacteriology. Spleen weight is a marker of the immune response to the bacterial challenge, with splenomegaly being associated with extensive proliferation of immunoglobulin producing B cells(3). The *C. burnetii* challenge was given via an aerosol route yet the lung weight was not significantly different as a percentage of body weight in comparison to the uninfected control group of mice (Figure 3a.). The spleen was significantly enlarged compared to the uninfected mice at day 8, 12, 15, 19 and 35 (\( p < 0.05 \), Figure 3a.). The testicles were not significantly enlarged compared to the uninfected controls (Figure 3.).
Figure 3: Organ weight as a percentage of body weight for AJ mice challenged via the aerosol route with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii* and provided with water but no antibiotic therapy. Five mice were euthanised at day 5, 8, 12, 15, 19 and 35. (a) Lung and spleen weight as a percentage of body weight. The splenic weight at day 8, 12, 15, 19 and 35 were significantly greater than the splenic weight of the uninfected mouse spleens ($p < 0.05$). (b) Testicle weight as a percentage of body weight. Values that are significantly different by a one-way ANOVA with Tukey multiple comparisons from the organ weights of the baseline, uninfected mouse organs are marked with an asterisk (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$)
5.3.3. Bacterial dissemination in water treated control mice

Bacterial burden in multiple organ sites was assessed by culture and RT-PCR (bacterial counts displayed in Tables 1 to 9 – Appendix A). The sites examined were the lung, spleen, brain, adipose, bone marrow, and testicles. Due to the inability to culture C. burnetii in axenic media until 2009(168), publications have used PCR to quantify bacterial burden. Therefore, RT-PCR was performed on lung, spleen, blood and testicle samples for comparison to the viable counts.

In the water treated control groups bacterial colonisation, as assessed by viable counts, follows the inverse weight loss profile of the control mice. By viable counts, bacterial dissemination to all organ sites except the adipose tissue can be seen at day 5 (Figure 4), the beginning of weight loss. Maximal dissemination occurs by day 8, which is the peak of weight loss, with all sites showing viable counts in 20-100% of mice (Figure 4). Bacterial dissemination then starts to reduce with only 2/5 mice (40%) showing lung colonisation by viable counts at day 35 (Figure 4). By RT-PCR the lung and spleen remain colonised in 100% of mice at day 35 demonstrating the benefit of calculating bacterial colonisation by culture and RT-PCR.
**Figure 4:** Heat map demonstrating the dissemination of *C. burnetii* in AJ mice challenged via the aerosol route with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii*. There were 5 mice per group. The groups were provided with water but no antibiotic therapy. Bacterial colonisation was assessed by culture and RT-PCR for comparison.

5.3.4. Bacterial burden in lungs, spleen and blood of water treated control mice

The mice were challenged via the aerosol route with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii* and a median count of $4 - 5 \times 10^6$ counts/g were found within the lungs at day 5 to 8 (Figure 5a). By day 35, 2 mice had viable counts remaining in the lungs, whilst the remaining 3 mice had cleared the infection (Figure 5a). By contrast the PCR results remain higher with DNA isolated from all mice in the lung at day 35 at a median of $5 \times 10^5$ PCR-CFU/g (Figure 5a). The viable counts were significantly higher at day 5-8 compared to the end of the study (day 35) (Figure 5a, $p < 0.01$). The RT-PCR counts are not significantly different at any timepoint.

Spread from the lungs to the spleen is seen in all water control groups, with viable counts being seen in 100% of mice from day 5 to 19. The median bacterial growth in the spleen ranged from $2 - 3 \times 10^5$ at day 5 to 8. These values are significantly higher than the counts at day 35, where none of the mice have viable bacteria in their spleens ($p < 0.01$, Figure 5b). To confirm splenic clearance by day 35 the spleens at day 35 were placed in ACCM-2 broth and incubated for 7 days at 2.5% $O_2$, 5% $CO_2$. No growth was seen when plated out after 7 days. This method had been confirmed with spleen from non-exposed control mice spiked with known concentrations of *C. burnetii* to ensure splenic tissue did not inhibit growth. Bacterial growth was demonstrated on these test plates (data not included). Bacterial DNA was quantifiable by PCR at all timepoints in the spleen. The highest count by PCR was seen at day 8 with a median of $1.7 \times 10^6$, this was significantly higher than the median count at day 35 of $2.3 \times 10^4$ ($p < 0.05$, Figure 5b).
Figure 5: Bacterial colonisation in the lung and spleen of AJ mice challenged via the aerosol route with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of \textit{C. burnetii}. There were 5 mice per group. The groups were provided with water but no antibiotic therapy. Bacterial counts were enumerated by culture and RT-PCR. (a) The bacterial count in the lung. (b) The bacterial count in the spleen. The individual results are displayed along with the median and interquartile range. Values within groups that are significantly different by the non-parametric Kruskall Wallis test are marked with an asterisk ($** p < 0.001$, * $p < 0.01$, * $p < 0.05$).
Blood is an important diagnostic marker for clinical practice. Serology and PCR are the tests available for a diagnosis of Q fever in humans. Serology will not be positive for the first 2 weeks and requires an acute and convalescent sample for confirmation (106). This can delay diagnosis for up to 4 weeks. Therefore, PCR in the early stages of disease would reduce the diagnostic delay and consequently any delay in antibiotic initiation.

Bacteria in the blood was enumerated by viable counts and PCR from day 5 to 19 for comparison. Bacteria are known to remain in the blood in untreated mice for up to 12 days. At day 5 to 8 100% of mice had bacteria isolated in the blood by both culture and PCR (Figure 6). By day 12 only 40% have viable counts and 60% have DNA isolated by PCR (Figure 6). By culture the bacteria in the blood at day 8 was significantly higher than at day 12, 15 and 19 ($p < 0.05$, Figure 6). By RT-PCR the bacteria in the blood at day 5 and 8 are significantly higher than at day 15 and 19 ($p < 0.05$, Figure 6).

**Figure 6:** Quantification of bacteria in the blood of AJ mice following an aerosol challenge with a mean retained dose of $6.7 \times 10^4$ of *C. burnetii*. There were 5 mice per group. The groups were provided with water, but no antibiotic therapy. Bacterial counts were assessed by culture and PCR. The individual results are displayed along with the median and interquartile range. Values within groups that are significantly different by the non-parametric Kruskall Wallis test are marked with an asterisk ($*** p < 0.001$, ** $p < 0.01$, * $p < 0.05$).
5.3.5. Isolation of bacteria from the reproductive system of water treated control mice

Homogenised testicular tissue containing seminal fluid, testicular cells and spermatozoa were cultured from all mice on ACCM-2 plates in quadruplicate and viable counts observed in 1/5 (20%) mice at day 5 and 2/5 (40%) mice at day 8, all other samples showed no detectable growth. (Figure 18). By PCR, 2/5 mice (40%) had DNA isolated at day 8 (Figure 7). These were the same mice that had viable counts on bacterial culture. At day 12, 1/5 (20%) had bacterial DNA isolated (Figure 7). All mice at days 5, 15, 19 and 35 were negative by PCR.

Figure 7: Quantification of bacteria in the testicles of AJ mice following an aerosol challenge with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii*. There were 5 mice per group. The groups were provided with water, but no antibiotic therapy. Bacterial counts were assessed by culture and PCR. The individual results are displayed.

Spermatozoa were isolated from the testicles by passive transfer into PBS after the testicle was gently diced in a sieve. The samples were stained using the primary antibody (anti *C. burnetii*-mouse LPS antibody), the secondary antibody (goat anti-mouse IgG-FITC) and a DNA stain (Dapi) and imaged using a confocal microscope (Figure 8). In contrast to uninfected mice, cellular debris could be seen over the head and midpiece of the sperm, suggestive of an inflammatory process but with no associated bacteria (Figure 8 a,b). *C. burnetii* could be seen in adjacent cells under microscopy from the 2 mice with viable growth at day 8 (Figure 8 c,d).
a. Abnormal inflammatory debris around the sperm head and midpiece.

Mitochondria sheath

Nucleus

5 μm

b. Flagellum

Mitochondrial sheath around mid-piece

Nucleus

Smooth head of normal sperm

5 μm
Figure 8: Spermatozoa from male AJ mice visualised by confocal microscopy. (a) Spermatozoa from an unexposed AJ mouse. The head and midpiece are smooth and no evidence of *C. burnetii* is present. (b) Spermatozoa from a mouse exposed to an aerosol challenge of a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii*. There is abnormal inflammatory debris over the head and midpiece of the spermatozoa, but no evidence of *C. burnetii*. (c) A collection of spermatozoa and surrounding cells from within the testicles of a mouse exposed to an aerosol challenge with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii*. *C. burnetii* can be seen within an adjacent cell but no *C. burnetii* attached to the spermatozoa. (d) This image is the same as (c) with the grey scale removed to clearly demonstrate the *C. burnetii* infection in the adjacent cell and not attached to the spermatozoa. (*Blue = DAPI, DNA stain, Green – mouse anti *C. burnetii* LPS monoclonal FITC stain*)

5.3.6. Cytokine response to *C. burnetii* exposure in water treated control mice

Macrophages are an essential part of the immune response to *C. burnetii*. Polarisation towards the M1 phenotype leads to the release of inflammatory cytokines(141), IFN-γ, TNF-α, IL-6 and IL-12 which triggers the release of nitric oxide (NO) and reactive oxygen intermediates (ROI) leading to cell death(140). Macrophages stimulated towards an M2 phenotype excrete anti-inflammatory cytokines such as IL-10 which promotes tissue remodelling and repair(140,141).

Serum from mice exposed to an aerosol challenge with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii* and provided with water but no antibiotic treatment were tested for cytokines known to be involved in the acute response to *C. burnetii* infection. IFN-γ, TNF-α, IL-6 and IL-12 were all significantly raised at day 5 compared to the control group of 5 mice that were not exposed to *C. burnetii* ($p < 0.05$, Figure 9 a-e). IFN-γ, TNF-α were also significantly raised at day 8 compared to the unexposed control mice ($p < 0.05$, Figure 9 a,b), and IFN-γ remained significantly raised at day 12 compared to the unexposed control mice ($p < 0.01$, Figure 9a).
a. IFN-y (ng/ml) over different days following exposure to C. burnetii.

b. TNF-alpha (ng/ml) over different days following exposure to C. burnetii.

Significance levels indicated by asterisks:** p < 0.01, *** p < 0.001.
c. Days/Treatment Group

IL-2 (ng/ml)

5
No C. burnetii exposure
5 8 12 15 19 35

*d*

1
10
100

DAYS/TREATMENT GROUP

IL-4 (ng/ml)

5
No C. burnetii exposure
5 8 12 15 19 35

*c.

1
10
100

DAYS/TREATMENT GROUP
Figure 9: Quantification of the acute cytokine response seen in AJ mice following an aerosol challenge with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii*. There were 5 mice per group. The groups were provided with water, but no antibiotic therapy. One group of 5 mice who were not exposed to *C. burnetii* but provided with water are shown as a negative control for the cytokine response. (a) IFN-γ. (b) TNF-α. (c) IL-2. (d) IL-4. (e) IL-6. (f) IL-10 (g) IL-12. The individual results are displayed along with the mean +/- standard error of the mean (SEM). Values within groups that are significantly different by a the non-parametric Kruskall Wallis test are marked with an asterisk (** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).
5.4. AJ mouse antibiotic efficacy study 3

The aim of this study was to assess the efficacy of doxycycline monohydrate commenced 1 day pre challenge versus 5 days post challenge in comparison to their water control groups. As described in section 5.2, in this study doxycycline monohydrate at 105 mg/kg twice daily was commenced either 1 day pre or 5 days post an aerosol challenge with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of C. burnetii. Antibiotics were continued for either 7 or 14 days post challenge (Figure 1). This meant that pre challenge mice were treated for a total of 9 or 16 days to ensure they completed a full course of treatment post challenge. This allowed an adequate comparison to the post challenge groups treated for 7 or 14 days. There were 15 mice per antibiotic group, 5 mice were euthanised at the completion of therapy and 10 mice at the end of the study (day 35), alongside water treated control mice for comparison.

5.4.1. Percentage weight loss from baseline in groups of mice treated with doxycycline monohydrate commenced pre and post challenge versus water treated control groups.

The water control group start to lose weight at day 5 reaching maximal weight loss by day 8 and returning to baseline by day 12 (Figure 10). Doxycycline monohydrate commenced pre challenge for a total of 9 and 16 days significantly prevented weight loss from day 6 to 9 compared to the water control group ($p < 0.01$, Figure 10a). From day 16 to 28 the pre challenge group treated for 9 days showed a significant increase in weight compared to the water treated controls. The group treated for 16 days showed a significant weight gain compared to the water controls from day 16 to 19 (Figure 10a). The reduction in weight loss correlated with clinical signs. In the pre challenge group treated for 9 days, up to 2/15 (13%) mice showed ruffling at day 7 and 8. There were no clinical signs recorded in the group treated pre challenge for 16 days.

Doxycycline monohydrate initiated 5 days post challenge and treated for 7 days significantly reduced weight loss from day 7 to 9 compared to the water control group ($p < 0.05$, Figure 10b). The group treated for 14 days significantly reduced weight loss at day 8 only compared to the water controls ($p < 0.05$, Figure 10b). In the post challenge group treated for 7 days, clinical signs correlated with weight loss with up to 9/15 (60%) mice displaying ruffled fur from day 5 to 10, maximum signs
were seen at day 6. In the group treated post challenge for 14 days up to 10/15 (75%) mice displayed ruffled fur from day 5 to 10, with the maximum number of mice showing signs at the peak of weight loss from day 7 to 9. Clinical signs are greater in the water control groups compared to all of the treatment groups with up to 100% of mice displaying ruffled fur from day 5 to 12. One mouse was found immobile in his cage and was euthanised at day 7 post challenge in the group treated with water for 16 days initiated pre challenge.
Figure 10: Efficacy of doxycycline monohydrate (DM) 105 mg/kg twice daily commenced 1 day pre or 5 days post challenge in AJ mice exposed by aerosol to a mean retained dose of 6.7 x 10^4 PCR-CFU of C. burnetii. Data shown is mean percentage weight loss from baseline +/- standard error of the mean (SEM). Five mice were euthanised at each cull point marked. (a) Weight loss in the pre exposure treatment groups. DM was commenced 1 day prior to the C. burnetii challenge and continued for a total of 9 or 16 days. (b) Weight loss in the post challenge treatment groups. DM was commenced 5 days post C. burnetii challenge and continued for 7 or 14 days. Significantly different values as determined by a Two-way RM ANOVA with Tukey multiple comparisons are indicated by an asterisk. (*P<0.05, **P<0.01, ***P<0.001)

5.4.2. Organ weight as a percentage of body weight for doxycycline monohydrate treatment groups versus the water treated control group

Organ weights for lung, spleen and testicles for all treatment groups were measured at autopsy and compared to their water treated controls at the same timepoint and against the baseline mice that were not exposed to C. burnetii. Comparison between the water treated controls and the untreated mice are not shown in this section but can be seen in section 5.3.2. Spleen weight is a marker of the immune response to the bacterial challenge(3).

In the pre-challenge groups, 9 days of doxycycline monohydrate significantly protected the lungs and spleen from organ enlargement compared to the water treated controls at day 8 (p < 0.05, p < 0.0001 respectively, Figure 11 a, b). Treatment for 16 days commenced pre challenge significantly reduced splenomegaly compared to their water treated controls at day 15 (p < 0.0001, Figure 11 a, b), there was no difference in lung enlargement between the groups at day 15. This is reflective of the time point of this euthanisation which occurred at day 15 when the weight loss in water treated mice had returned to baseline (Figure 10a), and the assumption that the acute infection had resolved in the control mice. By the end of the study (day 35) there was no difference in lung or spleen enlargement in the treatment or water control groups consistent with resolution of acute infection (Figure 11 a, b).

In the post challenge treatment groups, there was no significant difference in lung enlargement between the treatment groups and water treated controls or uninfected mice at any time point (Figure 11c). This is again consistent with the return to
baseline weight of the water control group at the cull timepoints (day 12 and 19),
and likely resolution of the acute infection in the lung. Doxycycline treated for 7 and
14 days initiated 5 days’ post challenge significantly protected from splenomegaly
by completion of therapy in both groups ($p < 0.0001$, $p < 0.01$ respectively, Figure
11d).

There was no difference in testicular weight as a percentage of body weight
between any of the treatment groups and their water controls (data not shown).
a. **Mean lung weight (% of body weight)**

- Baseline (uninfected A/J mice) Day 8
- 9 days pre-challenge DM Day 8
- 9 days pre-challenge DM Day 10
- 16 days water control Day 8
- 16 days water control Day 10
- 0 days pre-challenge DM Day 20
- 0 days pre-challenge DM Day 25
- 16 days water control Day 25
- 14 days water control Day 35

b. **Mean spleen weight (% of body weight)**

- Baseline (uninfected A/J mice) Day 8
- 9 days pre-challenge DM Day 8
- 9 days pre-challenge DM Day 10
- 16 days water control Day 8
- 16 days water control Day 10
- 0 days pre-challenge DM Day 20
- 0 days pre-challenge DM Day 25
- 16 days water control Day 25
- 14 days water control Day 35
Figure 11: Organ weight as a percentage of body weight for AJ mice challenged via the aerosol route with a mean received dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii*. Mice were treated with 9 or 16 days of doxycycline monohydrate (DM) initiated 1 day prior to challenge or 7 or 14 days of DM initiated 5 days post challenge. Five mice were euthanised at the completion of therapy along with their water treated controls. Ten mice were euthanised in each treatment group and 5 mice in the water treated control group at completion of the study (day 35). (a) The lung weights from AJ mice treated with a total of 9 or 16 days of doxycycline monohydrate initiated 1 day pre challenge or their water treated controls. (b) The spleen weights from AJ mice treated with 9 or 16 days of doxycycline monohydrate initiated 1 day pre challenge or their water treated controls. (c) The lung weights from AJ mice treated with 7 or 14 days of doxycycline monohydrate initiated at 5 days post challenge or their water treated controls. (d) The spleen weights from AJ mice treated with 7 or 14 days of doxycycline monohydrate initiated at 5 days post challenge or their water treated controls. Significantly different values from the baseline, uninfected mouse organs as determined by a One-way ANOVA with Tukey multiple comparisons are indicated by an asterisk. (*$P<0.05$, **$P<0.01$, ***$P<0.001$)

5.4.3. Bacterial burden in doxycycline monohydrate treated groups compared to their water treated control group.

Bacterial burden in multiple organ sites was assessed by culture and RT-PCR (Appendix B, tables 1-9). The sites assessed by culture were lung, spleen, blood, bone marrow, brain, adipose and testes. The sites assessed by RT-PCR were lung, spleen and blood. As previously discussed, the majority of *C. burnetii* studies quantify the bacteria by RT-PCR due to the difficulty in culturing the bacteria. PCR detects viable and non-viable bacteria and therefore the results differ to viable counts. The PCR was therefore useful to describe the bacterial burden over the course of the disease and also for comparison to previous published studies. A comparison between culture and PCR is demonstrated in figures 12 a-c.

Mice treated for 9 days initiated pre challenge only had viable counts within the lungs, this is in comparison to the water treated control group where dissemination is seen at day 8 post challenge to all organ sites tested (Figure 12a). Mice treated for 7 days initiated 5 days' post challenge had viable counts in lung and spleen at day 12 post challenge. The number of mice with splenic colonisation at day 12 was reduced following 7 days of treatment in comparison to their water control (Figure 12a).
Mice treated for 16 days initiated pre challenge had viable counts only in their lungs at completion of therapy (day 15) (Figure 12b). In comparison, the water control group euthanised at day 15 had bacterial dissemination to spleen, brain and adipose tissue (Figure 12b). In addition, the 16 days of treatment had reduced the number of mice with lung colonisation as determined by viable counts at day 15 compared to their water controls (Figure 12b). By the completion of 14 days of antibiotics commenced post challenge mice had viable counts in the lung and spleen in both the treatment and water control group but no bacteria isolated from other organ sites (Figure 12b).

By day 35 the pre challenge treatment groups show the highest rate of bacterial colonisation in the lungs out of all of the groups by viable counts. Sixty percent (6/10) and 88% (7/8) of mice had viable counts in the lungs in the pre challenge group treated for 9 days and the pre challenge group treated for 16 days respectively (Figure 12c). The pre challenge group treated for 16 days also had viable counts in the spleens of 50% (4/8) mice (Figure 12c). In comparison the post challenge groups had only 20% of mice (2/10) with viable counts in their lungs at day 35 after both 7 and 14 days of treatment. This is in comparison to 40% (2/5) mice in the water control group with viable counts in the lung at day 35 (Figure 12c).

The benefit in performing both culture and PCR to assess the bacterial spread can be seen in the heat maps (Figure 12 a-c). By culture the mice have no viable counts in the pre challenge groups, and it would not be known if the bacteria had disseminated past the lungs by completion of antibiotics unless the PCR had been performed (Figure 12 a, b). The PCR shows that 100% of mice in the pre challenge groups had seen dissemination to their spleens (Figure 12 a, b).
### a. % mice with bacterial colonisation in different tissue sites

<table>
<thead>
<tr>
<th></th>
<th>Pre challenge</th>
<th>Post challenge</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Doxycycline 9 days</td>
<td>Doxycycline 7 days</td>
</tr>
<tr>
<td></td>
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<td>(call day 12)</td>
</tr>
<tr>
<td>Culture</td>
<td>Culture</td>
<td>Culture</td>
</tr>
<tr>
<td>PCR</td>
<td>PCR</td>
<td>PCR</td>
</tr>
<tr>
<td>Lung</td>
<td>100%</td>
<td>80.1 – 99.9%</td>
</tr>
<tr>
<td>Spleen</td>
<td>0%</td>
<td>0.1 - 20%</td>
</tr>
<tr>
<td>Blood</td>
<td>40.1 - 60%</td>
<td>20.1 - 40%</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>60.1 - 80%</td>
<td>40.1 - 60%</td>
</tr>
<tr>
<td>Brain</td>
<td>80.1 – 99.9%</td>
<td>60.1 - 80%</td>
</tr>
<tr>
<td>Adipose</td>
<td>100%</td>
<td>80.1 – 99.9%</td>
</tr>
<tr>
<td>Testes</td>
<td></td>
<td>100%</td>
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</tbody>
</table>

### b. % mice with bacterial colonisation in different tissue sites

<table>
<thead>
<tr>
<th></th>
<th>Pre challenge</th>
<th>Post challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Doxycycline 16 days</td>
<td>Doxycycline 14 days</td>
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<tr>
<td></td>
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<td>(call day 19)</td>
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<tr>
<td>Culture</td>
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<tr>
<td>PCR</td>
<td>PCR</td>
<td>PCR</td>
</tr>
<tr>
<td>Lung</td>
<td>100%</td>
<td>80.1 – 99.9%</td>
</tr>
<tr>
<td>Spleen</td>
<td>0%</td>
<td>0.1 – 20%</td>
</tr>
<tr>
<td>Blood</td>
<td>40.1 - 60%</td>
<td>20.1 – 40%</td>
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<tr>
<td>Bone marrow</td>
<td>60.1 - 80%</td>
<td>40.1 – 60%</td>
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<tr>
<td>Brain</td>
<td>80.1 – 99.9%</td>
<td>60.1 – 80%</td>
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<tr>
<td>Adipose</td>
<td>100%</td>
<td>80.1 – 99.9%</td>
</tr>
<tr>
<td>Testes</td>
<td></td>
<td>100%</td>
</tr>
</tbody>
</table>
Figure 12: Heat map demonstrating the dissemination of *C. burnetii* in AJ mice challenged via the aerosol route with a mean retained dose of 6.7 x 10^4 PCR-CFU of *C. burnetii*. There were 5 mice per group, with the exception of the antibiotic treatment groups at day 35 which contained 10 mice per group. The groups were treated with doxycycline monohydrate for 9 or 16 days commenced 1 day pre challenge, or 7 or 14 days of doxycycline monohydrate commenced 5 days post challenge. Control groups treated with water and not antibiotics were euthanised alongside the treatment groups for comparison. Bacterial colonisation was assessed by culture and RT-PCR for comparison. The colour coding corresponds to the percentage of mice with bacteria isolated in the organ investigated by either culture or RT-PCR. (a) Groups of mice treated with doxycycline monohydrate commenced 1 day pre challenge and continued for 9 days, compared to treatment commenced at 5 days’ post challenge and continued for 7 days alongside their water controls. (b) Groups of mice treated with doxycycline monohydrate commenced 1 day pre challenge and continued for 16 days, compared to treatment commenced at 5 days’ post challenge and continued for 14 days alongside their water controls. (c) Bacterial colonisation in all treatment groups and water control group at the end of the study (day 35).

In summary, by the end of the study (day 35) the groups of mice where treatment was initiated post challenge had a lower bacterial burden in their lung and spleen as assessed by viable counts and RT-PCR than the pre challenge groups. By viable
counts the pre challenge treatment groups also had higher bacterial colonisation in their lung and spleens compared to the water treated control group. There was no dissemination past the spleen in any group by the end of the study.

5.4.4. Bacterial colonisation in the lung, spleen and blood of AJ mice

The bacterial colonisation in the lung and spleen of AJ mice challenged via the aerosol route with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii* was quantified by viable counts and PCR. The groups were provided with doxycycline monohydrate for 9 or 16 days’ post challenge commenced 1 day pre challenge, or 7 or 14 days of doxycycline monohydrate commenced 5 days post challenge. Control groups treated with water and not antibiotics were euthanised alongside the treatment groups for comparison. The bacterial burden in the lung and spleen was reduced in the groups treated for the pre challenge groups treated for 9 or 16 days compared to their water controls. This was significant in the pre challenge group treated for 9 day by viable counts and RT-PCR ($p < 0.05$, Figure 13 a, b). The same trend is seen in the groups treated for 7 or 14 days post challenge with a reduction in bacterial burden in all treatment groups compared to their water controls (Figure 13 c, d). This reached significance in the lung by viable counts for the group treated for 7 days’ post exposure ($p < 0.05$, Figure 13c). At completion of the study (day 35) the group treated pre challenge for 16 days showed significantly higher bacterial burden by viable counts in their lungs and spleen compared to both post challenge treatment groups ($p < 0.05$, Figure 13 e, f). The pre challenge group treated for 16 days also had significantly higher viable counts in their lung compared to the pre challenge group treated for 9 days ($p < 0.05$, Figure 13e). DNA quantification by RT-PCR shows higher counts in the lung and spleen of the water control group at day 35 than all of the treatment groups (Figure 13 e, f). This difference is significantly higher in the spleen of the water control group compared to the groups treated pre challenge for 16 days and post challenge for 14 days ($p < 0.05$, Figure 13f).
LOD
Lung
counts/g
PCR
7D DM post challenge (cull D12)
7D water post challenge (cull D12)
14D DM post challenge (cull D19)
14D water post challenge (cull D19)
Viable counts PCR
c.
d.
Spleen}

LOD
Spleen
counts/g
PCR
7D DM post challenge (cull D12)
7D water control (cull D12)
14D DM post challenge (cull D19)
14D water control (cull D19)
Viable counts PCR
c.
d.
Figure 13: Bacterial colonisation in the lung and spleen of AJ mice challenged via the aerosol route with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii*. There were 5 mice per group, apart from the pre challenge water group treated for 16 days, which contained 4 mice. The groups were provided with doxycycline monohydrate (DM) for 9 or 16 days commenced 1 day pre challenge, or 7 or 14 days DM commenced 5 days post challenge. Control groups treated with water and not antibiotics were euthanised alongside the treatment groups for comparison. Bacterial counts were enumerated by culture and PCR. (a) The bacterial count in the lung for the groups treated 1 day pre challenge for 9 or 16 days. (b) The bacterial count in the spleen for the antibiotic groups treated pre challenge for 9 or 16 days. (c) The bacterial count in the lung for the antibiotic groups treated with 7 or 14 days of antibiotic initiated 5 days post challenge. (d) The bacterial count in the spleen for the antibiotic groups treated for a total of 7 or 14 days challenge with treatment initiated 5 days post challenge. (e) The bacterial count in the lung of all treatment groups in comparison to the water treated control at the end of the study (day 35). (f) The bacterial count in the spleen of all treatment groups in comparison to the water treated control at the end of the study (day 35). The individual results are displayed along with the median and interquartile range. Values within groups that are significantly different by Kruskall Wallis test are marked with an asterisk (**p< 0.001, *p< 0.01, * p < 0.05).
Doxycycline monohydrate treatment initiated either pre or post challenge prevented dissemination to the blood at completion of therapy in all groups when assessed by viable counts. All treatment groups commenced pre and post challenge had no bacteria isolated from blood by culture at completion of therapy. This difference was significant for the group treated for 9 days compared to the water treated control group ($p < 0.01$, Figure 14a). Assessing bacterial colonisation by RT-PCR within the blood suggests that doxycycline monohydrate commenced pre challenge for 9 days, or for 7 and 14 days post challenge did not prevent dissemination to the blood during the acute illness (Figure 14 a, b). At completion of therapy in the pre challenge groups, 2/5 (20%) had bacterial DNA isolated in the blood after 9 days of therapy whilst no mice had DNA isolated after 16 days of therapy (Figure 14a). In the post challenge group treated for 7 days, 4/4 (100%) had DNA isolated and in the 14 day post challenge group 1/5 (10%) had DNA isolated in the blood (Figure 14b).
Figure 14: Bacterial quantification in the blood of AJ mice challenged via the aerosol route with a mean retained dose of $6.7 \times 10^4$ cfu of *C. burnetii*. There were 5 mice per group. Groups were treated with 7 or 14 days of doxycycline monohydrate initiated either 1 day pre challenge or 5 days post challenge. (a) Bacterial quantification by viable counts and RT-PCR in the blood for mice treated with a total of 9 or 16 days of antibiotics commenced 1 day pre challenge (b) Bacterial quantification by viable counts and RT-PCR in the blood for mice treated with 7 or 14 days of antibiotics commenced 5 days’ post challenge. The individual results are displayed along with the median and interquartile range. Values within groups that are significantly different by Kruskall Wallis test are marked with an asterisk (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).
5.4.5. Cytokine response to *C. burnetii* exposure in untreated mice compared to mice treated with doxycycline monohydrate.

Serum from mice following an aerosol challenge with a mean retained dose of 6.7 x 10^4 PCR-CFU of *C. burnetii* and treated with doxycycline monohydrate for 9 or 16 days started 1 day pre challenge or 7 or 14 days commenced 5 days' post challenge were tested for their cytokine response to infection. Control groups of mice treated for the same duration with water alone were euthanised alongside the treatment groups for comparison. A group of 5 mice not exposed to *C. burnetii* but treated with doxycycline monohydrate for 5 days were included to investigate the immunosuppressive role of the antibiotic. In addition, a group of 5 mice not exposed to *C. burnetii* and given 5 days of water were tested as a negative control group for the experiment. The cytokines of note in the immune response to *C. burnetii* include; IFN-γ, TNF-α, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12.

Levels of IFN-γ in water treated mice at day 8 and 12 compared to both unchallenged groups were significantly higher (*p* < 0.01, Figure 15. a, b). The levels of IFN-γ at day 15 in challenged mice given water compared to the unchallenged mice given water were also significantly higher (*p* < 0.05, Figure 15a). Significant differences were found in levels of TNF-α between the challenged mice treated with water at day 8 and both groups of unchallenged mice (*p* < 0.05, Figure 16a). There was no significant difference seen between the cytokine levels of IFN-γ and TNF-α in all treatment groups compared to their water controls or the unexposed mice (Figure 15-16, Table 10, Appendix B).

No significant differences in the cytokine expression of IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12 were seen between unchallenged mice treated with doxycycline monohydrate or water, and the groups treated with 9 or 16 days of doxycycline pre challenge, or 7 and 14 days of doxycycline post challenge and their water controls (Table 10 and 11, Appendix B).

By the end of the study (day 35), no significant difference was seen between the treatment groups, their water controls or the unchallenged mice for all of the cytokines tested (Table 12, Appendix B).
No significant differences were found for any of the tested cytokines between the unchallenged mice given either water or doxycycline for 5 days.

Figure 15: The IFN-γ levels in AJ mice following an aerosol challenge with a mean retained dose of 6.7 x 10⁴ PCR-CFU of *C. burnetii*. Mice were treated with 9 or 16 days of doxycycline monohydrate commenced 1 day pre challenge, or 7 or 14 days of doxycycline monohydrate commenced 5 days post challenge. The results are compared to groups of mice not challenged with *C. burnetii* and treated with either 5 days of doxycycline monohydrate or 5 days of water prior to culling. (a) IFN-γ levels in mice treated with 9 or 16 days of doxycycline monohydrate commenced 1 day pre challenge. (b) IFN-γ levels in mice treated with 7 or 14 days of doxycycline monohydrate commenced 5 days post challenge. Values within groups compared to the unchallenged groups or their water controls that are significantly different by Kruskall Wallis test are marked with an asterisk (***, p< 0.001, ** p< 0.01, * p < 0.05).
Figure 16: The TNF-α levels in AJ mice following an aerosol challenge with a mean retained dose of $6.7 \times 10^4$ PCR-CFU of *C. burnetii*. Mice were treated with 9 or 16 days of doxycycline monohydrate commenced 1 day pre challenge, or 7 or 14 days of doxycycline monohydrate commenced 5 days post challenge. The results are compared to groups of mice not challenged with *C. burnetii* and treated with either 5 days of doxycycline monohydrate or 5 days of water prior to culling. (a) TNF-α levels in mice treated with a total of 9 or 16 days of doxycycline monohydrate commenced 1 day pre challenge. (b) TNF-α levels in mice treated with 7 or 14 days of doxycycline monohydrate commenced 5 days post challenge. Values within groups that are significantly different by Kruskall Wallis test are marked with an asterisk ($*** p<0.001$, ** $p<0.01$, * $p<0.05$).
5.4.6. Serology titres in treatment and water control groups at completion of the study

The IgG antibody levels in serum that reacted to inactivated (killed) Phase 1 *C. burnetii* in Coxevac were tested at the end of the study (day 35) in groups of 5 AJ mice exposed to an aerosol challenge with a mean retained dose of $6.7 \times 10^4$ cfu of *C. burnetii*. The mice were treated with doxycycline monohydrate for 9 or 16 days of doxycycline monohydrate commenced 1 day pre challenge, or 7 or 14 days of doxycycline monohydrate commenced 5 days post challenge. A control group of 5 mice challenged with *C. burnetii* and provided with water, along with 5 mice that were not exposed to *C. burnetii* were tested for comparison. All groups of challenged mice had significantly higher P1 IgG titres compared to the unexposed mice ($p < 0.001$, Figure 17). Mice treated for 14 days commenced pre challenge had lower titres compared to the water treated controls ($p < 0.05$, Figure 17).

![Graph showing serology titres](image)

**Figure 17**: IgG antibody level to inactivated (killed) Phase 1 *C. burnetii* in coxevac for AJ mice following an aerosol challenge with a mean retained dose of $6.7 \times 10^4$ cfu of *C. burnetii*. 
Mice were treated with 9 or 16 days of doxycycline monohydrate commenced 1 day pre challenge, or 7 or 14 days of doxycycline monohydrate commenced 5 days’ post challenge. Serology titres were assessed at the end of the study (day 35). Five mice were tested from each group. Significance between the groups was assessed using the non parametric Kruskall Wallis test with Dunn’s multiple comparisons. Groups that are significantly different are marked with an asterisk (*** p< 0.001, ** p< 0.01, * p < 0.05)

5.4.7 Splenocyte stimulation assay

The splenocytes produced high levels of IFN-\(\gamma\) (> 1000 ng/ml) in response to the general polyclonal Concanavalin A (ConA) antibody. However, there was no production of IFN-\(\gamma\) in response to the test antigen above the level produced in the unstimulated control (data not included).

5.5 AJ mouse efficacy study 4

The weight loss seen with doxycycline hyclate in AJ mouse efficacy study 1 and 2 considered to be as a consequence of the drug was not present in study 3 using doxycycline monohydrate. As the tolerability study performed by Dstl, Porton Down suggested, doxycycline monohydrate appears to be a better tolerated preparation than doxycycline hyclate in the AJ mouse. In addition, the relapse in weight loss in the doxycycline hyclate groups in study 1 and 2 was not seen with doxycycline monohydrate in study 3. The results from study 3 indicated that doxycycline monohydrate initiated pre challenge prevented weight loss compared to the water control group, but by the end of the study the lungs and spleen had higher bacterial burden than the post challenge treatment groups and water control. This increase in bacterial burden was in the absence of a relapse in weight loss. The hypothesis from this study is that initiation of treatment post challenge at the point of symptom onset (day 5) appears to allow the innate immune system to function. The priming of the immune system in this group in combination with the doxycycline monohydrate appears to reduce the bacterial burden at study completion compared with treatment as prophylaxis.

Study 4 aimed to investigate further the optimum timing of antibiotic initiation to optimise the outcome in the AJ mouse model with regards to reduction in bacterial burden. Doxycycline monohydrate was directly compared to doxycycline hyclate to evaluate if the preparation of doxycycline affects the disease outcome. Further
investigations into the immunomodulatory effects of antibiotic treatment when initiated at different time points were carried out.

In study 4 AJ mice were exposed to an aerosol challenge with a mean retained dose of $2.9 \times 10^4$ PCR-CFU of *C. burnetii*. Groups of 6 mice were treated with either 105 mg/kg of doxycycline monohydrate or 105 mg/kg of doxycycline hyclate commenced 1 day prior to challenge and continued for 16 days, or commenced 2, 5 or 10 days’ post challenge and continued for 14 days (Figure 18). Mice were euthanised 14 days after completion of therapy. A water treated control group were given 50μl of distilled water (diH₂O) twice daily from 1 day prior to exposure for 16 days and euthanised at completion of the study (day 37 for comparison). Daily weights and clinical signs were recorded. At post mortem, lung and spleen weight were recorded for analysis. Weight loss data and organ weight data will be presented below. The bacteriology and splenocyte stimulation investigations will be completed after thesis submission.

**Figure 18**: Study plan for AJ mouse efficacy study 4. Mice were exposed to an aerosol challenge with a mean retained dose of $2.9 \times 10^4$ PCR-CFU of *C. burnetii*. Treatment was with doxycycline monohydrate 105 mg/kg or doxycycline hyclate 105 mg/kg twice daily commenced 1 day prior to challenge and continued for 16 days, or 2, 5 or 10 days after challenge and continued for 14 days. All mice were euthanised 14 days after completion of antibiotics. A control group of mice treated with water commenced 1 day prior to challenge and continued for 16 days were euthanised at study completion (day 37) for comparison. There were 6 mice per treatment group. The dashed line indicates the time period where mice are being observed and no longer receiving antibiotics or water.
5.5.1. Percentage body weight loss from baseline during the study for doxycycline monohydrate and doxycycline hyclate treated groups and the water control

The water treated control group were administered water 50μl twice daily via the oral route for the 16 days (D-1 to D14). Healthy juvenile mice would normally gain weight daily and in the water control group from the day of challenge some weight loss is displayed and at day 6 the increased trajectory of weight loss is seen with maximal weight loss at day 8-9 and the mice return to baseline by day 22 (Figure 19). Following recovery from the weight loss the AJ mice continue to gain weight exceeding their baseline weight as would be seen in young healthy mice. Some of the initial weight loss seen in the water control group is likely due to manipulation and administration of water. This is seen in a group of 6 mice who were not exposed to C. burnetii but were given the same regimen of water as the control group. These mice also lose some initial weight and fail to gain weight until after the water administration is complete at day 14 (Figure 19). Both groups of mice started to gain weight from day 16, 2 days after water administration stopped and then gained weight at the same trajectory.

![Graph showing weight loss as a percentage of baseline in water treated AJ mice following an aerosol challenge with a mean retained dose of 2.9 x 10^4 PCR-CFU of C. burnetii and for unchallenged mice to control for weight loss associated with manipulation and water administration. Mice were given 50μl of distilled water (dH2O) twice daily for 16 days commencing 1 day prior to challenge. There were 6 mice in each group. The mean weight change from baseline with standard error of the mean (SEM) are presented.](image-url)
Doxycycline monohydrate (DM) commenced 1 day prior to exposure significantly reduced weight loss compared to the water control group at days 8 and 9 (Figure 20a, \( p < 0.05 \)). The mice in the DM group weighed significantly less than the water control group at day 0 (the day of challenge) and day 6 (Figure 20a, \( p < 0.05 \)). Both doxycycline monohydrate (DM) and doxycycline hyclate (DH) initiated 1 day prior to challenge led to weight loss over the 1st 7 days in these mice, greater than that seen in the water treated group (Figure 20a). This could have been a drug side effect with both preparations. The weight loss for the doxycycline hyclate group is hard to interpret because 4/6 mice were euthanised at days 3, 5, 16 and 25 after reaching a humane end point.

Treatment commenced 2 days' post challenge significantly reduced weight loss from days 8 to 10 compared to the water control (Figure 20b, \( p < 0.01 \)). Treatment commenced at day 5 post challenge significantly reduced weight loss from days 4 to 6 (\( p < 0.05 \)) and days 8 to 10 (\( p < 0.01 \)) in the DH group, and from days 8 to 10 (\( p < 0.01 \)) in the DM group (Figure 21c). 1 mouse was euthanised at day 17 for reaching the humane end point in the DH treatment group commenced at day 5.

Weight loss was significantly reduced at days 8, 10, 11 and 13 in the DM treatment group commenced at day 10 post challenge (Figure 20d, \( p < 0.05 \)). Whereas no significant reduction in weight was displayed in the DH treated group. Mice treated with DH and DM commenced at day 10 did not display the same level of weight gain as seen by the water treated group after initial weight loss had returned to baseline. This difference was significant at days 23, 25, 26 and 28-32 in the DH group (Figure 20d, \( p < 0.05 \)).

The weight loss as a percentage of baseline weight was compared between the DM and DH treated groups for each regimen using a 2-way ANOVA with Tukey multiple comparisons. The only groups that differed with regards to weight loss were the groups treated with 16 days of antibiotics commenced 1 day prior to challenge (DM/DH -1). At days 17, 21-24 and 26-27 the mice in the DH treated group weighed significantly less that the DM treated group (\( p < 0.05 \)). This is likely due to greater weight loss as a drug side effect in the DH group. In addition, 4/6 mice were euthanised during the study for reaching a humane end point leaving only 2 mice for weight loss analysis by the end of the study. The weight loss has been included up to the point of humane euthanisation for all analyses.
Figure 20: Weight loss as a percentage of baseline in doxycycline monohydrate and hyclate treated groups of AJ mice following an aerosol challenge with a mean retained dose of 2.9 x 10^4 PCR-CFU of C. burnetii. Treatment was commenced at 1 day prior to challenge and continued for 16 days or commenced at days 2, 5 or 10 post challenge and continued for 14 days. One group of mice were given 50µl of distilled water (diH2O) twice daily for 16 days commencing 1 day prior to challenge as a positive control. There were 6 mice per group. (a) Doxycycline monohydrate (DM) and doxycycline hyclate (DH) commenced 1 day prior to challenge and continued for 16 days. 4 mice were euthanised due to reaching the humane end point in the DH treated group. (b) DM and DH commenced 2 days’ post challenge and continued for 14 days. (c) DM and DH commenced 5 days’ post challenge and continued for 14 days. 1 mouse in the DH treated group was euthanised at day 17 for reaching the humane end point. (d) DM and DH commenced 10 days’ post challenge and continued for 14 days. The mean weight change from baseline with standard error of the mean (SEM) are presented. A 2-way ANOVA with Tukey multiple comparisons was
performed to compare weight loss. The arrows along the x-axis represent the time point where mice were euthanised due to reaching a humane end point. The colour of the arrow corresponds to the group. Significant differences between the treatment groups and water control are marked with an asterisk. The colour corresponds to the treatment group (* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \)).

The clinical signs in the treatment groups are summarised as a heat map in figure 21. In the water treated group clinical signs began at day 3 which is 3 days prior to the peak of disease as displayed by weight loss (Figure 19). Ruffled fur remains present in 100% of mice up until day 11 which is when the mice are regaining weight. Ruffled fur continues to be intermittently present until day 29 which is after the mice have regained weight past their baseline weight and appear to be thriving with continued weight gain (Figure 19). Mice in the unchallenged group that were administered water appeared healthy throughout the study.

In the groups where treatment is commenced 1 day prior to the challenge clinical signs begin at a similar point to the water treated control (D3 for DM and D2 for DH). Clinical signs last the duration of the peak of weight loss as seen in the water treated control group for the DM group (D3 – 16). In the DH treated group clinical signs lasted for the duration of the study, up until day 28. The signs are more severe in this group with 25% of mice displaying hunching and 20-33% of mice appearing very hunched at points throughout the study. Within this group 4/6 mice were euthanised due to reaching a humane end point at days 3, 5, 16 and 25. The severe signs and weight loss requiring humane euthanisation at interim time points are only seen in the DH group and are likely due to side effects from the drug as seen in study 1 and 2.

Clinical signs in the treatment groups commenced 2 days’ post challenge began 1-2 days after the water control group and 2-3 days after commencing antibiotics. Clinical signs last the duration of the peak of weight loss as seen in the water treated group, and again the signs appear to be more severe, affecting more mice and for a longer duration in the DH treated group. The DM treated group display signs only intermittently from D4 – 27, with mice showing no signs between D17 - 25. In contrast the DH treated groups show signs for the duration of D5 – 26.
Mice in both treatment groups commencing antibiotics at day 5 post challenge show signs for the duration of D7 – 30. More mice are affected in the DH treated group, and 1 mouse is euthanised in this group for reaching a humane end point.

Clinical signs start at a similar time point to the water treated group for mice where treatment was initiated at day 10 post challenge (D2 for DH group and D3 for DM group). Signs were present for longer in the DM group, up to D36 compared to D25 in the DH group. Although the DH treated mice had a higher percentage of affected mice, up to 100% intermittently from D3 – 25. The D+10 treatment group were not receiving any therapy at the peak of weight loss and therefore as seen in the water treated groups these mice showed more severe ruffling of their fur at the peak of weight loss compared to the mice treated at D+2 and D+5.

The heat map (Figure 21) summarising the severity of clinical signs within each treatment group shows that the groups treated with DH post challenge all display a greater percentage of mice affected than the DM treated groups on the same regimen. Groups where treatment was initiated at D-1, D+10 and the water control all show more severe ruffling compared to the groups initiating treatment at D+2 and D+5. The group treated with DH started 1 day prior to challenge display the most severe signs seen in this study of hunching. Hunching over the course of the study was seen in 5 mice, 3 of which were later euthanised for reaching a humane end point.
**Figure 21:** Heat map representing the clinical signs seen in AJ mice over the course of the study following an aerosol challenge of $2.9 \times 10^4$ PCR-CFU of *C. burnetii*. Groups of 6 mice were treated with either doxycycline monohydrate (DM) or doxycycline hyclate (DH) for 16 days commenced 1 day prior to challenge (DM/DH -1) or for 14 days commenced 2, 5 or 10 days post challenge (DM/DH +2, DM/DH +5, DM/DH +10). A water control treated group was included for comparison. The percentage displayed is the maximum percentage of mice exhibiting the clinical sign at the same time point during the study. Mice in groups DH -1 and DH +5 were euthanised during the study after meeting humane end points. The percentages showed are as a proportion of all remaining mice in the study.

### 5.5.2. Organ weight as a percentage of body weight for doxycycline monohydrate and doxycycline hyclate treated groups and the water control

Lung and spleen weights were recorded for all treatment groups at post mortem and compared to the water control. The organ weights from a group of uninfected male AJ mice were provided as a comparison of baseline organ weight. Spleen weight is a marker of the immune response to the bacterial challenge(3).

The mean lung weights were greater in both groups where treatment was commenced 2 days’ post challenge in comparison to uninfected mice. There was
no significant difference between lung weights in any of the treatment groups compared to the water control group (Figure 22a). The spleen weights were all greater in the treatment and water groups compared to the uninfected controls, demonstrating that the disease process has affected the spleen in all treatment groups with either inflammatory infiltrate (Figure 22b). The spleen weights in baseline unchallenged mice were significantly lower in the D-1 DM group and D+10 DM and D+10 DH group. The spleen weight was significantly greater in the D+10 DM treated group compared to the D+2 DM group (Figure 22b).
**Figure 22:** Organ weight as a percentage of body weight for AJ mice challenged via aerosol route with a mean retained dose of $2.9 \times 10^4$ PCR-CFU of *C. burnetii*. Treatment with doxycycline monohydrate (DM) or doxycycline hyclate (DH) was commenced 1 day prior to challenge and continued for 16 days or commenced at days 2, 5 or 10 post challenge and continued for 14 days. One group of mice were given 50μl of distilled water (dH₂O) twice daily for 16 days commencing 1 day prior to challenge as a positive control. There were 6 mice per group except DH-1 = 2 mice, DH+5 = 5 mice due to humane euthanisation at interim time points. 

(a) Lung weights as a percentage of body weight. 

(b) Spleen weights as a percentage of body weight. Significant differences as determined by One-Way ANOVA with Tukey’s multiple comparisons tests are shown on the graph with significance values (* = $p < 0.05$)

5.6. Discussion

One of the key findings from study 3 was the potentially deleterious effect of using doxycycline as prophylaxis compared to treatment initiation post infection with regards to bacterial burden by the end of the study. Treatment for 9 or 16 days with doxycycline monohydrate commenced 1 day prior to challenge prevented weight loss, but bacteriology at the end of the study showed a higher burden of *C. burnetii* by culture in the lungs of both groups, and the spleen of the group treated for 16 days. This was despite only finding viable counts in the lungs of the pre challenge groups at completion of therapy. This is in contrast to the groups treated for 7 or 14 days commenced around the start of weight loss and clinical signs (day 5 post challenge). At completion of therapy both groups had viable counts isolated in some of the spleens, yet by the end of the study the spleen showed clearance. If infection is via the inhaled route, *C. burnetii* initially multiplies in alveolar macrophages. Late in the incubation period, from 2 days prior to symptoms and 12 days post symptoms, large numbers of bacteria are released into the blood leading to a transient bacteraemia(96). This bacteraemia facilitates the spread of infection to other organs sites such as spleen, liver, bone marrow and the reproductive tract. The onset of symptoms is associated with the emergence of the hosts adaptive immune response which includes an antibody and cell mediated immune response(96). These immune responses work synergistically to restrict and terminate the bacteraemia in conjunction with antibiotics(96). Antibodies facilitate the phagocytosis of the bacteria, but the cell mediated response is critical for clearance of the infection(3)). Doxycycline hyclate as prophylaxis in studies 1 and 2 reduced weight loss whilst on treatment, but after cessation of therapy the mice started to lose weight again along with the group where doxycycline hyclate was
initiated early in the incubation period (1 day post challenge). Doxycycline monohydrate as prophylaxis in study 3 prevented weight loss compared to the group where treatment was started late in the incubation period at symptom onset. Yet, the DM prophylaxis group had a higher disease burden at study completion compared to the post challenge treated mice. Doxycycline has been shown to reduce the pro-inflammatory cytokines, TNF-α, IL1, IL6, IL8 and IFN-γ, increase the anti-inflammatory cytokine, IL10 and reduce the release of ROIs and NO(231,236,239,240,251-255). These cytokines form the basis of the cell mediated response to control the infection, prevent replication and aid eradication from the intracellular compartment. The hypothesis to explain the results from the AJ mouse antibiotic efficacy studies 1 to 3 is that the cell mediated immune response is decreased prior to exposure to the bacteria, then once challenged with C. burnetii antigens the innate and consequent adaptive immune response may be decreased leading to C. burnetii immune evasion. Doxycycline is bacteriostatic not bactericidal and therefore once the antibiotic is stopped the bacteria can continue to replicate. The antibiotic associated immunosuppression may mean that the cell mediated response to infection had not been primed, and whilst the immune response is being activated after the antibiotics stop there may now be a higher bacterial burden ready for dissemination to various organ and tissue sites than if the immune system had been primed at the point of exposure to the bacteria. If onset of symptoms signals the emergence of the adaptive immune response, it may be that the development of symptoms is preferential because this means that the immune system has recognised the intracellular pathogen and is mounting an immune response, which ultimately along with antibiotics will limit replication, dissemination and eventually lead to eradication of the bacteria. There are no published in vivo studies investigating the effect of antibiotics as prophylaxis against C. burnetii, therefore this theory is based on the findings from the 3 AJ mouse studies discussed in this thesis. Further in vivo work is required to assess the immunomodulation of antibiotics and its effect on C. burnetii pathogenesis.

In study 3 the cytokine analysis and splenocyte stimulation assay did not show a difference between the groups. The postulated cytokine suppression was not seen in the unexposed mice given doxycycline in comparison to those given water. Yet, published literature provides evidence that doxycycline decreases the cytokines, ROIs and NO required for prevention of intracellular replication and eradication of this bacteria(231,236,253-255). These findings may not have been replicated in this study because to see the effect of immune downregulation secondary to
doxycycline the immune system first needs to be activated by exposure to a pathogen. Without exposure to C. burnetii there are negligible cytokines present to be suppressed by the antibiotic. Another reason why a difference in cytokines may not be seen is as a consequence of the animal model chosen, different strains of genetically modified mice display different immune functions (10,185). Within the innate immune system AJ mice have a reduction in functioning complement component 5 (C5), Nod-Like Receptor proteins (NLRp) and Neuronal Apoptosis Inhibitory proteins (NAIPs) (185). These make the mouse more susceptible to infection and reduces elimination of the bacteria. The adaptive immune system is also modified in AJ mice, when stimulated by bacteria the immune response tends towards a TH2 helper T cell response. The TH1 response is important in clearing intracellular pathogens and the TH2 response is more commonly associated with exposure to parasitic and other extracellular infections(185). M2 macrophages are more active in TH2 responders, and clearance of intracellular organisms is dependent on IFN-\(\gamma\) induced IL-12 expression which promotes a TH1 response. The genetic modification seen in AJ mice accounts for why they are highly susceptible to C. burnetii, and is why this strain of mice was selected for the C. burnetii mouse model(184). Yet, the genetic modifications will affect the immune function in response to this bacterium and has to be taken into account when interpreting the data. Mice are often used to study the immune system, however there are discrepancies between the innate and adaptive immune system of mice compared to humans which needs to be considered(256). The cytokine profile seen in the water pathogenesis study for mice following C. burnetii challenge is consistent with the profile seen in humans following exposure. A rise in IFN-\(\gamma\), TNF-\(\alpha\), IL-2, IL-4, IL6, IL10 and IL12 was seen at day 5 compared to the uninfected mice, the increased levels decreased over the course of study as the burden of disease lessened. In addition, the preparation of the blood for cytokine analysis was undertaken as per manufacturer’s instructions, but prior to analysis the samples had to be left in 4% paraformaldehyde for 40 hours at 4\(^{\circ}\)c before removal from the level 3 laboratory. This may have affected the results due a heightened level of background interference effectively decreasing the magnitude of cytokine changes. For many of the cytokines tested the results were at very low levels and making it less likely that a significant difference would be seen between the groups. Schoffelen et al(234) also found the cytokine response in mouse whole blood difficult to detect due to technical difficulties. The splenocyte stimulation assay did not display any differences in IFN-\(\gamma\) production between the groups because the
test antigen concentration used to stimulate the splenocytes was too low to provide sufficient stimulation.

The IgG antibody titres to phase I *C. burnetii* in the group treated for 16 days commenced 1 day prior to infection were lower than all other treatment groups and the water control group at completion of the study. This difference was significant in comparison to the water control group. All the groups had titres significantly higher than the unchallenged mice. *C. burnetii* serology is used clinically for Q fever diagnosis and to monitor progression of the disease aiding diagnosis of chronic infection. The antibody titre is a measurement of how much antibody has been produced in response to a specific antigen. It is not clear if antibody titres correlate with protection against infection. For intracellular bacteria induction of cellular and humoral immunity is necessary for maximum protection(257). The size of the innate immune response is proportional to the adaptive cellular response(257). Therefore, if doxycycline as prophylaxis reduces the innate immune response it can be expected that the antibody response to the bacteria will also be reduced. The role and importance of antibodies is unclear, for example in Mycobacterium TB the BCG vaccine induces the cellular immune response which is thought to be the protective response against infection as demonstrated in mouse studies(257). However, the antibodies induced by the vaccine enhance the uptake of mycobacteria by macrophages(257). It is suggested that antibodies prevent infection whereas the cellular immune responses control infection once replication has been established(257). Vaccine studies have shown that antibody titres following vaccination can last for greater than 10 years, and there is no decrease in the titres over the course of this time period(105). In conclusion, the lower titres of PI IgG seen in mice given 16 days of antibiotics initiated pre challenge are of uncertain significance. The humoral and cell mediated immunity following infection are both important for prevention of reinfection and control of the spread of disease, but it is not clear whether the magnitude of the response is important. The common belief is that reinfection does not occur following infection with *C. burnetii*(258). Although, Bernit *et al.* found that patients exposed with occupational exposure to *C. burnetii* had a significantly increased risk of neurological involvement and they postulated that this was due to multiple exposures and reinfection(246). Further work needs to be completed to review the role of antibody titres in control and prevention of reinfection with this bacterium.
Serology is the gold standard for a diagnosis of Q fever (105), but there is a delay of up to 2 weeks for antibody production which can delay diagnosis and consequent treatment. *C. burnetii* can be detected in blood by PCR prior to antibodies, the clearance of bacteria from the blood correlates with the development of antibodies (105). The use of PCR in the early stages of disease can reduce the diagnostic delay. In mouse study 3 the bacteria could be identified in early disease (day 5 and 8) by culture and PCR in 100% of infected mouse provided with water only. The number of mice with detectable DNA then reduced until clearance from the blood at day 19. Bacteria in the blood can therefore be seen to correlate with weight loss and clinical signs in this model. As the weight loss and clinical signs begin to recover in the mice, so does the bacterial burden in the blood. In the groups treated with antibiotics, DNA was detected in some of the blood samples, but this was not as reliable as in the untreated mice. It appears that PCR to diagnose Q fever is useful in the absence of treatment with antibiotics and can be useful once treatment has been initiated but there is a lower sensitivity for diagnosing Q fever. The blood PCR results cannot be relied on to diagnose Q fever infection, but it is useful diagnostic test within the clinical armamentarium.

There are case reports in published literature of possible sexual transmission from men following acute infection to their female partners (42, 259, 260). *C. burnetii* DNA in semen has been identified by PCR and the timing of infection in their sexual partners is inkeeping with sexual transmission. Although in humans it is an association and not a confirmed mechanism of transmission. The female partners could all have been exposed to the bacteria independently of their partners. Studies in animals have demonstrated sexual transmission (261). Kruszewska et al. demonstrated infection in organs and antibody production in female mice following mating with infected male mice. Uninfected male mice in close contact with the infected male mice did not show any signs of infection ruling out environmental spread of the bacteria (261). In this study Kruszewska performed electron microscopy on the spermatozoa and concluded that *C. burnetii* was seen attached to the head of the sperm. This study was completed in 1993 when the organism could not be confirmed by culture or PCR, and specific stains were not used to confirm the presence of *C. burnetii*. The results of the confocal microscopy in study 3 showed a presence of inflammatory debris over the head and midpiece of the sperm from infected mice, in contrast to the smooth head of uninfected mice. Yet, *C. burnetii* specific stains showed that bacteria were not attached to the spermatozoa. The bacteria was found within cellular debris in seminal fluid, and
this could be the mechanism for sexual transmission. The imaging results in study 3 do not support the conclusion by Kruszewska et al that C. burnetii attach to spermatozoa and “hitchhike” leading to passive transferal to their sexual partners(261).

Tigertt et al(189) demonstrated in a small study on humans exposed to aerosolised C. burnetii that treatment with oxytetracycline within 24 hours of fever onset led to cessation of symptoms 24-48 hours after antibiotic administration. Treatment with oxytetracycline late in the incubation period (time undefined) prevented symptoms in all cases, and initiation of treatment early in the incubation phase, 24 hours after exposure, in 4/5 cases led to disease after a longer incubation period of 8-10 days. This study led to guidance in the UK and USA that following a known exposure to C. burnetii treatment should be delayed for 8-10 days (73,262). There are no other published studies investigating timing of antibiotics following infection with C. burnetii, and no studies assessing the utility of antibiotics as prophylaxis. The findings demonstrated by Tigertt et al(189) are consistent with the results from AJ mouse efficacy study 3 suggesting that treatment initiation late in the incubation period or around symptom onset appeared to have a favourable outcome compared to treatment initiated as prophylaxis.

Study 4 further investigated the timing of antibiotic initiation. Treatment was started as prophylaxis (1 day prior to challenge), early in the incubation period (2 days’ post challenge), late in the incubation period (day 5 post challenge), and following recovery from the acute infection (day 10 post challenge). Only the results of weight loss and organ weight were available at the point this thesis was written. They show that the best outcome with regards to weight loss was when treatment was initiated at 2 and 5 days’ post challenge. These groups also had the lowest spleen weight suggestive of a lower inflammatory response in this organ site. Further work is required to look at the bacteriology in the lungs and spleen of these groups in order to ascertain the bacterial burden following the different treatment regimens. Weight loss and organ weight are just 2 markers of disease severity in this model and need to be taken into account with the immunological and bacteriological markers once performed. This study has shown that doxycycline hyclate is not as well tolerated as doxycycline monohydrate which is consistent with findings in human studies using doxycycline as malaria prophylaxis(250). A relapse in weight was not seen after completion of antibiotics in study 4 unlike studies 1 and 2 which may be as a consequence of the extension to the length of treatment.
Bacteriology is required to ascertain whether there is a difference in efficacy between the 2 doxycycline preparations.

The limitations of these in vivo studies are based on model selection. The AJ mouse model was selected because it is an aerosolised, non-lethal model which has a close representation to human disease compared to other mammalian models. AJ mice are the most susceptible strain to C. burnetii and this is due to the genetic modifications which have altered the innate and adaptive immune system. Whilst this increases susceptibility and the ability to use this mouse to model Q fever, it also modifies the immune response when exposed to bacterial challenge and antibiotics. Future work will ideally examine the findings and hypotheses from these AJ studies in a higher order non-human primate model to test for reproducibility. Further suggested work would be to perform PCR analysis on all of the organ sites sampled in study 3. Whilst no viable bacteria were seen during the interim and final euthanisation points in the antibiotic treated mice, it would be valuable to understand whether the bacteria had disseminated to these sites and whether it persisted at the culmination of the study. There is a suggestion that the adipose and bone marrow act as a sanctuary site for C. burnetii which can lead to disease recrudescence if the patient becomes immunosuppressed (248,249). Also, it has been postulated the C. burnetii DNA fragments could stimulate the immune system leading to Q fever fatigue syndrome (99). Finally, work will continue to complete the bacteriology and immunological analysis of the samples from study 4 to further increase the understanding of the optimum timing of antibiotic initiation in treatment and prevention of Q fever.

In summary, the key findings from the pathogenesis study completed in study 3 are;

- Weight loss in challenged, untreated mice commenced at day 5 and returned to baseline by day 11, with peak weight loss at day 8.
- From day 8 – 35 the spleens in challenged, untreated mice were significantly heavier compared to the uninfected mice.
- Bacterial dissemination to all organ sites tested (lung, spleen, testes, adipose, blood, bone marrow and brain) was demonstrated. The greatest dissemination of infection was seen by day 8 which corresponds to the peak of weight loss.
• By the end of the study (day 35) viable counts were still isolated in the lung of 40% of the mice, and 100% of mice had DNA isolated by RT-PCR in their lungs.
• Bacteria were isolated from the blood in 100% of mice at day 5 and 8 by viable counts and RT-PCR.
• Significant increases were seen in IFN-γ (D5 – 15), TNF-α (D5 – 8), IL6 (D5), IL10 (D5) and IL12 (D15) compared to the unchallenged mice. These changes were consistent with previous publications (146) (234)

The doxycycline monohydrate (DM) efficacy study 3 demonstrated that;

• DM initiated 1 day pre challenge significantly prevented weight loss compared to the water control group.
• DM initiated 5 days post challenge stopped further weight loss 24 hours after treatment initiation, which led to a significant reduction in weight loss compared to the water control group.
• At all interim euthanisation points the lung and spleen weight of treated mice were lower than the water treated control groups suggesting that the antibiotics reduced the inflammatory response in these organ sites whilst treatment was ongoing.
• By day 35 the organ weights for the treatment groups and water controls were comparable.
• At completion of therapy no viable bacteria were found outside of the lungs in the pre challenge treatment groups. Viable bacteria were found in the lungs and spleens of the post challenge groups but not in any of the other organ sites. DNA was isolated in the spleen of pre and post challenge groups by RT-PCR demonstrating that the bacteria had disseminated to the spleen during the treatment course.
• By day 35 both pre-challenge treatment groups had a higher proportion of mice with viable bacteria in their lungs compared to the post challenge and water treated groups. The 16 day pre challenge treated group had the highest number of mice with viable bacteria in their lungs and were the only group to have viable bacteria in the spleen. This suggests that pre challenge treatment is not effective at preventing or reducing disease burden compared with treatment at the point of symptom onset.
• There are benefits to performing PCR and culture when investigating bacterial spread of disease. PCR detects viable and non-viable counts and therefore demonstrates spread, whereas culture provides an assessment of clearance of the bacteria.
• No viable bacteria were seen in the blood at completion of antibiotics in the pre and post challenge groups. DNA was found in blood samples at completion of therapy in some of the blood samples from all treatment groups except the 16 day DM treatment group initiated at 1 day pre challenge. This demonstrates the potential utility of performing PCR for diagnosis even after antibiotic initiation.
• The PI IgG antibody titres to C. burnetii were significantly raised in all of the treatment groups and water control group at completion of the study (day 35) compared to the unchallenged mice.

Mouse efficacy study 4 comparing the timing of treatment with doxycycline monohydrate and doxycycline hyclate showed that;

• In the D -1 treatment groups, both DM + DH groups had weight loss greater than the water control for the first 7 days of treatment. This may have been a drug related side effect. DM significantly reduced weight loss compared to the water control during the peak of weight loss.
• Treatment initiated D +2 and D +5 prevented weight loss compared to the water control group. This was significant over the peak of disease.
• Once the antibiotics started in the D+10 group there was a reduction in weight loss compared to the water group. The treatment groups failed to gain weight during recovery whilst they remained on antibiotics and for 5 days following completion.
• Clinical signs and weight loss were seen in a greater percentage of mice in all DH treated groups compared to the DM group on the same regimen, suggesting that DH is not as well tolerated as DM.
• There was no significant difference in weight loss or organ weights between the DM and DH treated groups for D+2, D+5 and D+10 regimen.
• Further bacteriology and immunological experiments will be carried out to investigate the difference between each treatment regimen and between the 2 drug preparations.
Chapter 6: Q fever in military personnel – A retrospective cohort study

6.1. Introduction

Q fever was first known to have affected military troops during World War II when thousands of Allied and German troops were infected with *C. burnetii* in the Mediterranean countries, Balkans and Southern Europe(1). More recently, Q fever has affected British military personnel on deployments to Afghanistan(67,68). Some of the military patients subsequently suffered from debilitating fatigue following the acute infection, which has led to medical discharge from the services. A number of medico-legal cases against the Ministry of Defence have ensued from service personnel who claim there was substandard care or that doxycycline prophylaxis could have reduced the severity of disease(263). There is no published evidence regarding the efficacy of chemoprophylaxis for the prevention or reduction of the severity of Q fever, but this has been explored by the *in vivo* work in chapters 4 and 5. One small human study in the 1950’s demonstrated that treatment late in the incubation period for Q fever prevented development of symptoms but antibiotics early in the incubation period only prolonged the incubation period(189).

Previous outbreaks suggest there is an increased risk of acute Q fever (AQF) acquisition associated with gender, age and smoking(57,79,80). Chronic Q fever (CQF) occurs in 1-5% of all patients following infection with Q fever(86). Most commonly this presents as endocarditis or vascular infection(15). Previous valvular surgery or valvular abnormalities, the presence of vascular prostheses and aneurysms, mild renal insufficiency, older age and non-haematological malignancy are predictors of CQF(79,88,89). Patients with CQF commonly will not recall a preceding episode of AQF(86).

*C. burnetii* can resist harsh environmental conditions in the small cell variant (SCV) form(15). Dry soil and high particle matter concentration in the air are associated with acquisition, whilst vegetation is inversely associated(83). These environmental conditions are commonly present for British military personnel working in Afghanistan if they are near infected ruminant animals.

Post infection fatigue lasting 6 to 12 months is common following AQF(264). These symptoms will often resolve, but 10-30% will have persistent fatigue for greater than
a year, and has been reported to last for greater than 10 years, this is termed Q fever fatigue syndrome (QFS) (78,93). The cause of QFS to date is unknown(265). The severity of acute illness is a predictor of QFS(91,93), and it appears that asymptomatic seroconversion does not lead to QFS(96). Retrospective analysis of a Canadian outbreak did not find an association between initial symptoms or antibiotics during acute disease and the development of persistent symptoms(97). Other factors possibly associated with development of QFS are young age, female gender and smoking(93), an increase in IL-6 and IFN-\( \gamma \) and a decrease in IL-2(99). Published literature around the causes and predictors of QFS are often contradictory leading to no firm conclusions(93). Some of the difficulties arise from different definitions of QFS and the subjectiveness of symptoms(93). In addition, data is based on retrospective analysis of outbreak cohorts. These outbreaks are geographically varied and different strains of C. burnetii are responsible. It is known that different strains lead to different clinical presentations and severity of symptoms(17) which may impact on the disease outcome.

Eighty-six patients were followed up in the UK Military Infectious Diseases & Tropical Medicine Clinic at Birmingham Heartlands Hospital following Q fever infection in Afghanistan. The patients had a detailed set of notes both from their patient care in Afghanistan and back in the UK. The aim of this retrospective review of the military patients’ notes was to identify factors associated with progression to QFS, so that patients who were identified as high risk during their acute infection could be targeted for rehabilitation to reduce the likelihood of developing fatigue, or some of these predictive factors could be reversed. A recent study from the Netherlands found that cognitive behavioural therapy (CBT) is effective in reducing fatigue severity(120). Although the beneficial effects of CBT were not maintained 1 year after completion of therapy(121), further work in this area is ongoing to direct rehabilitation in QFS.

6.2. Demographics

Eighty-six military patients were identified as having C. burnetii infection from 2007 to 2015, the details were held at the UK Military Infectious Diseases & Tropical Medicine Clinic at Birmingham Heartlands Hospital. The majority of patients (83) acquired the infection when deployed in Afghanistan, 2 acquired the infection when on exercise in Kenya, and 1 patient from within the UK.
In 2018, 10.4% of the UK Armed Forces were female, and the average age of officers was 30 years and other ranks (Private through to Warrant Officer), was 37 years (266). Within the Q fever cohort, 85 patients (98.8%) were male and 1 patient (1.2%) was female. The majority of patients were aged between 18 and 29 years (68.6%; Table 1). Within the cohort the mean age of officers was 36 years (n=4), and of other ranks 28 years (n=80).

**Table 1**: The age distribution of the Q fever patient cohort followed up at the UK Military Infectious Diseases & Tropical Medicine Clinic at Birmingham Heartlands Hospital. The majority of patients (68.6%) were between 18 and 29 years of age.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Frequency (n=86)</th>
<th>Percent %</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 – 24</td>
<td>24</td>
<td>27.9</td>
</tr>
<tr>
<td>25 – 29</td>
<td>35</td>
<td>40.7</td>
</tr>
<tr>
<td>30 – 34</td>
<td>10</td>
<td>11.6</td>
</tr>
<tr>
<td>35 - 39</td>
<td>7</td>
<td>8.1</td>
</tr>
<tr>
<td>40 – 44</td>
<td>7</td>
<td>8.1</td>
</tr>
<tr>
<td>45 – 49</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50 – 55</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Missing data</td>
<td>2</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Within the military cohort, 80.2% of cases were from ground combat troops (Table 2), and 80.2% were junior ranks (Private to Corporal, Table 3).
Table 2: The military trade groups within which the Q fever patient cohort work. The majority of patients (80.2%) were from ground combat troops (* indicates ground troops).

<table>
<thead>
<tr>
<th>Trade</th>
<th>Number of patients (Total=86)</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infantry*</td>
<td>53</td>
<td>61.6</td>
</tr>
<tr>
<td>Royal Marines*</td>
<td>7</td>
<td>8.1</td>
</tr>
<tr>
<td>Corps of Royal Engineers</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Army Medical Services</td>
<td>4</td>
<td>4.7</td>
</tr>
<tr>
<td>Royal Regiment of Artillery*</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>Explosive Ordnance Disposal/Army</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>Technical Operators*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parachute Regiment*</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>Royal Electrical and Mechanical</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>Engineers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Royal Corps of Signals</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>Army Air Corps</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Adjutant General Corps</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Cavalry*</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Special Forces*</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Royal Tanks Regiment*</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Royal Army Veterinary Corps</td>
<td>1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table 3: The rank distribution of the Q fever patient cohort. The majority of patients (80.2%) were from junior ranks (Private to Corporal).

<table>
<thead>
<tr>
<th>Rank</th>
<th>Number of patients (total = 86)</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Private</td>
<td>36</td>
<td>41.8</td>
</tr>
<tr>
<td>Lance Corporal</td>
<td>11</td>
<td>12.8</td>
</tr>
<tr>
<td>Corporal</td>
<td>22</td>
<td>25.6</td>
</tr>
<tr>
<td>Sergeant</td>
<td>7</td>
<td>8.1</td>
</tr>
<tr>
<td>Staff Sergeant</td>
<td>3</td>
<td>3.5</td>
</tr>
<tr>
<td>Warrant Officer</td>
<td>3</td>
<td>3.5</td>
</tr>
<tr>
<td>Lieutenant</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Captain</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>Major</td>
<td>1</td>
<td>1.2</td>
</tr>
</tbody>
</table>
From 2010 to 2013 between 29 - 34% of males in the British Army smoked (267). The Q fever cohort has a higher percentage of smokers than the Army average with 68.6% of the cohort smoking at the time they were infected (Table 4).

**Table 4:** The smoking status of the Q fever patients.

<table>
<thead>
<tr>
<th>Smoking History</th>
<th>Number of patients (Total=86)</th>
<th>Percentage of total patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current smoker</td>
<td>59</td>
<td>68.6</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>18</td>
<td>20.9</td>
</tr>
<tr>
<td>Missing data</td>
<td>3</td>
<td>3.5</td>
</tr>
</tbody>
</table>

6.3. Clinical data

6.3.1. Signs and symptoms

The most common symptoms at presentation to a medical professional seen in 50% or more of patients were fever (93.0%), headache (74.4%) and myalgia (50.0%) (Table 5).
Table 5: The presenting symptoms or signs of the military patients in the Q fever cohort. Symptoms were recorded from the patients earliest account of their symptoms from either the Military Primary Health Care records or within hospital records.

<table>
<thead>
<tr>
<th>Symptom or sign at onset</th>
<th>Number of patients (Total = 86)</th>
<th>Percentage of total patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>80</td>
<td>93.0</td>
</tr>
<tr>
<td>Headache</td>
<td>64</td>
<td>74.4</td>
</tr>
<tr>
<td>Myalgia</td>
<td>43</td>
<td>50.0</td>
</tr>
<tr>
<td>Malaise</td>
<td>40</td>
<td>46.5</td>
</tr>
<tr>
<td>Diaphoresis</td>
<td>36</td>
<td>41.9</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>31</td>
<td>36.0</td>
</tr>
<tr>
<td>Vomiting</td>
<td>25</td>
<td>29.1</td>
</tr>
<tr>
<td>Anorexia</td>
<td>24</td>
<td>27.9</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>22</td>
<td>25.6</td>
</tr>
<tr>
<td>Cough</td>
<td>22</td>
<td>25.6</td>
</tr>
<tr>
<td>Nausea</td>
<td>20</td>
<td>23.3</td>
</tr>
<tr>
<td>Photophobia</td>
<td>12</td>
<td>14.0</td>
</tr>
<tr>
<td>Sore throat</td>
<td>12</td>
<td>14.0</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>11</td>
<td>12.8</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>9</td>
<td>10.5</td>
</tr>
<tr>
<td>Shortness of breath</td>
<td>9</td>
<td>10.5</td>
</tr>
<tr>
<td>Chest pain</td>
<td>5</td>
<td>5.8</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>4</td>
<td>4.7</td>
</tr>
<tr>
<td>Weight loss</td>
<td>4</td>
<td>4.7</td>
</tr>
<tr>
<td>Confusion</td>
<td>1</td>
<td>1.2</td>
</tr>
</tbody>
</table>
6.3.2. Treatment

Doxycycline 200 mg daily was given to 70/86 (81.4%) patients. The days from symptom onset to initiation of doxycycline were recorded and ranged from 0 – 324 days with a median of 5.5 days and a mode of 6 days. Within a week of symptom onset, 59.4% of patients had received doxycycline (Table 6).

Table 6: Number of days from symptom onset to initiating doxycycline treatment for acute Q fever.

<table>
<thead>
<tr>
<th>No of days from symptom onset to doxycycline initiation</th>
<th>No of patients initiating doxycycline</th>
<th>% of cohort initiating doxycycline</th>
<th>Accumulative % of the cohort receiving doxycycline treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>4.7</td>
<td>5.9</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>3.5</td>
<td>9.4</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>10.5</td>
<td>19.9</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>11.6</td>
<td>31.5</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>9.3</td>
<td>40.8</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>12.8</td>
<td>53.6</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>5.8</td>
<td>59.4</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>3.5</td>
<td>62.9</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>2.3</td>
<td>65.2</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>1.2</td>
<td>66.4</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>1.2</td>
<td>67.6</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>2.3</td>
<td>69.9</td>
</tr>
<tr>
<td>35</td>
<td>1</td>
<td>1.2</td>
<td>71.1</td>
</tr>
<tr>
<td>41</td>
<td>1</td>
<td>1.2</td>
<td>72.3</td>
</tr>
<tr>
<td>44</td>
<td>1</td>
<td>1.2</td>
<td>73.4</td>
</tr>
<tr>
<td>49</td>
<td>1</td>
<td>1.2</td>
<td>74.6</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>1.2</td>
<td>75.8</td>
</tr>
<tr>
<td>53</td>
<td>1</td>
<td>1.2</td>
<td>77.0</td>
</tr>
<tr>
<td>63</td>
<td>1</td>
<td>1.2</td>
<td>78.3</td>
</tr>
<tr>
<td>93</td>
<td>1</td>
<td>1.2</td>
<td>79.4</td>
</tr>
<tr>
<td>284</td>
<td>1</td>
<td>1.2</td>
<td>80.7</td>
</tr>
<tr>
<td>324</td>
<td>1</td>
<td>1.2</td>
<td>81.4</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>81.4</td>
<td>81.4</td>
</tr>
<tr>
<td>Doxycycline not given</td>
<td>16</td>
<td>18.6</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
6.3.3. Admission to hospital and length of stay

During their acute illness 74.4% of the military cohort were admitted into hospital with a median length of stay of 3 days (Table 7).

**Table 7:** The length of stay in days for patients admitted to the hospital in Camp Bastion with acute Q fever.

<table>
<thead>
<tr>
<th>Hospital length of stay</th>
<th>Number of patients (total=86)</th>
<th>Percentage of total patient number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>9.3</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>17.4</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>20.9</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>9.3</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>11.6</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>3.5</td>
</tr>
<tr>
<td>No hospital admission</td>
<td>22</td>
<td>25.6</td>
</tr>
</tbody>
</table>

6.3.4. Laboratory results

In the first 15 days following initial presentation the patients exhibited a marked transaminitis, 28/77 (36.4%) patients had an alanine aminotransferase (ALT) greater than 5 times the upper limit of normal (greater than 200 IU/L; Figure 2a). Only 8/77 patients (10.4%) at their peak had an ALT within the normal range, defined as less than 40 IU/L (Figure 2b). Alkaline phosphatase (ALP) and gammaglutamyltransferase (GGT) were also raised during the first 15 days of illness (Figure 1c&d). During the acute illness 24/71 (33.8%) of patients with a recorded result had a raised ALP and 38/52 (65.4%) had a raised GGT. ALP and GGT are markers of cholestasis suggestive of a blockage to the flow of bile. This was generally not associated with a rise in bilirubin (Figure 1e). Hypoalbuminaemia (less than 35 g/L) was present in 39/69 (56.5%) of the cohort with recorded results during acute illness (Figure 1f).
Figure 1: Scatter plots of liver function tests over the course of the disease from symptom onset in days. (a) Alanine aminotransferase (ALT) (b) Aspartate aminotransferase (AST). (c) Alkaline phosphatase (ALP) and (d) Gammaglutamyltransferase (GGT). (e) Total bilirubin (f) Albumin. The reference lines on the Y axis reflect the normal range for the blood test, or the upper limit of normal where there is only 1 line. The reference ranges are taken from the military deployed laboratory at the Role 3 Hospital, Camp Bastion, Afghanistan.
Renal function, as demonstrated by urea and creatinine, was generally within the normal range for all patients in the Q fever cohort (Figure 2a&b). Hyponatraemia was seen within the first 10 days, 26/72 (30.2%) of patients with blood tests for sodium had a hyponatraemia of less than 135 mmol/L during their hospital admission. C-reactive protein (CRP) is a marker of infection or inflammation and was raised in the majority of acute Q fever cases (Figure 2d) with 68/72 (94.4%) of patients displaying a raised CRP of greater than 10 mg/L, and 24/72 (33.3%) with a CRP raised greater than 100 mg/L during their admission.

Figure 2: Scatter plots of renal function and C-reactive protein (CRP) over the course of the disease from symptom onset in days. (a) Urea (b) Creatinine (c) Sodium (d) CRP. The reference lines on the Y axis reflects the upper limit of normal or the normal range for the blood test. The reference ranges are taken from the military deployed laboratory at the Role 3 Hospital, Camp Bastion, Afghanistan.

The total white cell count and neutrophil count were often normal, but the notable abnormalities were the lymphopenia and thrombocytopenia seen mainly in the first 10 days of illness (Figure 3a-d). During their admission 26/71 (30.2%) patients had
a low lymphocyte count of less than $1 \times 10^9$/L, whilst 48/68 (70.6%) had a platelet count of less than $150 \times 10^9$/L. The full blood count in acute Q fever demonstrated a slight anaemia during the first 10 days from initial presentation in some patients (Figure 3e). During their admission 9/71 (10.5%) of patients displayed a haemoglobin of less than 13.5 g/L.

![Figure 3](image)

**Figure 3**: Scatter plots of full blood counts over the course of the disease from symptom onset in days. (a) Total white cell count (b) Lymphocytes (c) Neutrophils (d) Platelets (e) Haemoglobin. The reference lines on the Y axis reflect the normal range for the blood test. The reference ranges are taken from the military deployed laboratory at the Role 3 Hospital, Camp Bastion, Afghanistan.

6.3.5. Disease outcome
Hospital notes and clinic letters from UK hospitals where patients were followed up were accessed to find the long-term outcome for each patient. This data is shown in Table 8. One patient was excluded from analysis because Q fever was an incidental finding during an admission for complex trauma and a long-term outcome secondary to Q fever could not be ascertained due to confounding factors. The definition of QFS has been adapted from the Dutch QFS guidelines (93). QFS in this study was defined as: severe fatigue causing significant disability in daily life for more than 12 months, with a temporal relationship with acute Q fever and not caused by co-morbidities. As stated by the Dutch, fatigue should be absent before acute Q fever or should have significantly increased since the infection (93).

Table 8: The disease outcome for the Q fever military patient cohort.

<table>
<thead>
<tr>
<th>Disease outcome</th>
<th>Number of patients (N=85)</th>
<th>Percentage of total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Q fever (AQF)</td>
<td>64</td>
<td>74.4</td>
</tr>
<tr>
<td>Chronic Q fever (CQF)</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Q Fever Fatigue Syndrome (QFS)</td>
<td>19</td>
<td>22.1</td>
</tr>
<tr>
<td>CQF and QFS</td>
<td>1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Following the large Q fever outbreak in the Netherlands from 2007 to 2010 the Dutch published a consensus guideline for diagnosis of Chronic Q fever (CQF) (85). This guideline has been applied to the military cohort and the results shown in table 9. Seventeen patients met the criteria for either proven, probable or possible CQF.

Table 9: The proven, probable and possible CQF cases in the military patient cohort using the case definitions suggested by the Dutch Consensus Group (85).

<table>
<thead>
<tr>
<th>CQF diagnosis using the Dutch Consensus Guideline</th>
<th>Number of patients (N=85)</th>
<th>% of total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proven</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Probable</td>
<td>6</td>
<td>7.1</td>
</tr>
<tr>
<td>Possible</td>
<td>10</td>
<td>11.8</td>
</tr>
</tbody>
</table>

The French National Reference Centre have also published guidelines on diagnosis of Q fever endocarditis and Q fever vascular infections (198). These guidelines have been applied to the military cohort and the results shown in Tables 10 and 11. Seven patients met the criteria for proven or possible CQF endocarditis (Table 10), and 2 patients for possible CQF vascular infection (Table 11).
Table 10: The proven, probable and possible cases of CQF endocarditis using the French guidelines for case definitions (198,268).

<table>
<thead>
<tr>
<th>Q fever endocarditis diagnosis using the French guidelines</th>
<th>Number of patients (N=85)</th>
<th>% of total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proven</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Probable</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Possible</td>
<td>6</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Table 11: The proven, probable and possible cases of Q fever vascular infections using the French guidelines for case definitions (198).

<table>
<thead>
<tr>
<th>Q fever vascular infection diagnosis using the French guidelines</th>
<th>Number of patients (N=85)</th>
<th>% of total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proven</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Probable</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Possible</td>
<td>2</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Fifty-five (55/85, 64.7%) of the Q fever cohort underwent echocardiography as Q fever endocarditis risk stratification and surveillance. 8/55 (14.5%) were abnormal. None of the patients with abnormal echocardiograms developed endocarditis. The abnormalities included; perimyocarditis x 2, thickened aortic or mitral valves x 3, bicuspid aortic valve x 1, mild tricuspid, aortic or mitral valve regurgitation x 2.

6.4. Analysis of Q fever cohort for factors associated with Q fever fatigue syndrome

Following univariate logistic regression the variables with an association with QFS that approached significance with a p value of less than 0.1 were: a delay to initiating doxycycline of greater than 5 days from symptom onset (OR 2.91, 95% CI 0.95 – 8.91, p = 0.062), a haemoglobin of less than 13.5 g/L (OR 4.11, 95% CI 0.97 – 17.41, p = 0.055), an ALP greater than the upper limit of normal (>140 IU/L) (OR 3.16, 95% CI 1.05 – 9.50, p = 0.04), and a GGT greater than the upper limit of normal (> 48 IU/L) (OR 4.80, 95% CI 0.94 – 24.62). The results are shown in Table 12.
Table 12: Univariate logistic regression analysis for each variable of interest possibly associated with Q fever fatigue syndrome.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>OR</th>
<th>95% CI</th>
<th>p - value</th>
<th>Number of patients (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>1.004</td>
<td>0.933 – 1.081</td>
<td>0.906</td>
<td>82</td>
</tr>
<tr>
<td>Current smoker</td>
<td>0.718</td>
<td>0.245 – 2.104</td>
<td>0.545</td>
<td>80</td>
</tr>
<tr>
<td>Smoking (ex and current combined)</td>
<td>1.750</td>
<td>0.556 – 5.504</td>
<td>0.338</td>
<td>81</td>
</tr>
<tr>
<td>Temperature on admission</td>
<td>1.377</td>
<td>0.744 – 2.549</td>
<td>0.308</td>
<td>64</td>
</tr>
<tr>
<td>Temperature &gt;= 38 on admission</td>
<td>3.150</td>
<td>0.366 – 27.144</td>
<td>0.296</td>
<td>64</td>
</tr>
<tr>
<td>Respiratory rate on admission</td>
<td>1.077</td>
<td>0.921 – 1.260</td>
<td>0.351</td>
<td>45</td>
</tr>
<tr>
<td>Tachypnoea &gt;=20 on admission</td>
<td>0.848</td>
<td>0.212 – 3.391</td>
<td>0.816</td>
<td>45</td>
</tr>
<tr>
<td>Heart rate on admission</td>
<td>1.017</td>
<td>0.981 – 1.054</td>
<td>0.350</td>
<td>63</td>
</tr>
<tr>
<td>Tachycardia &gt;=100 on admission</td>
<td>1.467</td>
<td>0.442 – 4.869</td>
<td>0.532</td>
<td>63</td>
</tr>
<tr>
<td>Doxycycline given</td>
<td>0.368</td>
<td>0.076 – 1.777</td>
<td>0.214</td>
<td>84</td>
</tr>
<tr>
<td>Delay to doxycycline (for each day)</td>
<td>1.005</td>
<td>0.995 – 1.014</td>
<td>0.321</td>
<td>68</td>
</tr>
<tr>
<td>Delay to doxycycline &gt; 3 days</td>
<td>2.969</td>
<td>0.619 – 14.243</td>
<td>0.174</td>
<td>84</td>
</tr>
<tr>
<td>Delay to doxycycline &gt; 5 days</td>
<td>2.909</td>
<td>0.950 – 8.910</td>
<td>0.062</td>
<td>84</td>
</tr>
<tr>
<td>Delay to doxycycline &gt; 7 days</td>
<td>1.140</td>
<td>0.419 – 3.101</td>
<td>0.797</td>
<td>84</td>
</tr>
<tr>
<td>Abnormal Hb (&lt;13.5 g/L, &gt;17.5 g/L)</td>
<td>3.385</td>
<td>0.932 – 12.292</td>
<td>0.064</td>
<td>69</td>
</tr>
<tr>
<td>High Hb &gt; 17.5 g/L</td>
<td>2.824</td>
<td>0.368 – 21.639</td>
<td>0.318</td>
<td>69</td>
</tr>
<tr>
<td>Low Hb &lt; 13.5 g/L</td>
<td>4.107</td>
<td>0.969 – 17.413</td>
<td>0.055</td>
<td>69</td>
</tr>
<tr>
<td>Lymphopenia (&lt;1 x10⁹/L)</td>
<td>0.368</td>
<td>0.107 – 1.270</td>
<td>0.114</td>
<td>69</td>
</tr>
<tr>
<td>Neutropenia (&lt;1.5 x10⁹/L)</td>
<td>3.0</td>
<td>0.549 – 16.379</td>
<td>0.205</td>
<td>70</td>
</tr>
<tr>
<td>Abnormal WCC (&lt;4 x10⁹/L, &gt;11 x10⁹/L)</td>
<td>2.222</td>
<td>0.760 – 6.494</td>
<td>0.144</td>
<td>70</td>
</tr>
<tr>
<td>Lowest platelet count</td>
<td>0.996</td>
<td>0.987 – 1.005</td>
<td>0.389</td>
<td>67</td>
</tr>
<tr>
<td>Thrombocytopenia &lt; 150 x10⁹/L during admission</td>
<td>1.147</td>
<td>0.347 – 3.797</td>
<td>0.822</td>
<td>67</td>
</tr>
<tr>
<td>Thrombocytopenia &lt; 100 x10⁹/L during admission</td>
<td>1.378</td>
<td>0.460 – 4.123</td>
<td>0.567</td>
<td>67</td>
</tr>
<tr>
<td>Highest ALT</td>
<td>1.001</td>
<td>0.999 – 1.002</td>
<td>0.591</td>
<td>75</td>
</tr>
<tr>
<td>&gt; 3x max ALT (&gt;168 IU/L)</td>
<td>2.031</td>
<td>0.844 – 4.887</td>
<td>0.114</td>
<td>75</td>
</tr>
<tr>
<td>&gt; 5x max ALT (&gt;280 IU/L)</td>
<td>1.474</td>
<td>0.638 – 3.403</td>
<td>0.363</td>
<td>75</td>
</tr>
<tr>
<td>AST 3x ULN (&gt;135 IU/L)</td>
<td>1.158</td>
<td>0.344 – 3.899</td>
<td>0.813</td>
<td>55</td>
</tr>
</tbody>
</table>
Variables with an odds ratio (OR) associated with a p-value of less than 0.1 are highlighted in red. * The definition of hepatitis is taken from Wielder et al (77). Of the 85 patients included for analysis the number of patients that had a data entry for each individual variable is shown in the table.

Forty-eight patients had data available for all 4 variables approaching significance (Low Hb, high ALP, high GGT and a delay in doxycycline of greater than 5 days). Univariate analysis of these 48 patients looking at these variables was completed (Table 13: columns 2&3) and multivariate logistic regression was carried out on the same data to control for confounding within the dataset (Table 13: columns 4&5). ALP and GGT are both markers of liver dysfunction, in particular cholestasis. To confirm a relationship between ALP and GGT in this dataset a 2-tailed bivariate Pearson Correlation was performed which showed a close positive correlation between ALP and GGT, \( r = 0.837, p < 0.001 \), with an \( R^2 = 0.70 \). Of the two, because the predictive value of ALP was reduced in the multivariate model it was removed from the model leaving the remaining 3 variables (low Hb, raised GGT and delay to

<table>
<thead>
<tr>
<th>Exposure</th>
<th>OR</th>
<th>95% CI</th>
<th>( p )-value</th>
<th>Number of patients (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST 3x ULN (&gt;135 IU/L)</td>
<td>1.158</td>
<td>0.344 – 3.899</td>
<td>0.813</td>
<td>55</td>
</tr>
<tr>
<td>ALP &gt; ULN (&gt;140 IU/L)</td>
<td>3.162</td>
<td>1.053 – 9.502</td>
<td>0.04</td>
<td>69</td>
</tr>
<tr>
<td>GGT &gt; ULN (&gt;48 IU/L)</td>
<td>4.800</td>
<td>0.936 – 24.620</td>
<td>0.06</td>
<td>55</td>
</tr>
<tr>
<td>Highest bilirubin &gt; 2x ULN (&gt;34 umol/L)</td>
<td>1.333</td>
<td>0.224 – 7.920</td>
<td>0.752</td>
<td>80</td>
</tr>
<tr>
<td>* Hepatitis = 2xULN bilirubin (&gt;34 umol/L), 2xULN ALT (&gt;112 IU/L)</td>
<td>6.111</td>
<td>0.523 – 71.440</td>
<td>0.149</td>
<td>75</td>
</tr>
<tr>
<td>Abnormal urea (&gt;7.5 mmol/L)</td>
<td>0</td>
<td>0</td>
<td>0.999</td>
<td>71</td>
</tr>
<tr>
<td>Abnormal creatinine (&gt;110 umol/L)</td>
<td>0.594</td>
<td>0.147 – 2.392</td>
<td>0.463</td>
<td>69</td>
</tr>
<tr>
<td>Hyponatraemia &lt;135 g/L during admission</td>
<td>1.688</td>
<td>0.582 – 4.891</td>
<td>0.335</td>
<td>69</td>
</tr>
<tr>
<td>Hyponatraemia &lt;130 g/L on admission</td>
<td>0.958</td>
<td>0.093 – 9.885</td>
<td>0.971</td>
<td>66</td>
</tr>
<tr>
<td>Hypoalbuminaemia &lt;35 g/L during admission</td>
<td>1.577</td>
<td>0.530 – 4.694</td>
<td>0.413</td>
<td>67</td>
</tr>
<tr>
<td>Hypoalbuminaemia &lt;30 g/L during admission</td>
<td>2.528</td>
<td>0.776 – 8.233</td>
<td>0.124</td>
<td>67</td>
</tr>
<tr>
<td>CRP &gt;10 mg/L</td>
<td>6.06 x10^{8}</td>
<td>0 – \infty</td>
<td>0.999</td>
<td>70</td>
</tr>
<tr>
<td>CRP &gt;100 mg/L</td>
<td>0.726</td>
<td>0.223 – 2.362</td>
<td>0.595</td>
<td>70</td>
</tr>
<tr>
<td>Hospital admission</td>
<td>1.273</td>
<td>0.270 – 6.005</td>
<td>0.761</td>
<td>50</td>
</tr>
<tr>
<td>Length of stay</td>
<td>1.200</td>
<td>0.831 – 1.762</td>
<td>0.320</td>
<td>50</td>
</tr>
</tbody>
</table>
doxycycline administration of greater than 5 days). In the full dataset 49 patients had values for the 3 variables, running the multivariate model showed that all 3 variables are significant predictors of QFS (Table 13: columns 6&7). The Cox and Snell pseudo $R^2$ statistic for the 4 independent variables in the binomial logistic regression model is 0.256 and for the 3 independent variables is 0.247, showing that the 3 variables account for 24.7% of the factors leading to progression to QFS.

**Table 13:** Results from the multivariate logistic regression analysis for the variables that approached significance by univariate analysis.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>OR (95% CI) Univariate analysis (n=48)</th>
<th>$p$-value</th>
<th>OR (95% CI) Multivariate LR (n=48)</th>
<th>$p$-value</th>
<th>OR (95% CI) Multivariate LR (n=49)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Hb &lt; 13.5 g/L</td>
<td>4.13 (0.79 – 21.69)</td>
<td>0.093</td>
<td>9.049 (1.07 – 76.54)</td>
<td>0.043</td>
<td>8.95 (1.07 – 75.3)</td>
<td>0.044</td>
</tr>
<tr>
<td>ALP &gt; ULN (&gt;140 IU/L)</td>
<td>3.25 (0.87 – 12.09)</td>
<td>0.079</td>
<td>1.88 (0.359 – 9.87)</td>
<td>0.454</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGT &gt; ULN (&gt;48 IU/L)</td>
<td>4.737 (0.92 – 24.49)</td>
<td>0.064</td>
<td>5.33 (0.668 – 42.62)</td>
<td>0.114</td>
<td>7.37 (1.11 – 49.11)</td>
<td>0.039</td>
</tr>
<tr>
<td>Delay to doxycycline &gt; 5 days</td>
<td>4.04 (1.05 – 15.58)</td>
<td>0.043</td>
<td>5.61 (1.17 – 26.95)</td>
<td>0.031</td>
<td>5.75 (1.21 – 27.32)</td>
<td>0.028</td>
</tr>
</tbody>
</table>

Univariate and multivariate logistic regression on the 48 patients with values for all 4 of these variables are shown in columns 2&3 and 4&5 respectively. The predictive value of ALP was reduced in the multivariate model and was therefore removed leaving 3 variables of significance in the multivariate model (columns 6&7).

6.5. Analysis of serology titres

Within the military cohort Phase II (PII) IgM rises the earliest peaking at 1 to 3 months (Figure 4a), shortly followed by PII IgG which also peaks at 1 to 3 months (Figure 4b). Phase I (PI) IgA and PI IgG are negative in the first 2 weeks, and PI
IgA typically has very low titres, and in many cases negative results (Figure 4c). PI IgG increases at a slower rate than the PII antibodies and peaks at 6 – 9 months (Figure 4d). Persistent high PI titres are associated with chronic disease(75), therefore within the analysis of the cohort serology the titres for patients diagnosed with QFS were separated from the acute cases to see if the pattern of antibody response differed between the two groups. The mean titres are shown for the 2 antibody phases in Figure 5 and individual values are shown in Figure 6 to display the variation seen between patients.
Figure 4: Mean serology titres +/- standard error of the mean (SEM) for all patients with recorded values in the cohort tested in each time interval. The number of days are from date of symptom onset. (a) PII IgM serology (b) PII IgG serology (c) PI IgA serology (d) PI IgG serology. (note: the x-axis does not divide time evenly)
Figure 5: Mean serology titres +/- SEM for patients with AQF only & no QFS (green) versus patients with AQF who developed QFS (red). The number of days are from date of symptom onset. (a) PII IgM serology (b) PII IgG serology (c) PI IgA serology (d) PI IgG serology. (note: the x-axis does not divide time evenly)
Figure 6: Individual serology titres for patients with AQF only & no QFS (green) versus patients with AQF who developed QFS (red). The mean +/- SEM for each time point are shown on the graph. The number of days is from date of symptom onset. (a) PII IgM serology (b) PII IgG serology (c) PI IgA serology (d) PI IgG serology. (note: the x-axis does not divide time evenly)
To compare the mean values between the AQF only and QFS groups for each antibody titre between 1 and 12 months, an unpaired t-test or a Mann-Whitney U test was performed depending on whether the data had a normal distribution (Table 14). A statistically significant difference was found between the groups for PI IgA and PI IgG ($p < 0.05$, Table 14). The distribution of the mean titres for all antibodies were non-parametric and the ranks for their respective Mann Whitney U analysis is shown in Figure 7.

**Table 14:** Summary of the statistical analysis comparing the means of the different serological titres (PII IgM, PII IgG, PI IgA and PI IgG) for AQF only and QFS groups. The cube root of the mean titres for these antibodies was taken in an attempt to normalise the data distribution allowing parametric analyses to be performed.

<table>
<thead>
<tr>
<th></th>
<th>Distribution of mean titres</th>
<th>Test performed</th>
<th>$p$ value</th>
<th>Distribution following cube root of mean titres</th>
<th>Test performed</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PII IgM</td>
<td>Non-parametric</td>
<td>Mann Whitney U</td>
<td>0.39</td>
<td>Parametric</td>
<td>Unpaired t-test</td>
<td>0.39</td>
</tr>
<tr>
<td>PII IgG</td>
<td>Non-parametric</td>
<td>Mann Whitney U</td>
<td>0.06</td>
<td>Parametric</td>
<td>Unpaired t-test</td>
<td>0.23</td>
</tr>
<tr>
<td>PI IgA</td>
<td>Non-parametric</td>
<td>Mann Whitney U</td>
<td>0.0091</td>
<td>Non-parametric</td>
<td>Mann Whitney U</td>
<td>0.0091</td>
</tr>
<tr>
<td>PI IgG</td>
<td>Non-parametric</td>
<td>Mann Whitney U</td>
<td>0.0053</td>
<td>Non-parametric</td>
<td>Mann Whitney U</td>
<td>0.0053</td>
</tr>
</tbody>
</table>
Figure 7: Ranks of the individual mean serology titres with median rank marked. (a) PII IgM serology (b) PII IgG serology (c) PI IgA serology (d) PI IgG serology. AQF = Acute Q fever, QFS = Q fever fatigue syndrome.
6.6. Discussion

The military Q fever patients were a young, predominantly male cohort. The majority of infections were in deployed troops to Afghanistan. Deployed military personnel in general are healthy and have a high level of fitness. Being able to follow up this cohort for at least 2 years following acute infection has helped inform the long-term outcome of Q fever patients without the confounding factors of previous co-morbidities. The military operation in Afghanistan was an asymmetric combat operation which required a strong presence of combat troops which accounts for the high proportion of troops from the infantry and other frontline roles amongst our cohort. Anderson et al. have previously found amongst US troops in Iraq that working in a combat role was a risk factor for seroconversion to Q fever (OR = 1.8, 95% CI: 1.1 -2.8) (64). Exposure to C. burnetii is assumed to be highest amongst the ground troops that are working and living amongst ruminant animals which are reservoirs for this bacterium.

Within the military AQF cohort there was a higher rate of smokers compared with the general military population. A high rate of smoking has been seen in previous large outbreaks such as the largest UK outbreak in the West Midlands in 1989 (55% Q fever outbreak cohort versus 35% in the matched general population) (57) and the largest reported outbreak worldwide in the Netherlands from 2007 to 2010 (48.6% smokers in the Q fever cohort (07-08) versus 20.4% in the matched general population) (80). It is not known whether smoking predisposes a patient to Q fever infection or whether patients who smoke are more likely to develop symptomatic disease. Some theories for why the prevalence of smoking is higher amongst Q fever patients include; that smokers have a greater exposure to outdoor contaminated aerosols due to their smoking habits, the possibility of hand to mouth transmission, the increased risk of respiratory infections due to structural and immune changes secondary to smoking, and the possibility that smoking may be associated with an increased severity of disease prompting presentation to a medical professional (57,80). Data from admissions to hospital in the Netherlands with acute Q fever or an acute febrile illness with either respiratory symptoms or hepatitis also found an increased rate of smoking in the Q fever group (78% smokers in the Q fever group versus 57% in the acute febrile illness group, $p = 0.013$)(269). From the Netherlands outbreak, 12 to 26 months follow up of patients found that smoking was a significant predictor of a prolonged absence from work (> 1 month) following acute infection (OR 1.69, 95% CI 1.01 – 2.84, $p = 0.046$).
However smoking was not a statistically significant predictor of fatigue in this group (OR 1.29, 95% CI 0.19 – 2.10, p = 0.229) (270). These findings agree with the multivariate analysis completed on the military cohort where a history of current and previous smoking is associated with an increased chance of progressing to QFS, but this does not reach significance (OR 1.75, 95% CI 0.56 – 5.50, p = 0.338).

The range of presenting signs and symptoms within the military cohort are consistent with other outbreak reports, but the respiratory symptoms of cough, and shortness of breath are lower than seen in the Netherlands outbreak(77,95). In the Netherlands outbreak amongst hospitalised patients 86% had chest x-ray changes consistent with pneumonia(77). Q fever can present with a pneumonic or hepatitic picture. The variation is geographical for example France have reported a higher incidence of hepatitis(271), and French Guiana a high incidence of pneumonia(49). Many reasons are postulated for this geographical difference from strain variation to host immune status(134). The military cohort had a predominantly hepatitic presentation although it is important to note that following the 2008 fever study in Camp Bastion(67) military guidance was published recommending that Q fever should be screened for in patients with an undifferentiated febrile illness (UFI). This may have meant that patients presenting with pneumonia were not considered to have UFI and were therefore not screened for C. burnetii.

Acute Q fever patients do not often need admission to hospital, because in 60% of cases patients remain asymptomatic, 38% have acute self-limiting illnesses, and reportedly only 2% require hospital admission during the acute illness(14). Yet 74.4% of the military cohort were admitted into hospital during their acute illness compared to 28% in the Herpen outbreak in the Netherlands(272). The high percentage of admissions is likely due to the deployed environment in Afghanistan. Patients were frequently sent from Forward Operating Bases (FOBs) to the hospital or primary health care centre at Camp Bastion for investigation and convalescence. However, those patients who were admitted were much more likely to have their AQF diagnosed and hence were more likely to be recorded in this cohort. Hospitalisation during the acute admission was a significant predictor of both long term sick leave (greater than 1 month) and fatigue for greater than 12 – 26 months in follow up from the Netherlands outbreak(93). Within the military cohort admission to hospital was not significantly associated with progression to QFS (OR 1.273, 95% CI 0.273 – 6.005, p = 0.761). This is likely because hospitalisation is a marker of severity of disease normally, but in the military cohort hospitalisation was often
due to the austere environment and not severity of disease. Severity of acute Q fever is associated with worse long term health status including QFS(93). The median length of stay in hospital for the military cohort was 3 days which is comparable to 4 days in the Herpen outbreak in the Netherlands(272) and 5 days in another Dutch hospital(77). Length of admission was not a significant predictor of progression to QFS in the military cohort (OR 1.210, 95% CI 0.881 – 1.762, p = 0.320), but has previously been associated with fatigue(93).

The standard recommended treatment for acute Q fever is 100 mg of doxycycline twice daily for 14 days(106). Doxycycline reduces the duration of fever with a mean time to defervescence of 2-3 days following initiation of treatment versus a mean of 12.5 days of fever in untreated patients(106). An optimum time to start treatment following exposure to C. burnetii is not known. A small study from the 1950’s in humans following deliberate release of the bacteria found that oxytetracycline initiated at the point of fever onset stopped symptoms after 24-48 hours(189). Treatment started late in the incubation period prevented symptom onset, whilst treatment initiated early in the incubation period, within 24 hours of exposure merely prolonged the incubation period(189). Long term doxycycline treatment does not appear to reduce fatigue severity in QFS patients(120), but no data exists to show a relationship between treatment of acute infection and progression to QFS. A positive relationship between severity of disease and duration of symptoms with QFS has been shown(93). Doxycycline reduces the number of days with fever(24), and therefore the duration of symptoms so treatment could reduce progression to QFS, but this needs to be further investigated. Although, it has been suggested that to see a reduction of fever duration doxycycline needs to be started within the first 3 days of symptoms(273). The military cohort received antibiotics within a median of 5.5 days. Multivariate analysis of the military cohort suggest that initiation of doxycycline greater than 5 days after symptom onset is associated with progression to QFS (OR 5.75, 95% CI 1.21 – 27.32, p = 0.028). This finding supports early diagnosis and treatment of acute Q fever to prevent progression to QFS, with the caveat that this study was a small study conducted on 86 patients, and that multiple tests have been performed on the data thereby increasing the likelihood of a type I statistical error.

The pathology results seen in the military cohort are consistent with other published case series and outbreaks, with the exception of platelets(10,16,57,77). Within the military cohort, patients presented with a raised CRP (94%), lymphopenia (30%),
thrombocytopenia (71%), anaemia (11%), raised ALT (90%), GGT (65%) and ALP (34%), hyponatraemia (30%) and hypalbuminaemia (57%). The rate of thrombocytopenia was higher in the military cohort compared with other reviews where a thrombocytopenia was reported in 3-25% of patients (10,75,77). This may be as a consequence of a possible bias towards Q fever hepatitis presentations in the military cohort, or a strain variation. Patients from the Aztrakhan region of Russia are reported to have a high rate of haemostasis complications and possible platelet dysfunction associated with acute Q fever (274).

The hepatitis seen in AQF is well described with a marked transaminitis (deranged liver enzymes indicative of liver inflammation) in the absence of jaundice (16,275). This pattern was also seen in the military patients. In comparison to the hospitalised patients seen during the Netherlands outbreak the military cohort had a higher percentage of patients presenting with thrombocytopenia and transaminitis. The Netherlands hospital admission data shows only 3.3% of patients presenting with thrombocytopenia versus 71% in the military cohort, and 32.3% of patients presenting with an ALT greater than 45 U/L versus 90% in the military cohort (77). Eighty percent of patients included in the Netherlands analysis had a diagnosis of pneumonia, whereas in the military cohort no patients had a formal diagnosis of pneumonia which may account for the high representation of transaminitis in the military cohort. As previously discussed, guidance for consideration of a diagnosis of Q fever during the military deployment in Afghanistan focused on patients with a UFI. Patients with a pneumonia will likely not have been considered to have an UFI. By multivariate analysis both ALP and GGT were found to be associated with progression to QFS. The positive association between GGT and QFS was significant (OR 7.37, 95% CI 1.11 – 49.11, p = 0.039). A raised GGT and ALP is suggestive of hepatobiliary dysfunction, and the transaminitis is consistent with hepatic dysfunction. Therefore, it can be seen in the military cohort that patients acquiring infection from Afghanistan have a high proportion of liver dysfunction which appears to be transitory although hepatic biopsies were not part of routine follow up. Although there is likely a disproportionally high percentage of patients with liver dysfunction due to the screening protocol employed by the military in Afghanistan for Q fever as previously discussed. Fewer patients had raised ALP and GGT compared to ALT and AST in the cohort, which were not significantly associated with QFS. The difference in severity of disease and proportions of patients presenting with pneumonia or hepatitis in different outbreaks is postulated to be due to strain variation (15). The most virulent strain known to date is MST 17.
found in Cayenne, French Guiana, producing severe pneumonia(15,276) Little is known about the strain from Afghanistan, more work is required to isolate the strain and to fully identify Q fever patients from this region to discover any association between the strain and disease severity and hepatitis.

The hypoalbuminaemia seen in the military cohort in the context of AQF is likely secondary to acute inflammatory illness and may be related to the severity of disease. In the multivariate analysis a low haemoglobin of less than 13.5 g/L during the acute illness was associated with progression to QFS (OR 8.95, 95% CI 1.07 – 75.3, p = 0.044). Anaemia (Hb < 13.5 g/L) was seen in 11% of the military cohort, this is lower than seen in hospitalised patients during the Netherlands outbreak where 32% of patients were anaemic (Hb < 8 g/L) (77). The higher proportion of a more severe anaemia in the hospitalised Netherlands patients could be as a result of a more severe disease warranting hospital admission. Mild anaemia has been associated with acute infection and is referred to as anaemia of inflammation(277,278). Many theories are postulated to the cause of anaemia in this context, from mild idiopathic haemolysis and marrow inhibition, to the effect of pro inflammatory cytokines(277,278). Bone marrow involvement occurs during all stages of Q fever disease(247,248,279) C. burnetii DNA has been isolated in AQF, QFS and CQF patients(248), along with bone marrow granulomas on biopsy(279). It is possible that anaemia could be as a consequence of the marrow involvement. The severity of disease may be associated with a drop in haemoglobin which may explain the association with progression to QFS. Further research is required and as previously the results are presented with the caveat that this is on limited numbers of patients undergoing multiple tests increasing the chance of a type I error.

The results from the multivariate analysis of the selected variables in the military cohort suggest that; initiation of doxycycline greater than 5 days after symptom onset, a haemoglobin of less than 13.5 g/L during acute admission and a GGT above the upper limit of normal (greater than 48 IU/L) during acute admission are all associated with a significantly increased odds ratio of developing QFS. The Cox and Snell pseudo R-square values for these 3 variables in the multivariate model was 0.247 which suggests that these 3 variables together account for 24.7% of the reasons for progression to QFS. This means there are other variables not in the model that influence progression to QFS. There are only 86 patients in this cohort and only 49 had entries for all 3 variables. Multiple tests performed on a small
number of patients can introduce error leading to false positives, and therefore the correlations of these variables with progression to QFS needs to be tested within a larger independent cohort in the future.

The immunofluorescence assay (IFA) is regarded as the reference standard for Q fever diagnosis and provides results that are semi-quantitative. Seroconversion or a four-fold increase in titres between acute and convalescent serum is considered diagnostic of acute infection(280). There are 2 phases to the serological response, typically Phase II (PII) is associated with acute infection, and a persistently raised Phase I (PI) response is associated with chronic infection(281). The PII IgM and PII IgG response appears before the PI IgA and PI IgG response. PII IgM antibodies are the first to appear in acute infection and there is an overlap between DNA detection and these acute antibodies(282). Early studies suggested the PII IgM antibodies would become undetectable within 4 months, but publications from the Netherlands outbreak suggest these antibodies could persist at detectable levels for greater than 12 months(282) which is consistent with the findings from our cohort. PII antibody titres are typically higher than PI antibodies(280) which was seen within the military cohort. The decay rate of antibodies is slowest for PI IgG with a half-life of up to several years for PI and PII IgG leading to positive results for greater than a year(281). Serology titres for PI and PII antibodies were monitored over 2 years for evidence of progression to chronic disease. Comparing the means between the acute patients with resolved disease and the patients with QFS showed that QFS patients had significantly higher titres between 1 and 12 months for PI IgG and PI IgA. The cohort was not large enough to be able to predict progression to QFS from levels of PI IgG and PI IgA but it would be interesting to investigate this within larger cohorts. In a small study following up patients from the Q fever outbreak in South Wales, in 11 patients there was no relationship found between fatigue and serology titres at 6 years(283). This was a small study which may not have been powered to find a difference and leaves the question of the ability of serology titres to predict QFS open for further research.

The limitations of this study include the low number of patients and a recruitment bias towards AQF patients presenting with a UFI rather than respiratory symptoms. Multiple testing of the data looking for predictors of progression to QFS may have introduced a type I error meaning that the null hypothesis was falsely rejected. A further limitation is that this was a retrospective study which meant only opportunistic data could be captured. Future work will aim to enhance case finding
within the military including respiratory symptoms into the screening guidelines to ensure Q fever pneumonia patients are identified. Using the pseudo $R^2$ value, the 3 predictors of progression to Q fever account for approximately 25% of the reasons for progression from AQF to QFS. Future work in further outbreaks should seek to find other predictors and to test the possible factors identified in this study. In addition, ideally the *C. burnetii* strain from clinical samples from Afghanistan will be isolated and the strain correlated to the clinical presentation.

In summary, the main positive findings from this retrospective review of 86 patients following acute Q fever include;

- Smoking may be a risk factor for developing acute Q fever.
- A delay to initiation of empirical antibiotic therapy of greater than 5 days from symptom onset, a low haemoglobin of less than 13.5 g/L and a raised GGT greater than 48 IU/L are associated with progression to QFS.
- PI IgG and PI IgA are significantly higher in months 1 to 12 following disease onset in patients suffering from QFS.
Chapter 7: Summary and conclusions

The aims of the research undertaken for this PhD research degree were to:
evaluate the efficacy of a range of antibiotics using \textit{in vitro} and \textit{in vivo} models;
evaluate the efficacy of chemoprophylaxis for prevention or amelioration of acute
disease; and to characterize Q fever prevalence and outcome in a military cohort.

\textit{In vitro} experiments used axenic media and a THP-1 cell-based assay for the first
time to investigate the efficacy of doxycycline, levofloxacin, ciprofloxacin and co-
trimoxazole against \textit{C. burnetii}. Doxycycline is the recommended therapy for
 treatment of Q fever(106). In comparison to doxycycline, the quinolones performed
well with a bactericidal effect seen in both broth and the cell-based assay.
Doxycycline is bacteriostatic in the acidic environment required for \textit{C. burnetii}
replication(106), therefore a bactericidal treatment for Q fever may be
advantageous. In comparison co-trimoxazole, the recommended treatment for
pregnant patients, displayed high MICs in broth but it did perform better in the
intracellular environment.

\textit{In vivo} studies of antibiotic efficacy were performed in \textit{G. mellonella} larvae and AJ
mice. The key aims were to further assess the efficacy of these antibiotics in
different \textit{in vivo} models, and to assess the optimum window of opportunity for
treatment and prevention of disease. In \textit{G. mellonella} larvae and AJ mice
doxycycline hyclate and levofloxacin appear promising. A survival benefit was
demonstrated in larvae and weight loss reduction in AJ mice when doxycycline and
levofloxacin were used as treatment. When these antibiotics were used as
chemoprophylaxis a survival benefit was seen with levofloxacin in larvae, and
weight loss reduction for both antibiotics in AJ mice. These benefits were not seen
with ciprofloxacin and co-trimoxazole. Yet, in AJ mice treatment with ciprofloxacin
reduced spleen weight and bacterial burden.

Further AJ mouse efficacy studies were performed using doxycycline monohydrate
to investigate alternative treatment regimens. The results do not support the benefit
of using doxycycline for chemoprophylaxis. The studies suggest that initiating
antibiotics prior to \textit{C. burnetii} exposure, or early in the incubation period, may either
prolong the incubation period or lead to disease relapse when antibiotics are
stopped. The results of these efficacy studies support the findings from the small
human study conducted in the 1950s. In this study Tigertt \textit{et al.} found that
treatment late in the incubation period or at symptom onset is beneficial, but treatment early in the incubation period delayed symptom onset but did not prevent disease (189).

The main hypothesis postulated during this thesis for the potentially deleterious effect of antibiotics as chemoprophylaxis is that there is a reduction in activation of the innate and adaptive immune system when exposed to C. burnetii in the presence of antibiotics. A cell mediated immune response is required for clearance of this intracellular antibiotic (234). Antibiotics and the adaptive humoral response act as adjuncts, but if the cell mediated response is reduced when the patient is exposed to C. burnetii this may lead to bacterial immune evasion, which can lead to disease relapse when the antibiotics are stopped. Immune evasion may also lead to bacterial dissemination and storage in sanctuary sites, which may lead to disease relapse in the event of immune suppression. In addition, it is believed that all antibiotics used to treat Q fever are bacteriostatic at an acidic pH, thus preventing replication but not leading to eradication. If antibiotics are used as chemoprophylaxis the prevention of replication may also reduce the stimulation of the innate and adaptive immune response limiting the ability to eradicate this bacterium. To date there are no other in vitro or in vivo studies evaluating the efficacy of chemoprophylaxis for Q fever therefore this hypothesis is based on the results from the AJ mouse studies. Further work is required to investigate this proposed mechanism of immune downregulation and bacterial immune evasion when antibiotics are used as chemoprophylaxis.

Approximately 20% of the Q fever military cohort developed Q fever fatigue syndrome (QFS) following an acute infection. In some cases, this has led to debilitating fatigue and medical discharge from military service. Through treating these patients, the question of whether QFS could be prevented or reduced for future serving personnel arose. Published literature suggests that development of fatigue is related to severity of disease (93), therefore if the severity of disease is reduced this may reduce the impact of QFS. Analysis of the Q fever cohort suggested that treatment initiation greater than 5 days after symptom onset, mild anaemia and a raised GGT may increase the likelihood of progression to QFS. The relationship of these factors with QFS development could be due to an association with increased disease severity. It has been shown that doxycycline reduces fever duration, although for this effect it appears that treatment needs to start within 3 days of symptom onset (107). Published literature and the results of these studies
suggest there is an optimum time to antibiotic initiation following *C. burnetii* exposure and this appears to be around the time of symptom onset.

The results of these studies will augment the current published knowledge base on prevention and treatment of acute Q fever, and prevention or reduction of Q fever fatigue syndrome.

In conclusion, in the absence of a licensed Q fever vaccine in the UK, the novel data generated during this research degree suggest that to improve the clinical outcome for future Q fever patients in the military, timely diagnosis and early treatment at the point of symptom onset with an appropriate course of antibiotics needs to be achieved. It is suggested that this will reduce symptom duration, disease severity and the likelihood of developing long term fatigue.
Appendix A:

A1. Independent variables selected for data collection from the military Q fever cohort

Age
Gender
Rank
Trade group
Hospital admission and length of stay
Current/previous smoking history
Date of symptom onset
Disease outcome
  • Acute Q fever
  • Chronic Q fever
  • Q fever fatigue syndrome
Echocardiogram findings
Chest X-ray
Ultrasound abdomen
Antibiotics provided
Number of days from symptom onset until doxycycline initiation
Serology results
  • PII IgM
  • PII IgG
  • PI IgA
  • PI IgG
PCR results
Presenting signs
  • Temperature
  • Blood pressure
  • Heart rate
  • Respiratory rate
Clinical presentation
  • Fever
  • Headache
  • Arthralgia
• Myalgia
• Sore throat
• Chest pain
• Cough
• Anorexia
• Nausea
• Vomiting
• Diarrhoea
• Abdominal pain
• Confusion
• Shortness of breath
• Diaphoresis

Clinical signs
• Weight loss
• Splenomegaly
• Lymphadenopathy
• Hypoxia

Laboratory tests
• Alanine Aminotransferase (ALT)
• Aspartate Aminotransferase (AST)
• Alkaline Phosphatase (ALP)
• Gamma-Glutamyl Transpeptidase (GGT)
• Bilirubin
• Potassium
• Urea
• Creatinine
• Sodium
• Albumin
• Haemoglobin
• White cell count (WCC)
• Neutrophils
• Lymphocytes
• Platelets
• C-reactive protein (CRP)
Appendix B

B1. Mean bacterial counts by culture from lung, spleen, brain, adipose testes, blood and bone marrow from AJ mouse efficacy study 3

Tables 1-9: Mean bacterial counts by culture in the lung, spleen, brain, adipose, testes, blood and bone marrow of AJ mice following an aerosol challenge with a mean retained dose of $6.7 \times 10^4$ cfu of C. burnetii. The groups were provided with 9 or 16 days of doxycycline monohydrate (DM) commenced 1 day pre challenge, or 7 or 14 days of DM commenced 5 days post challenge and were euthanised either at completion of therapy or at the end of the study (day 35). The mean for the mice with viable counts detected are presented with the standard error of the mean (SEM). Where only one mouse had bacteria isolated the count in given and not the mean, these values are marked with an *. The limit of detection (LOD) for each tissue type was calculated taking into account the weight of the tissue, number of repeats performed, and the dilutions plated out. The average LOD was taken across each tissue type for these groups.

Table 1: The limit of detection for each tissue for mice euthanised between day 8 to 19.

<table>
<thead>
<tr>
<th>Limit of detection (LOD)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>140 cfu/g</td>
</tr>
<tr>
<td>Adipose</td>
<td>200 cfu/g</td>
</tr>
<tr>
<td>Testes</td>
<td>170 cfu/g</td>
</tr>
<tr>
<td>Blood</td>
<td>45 cfu/ml</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>90 cfu/g</td>
</tr>
</tbody>
</table>
Table 2: Viable counts in each tissue at day 8 in mice treated with doxycycline monohydrate (DM) or water for 9 days initiated 1 day pre challenge. One mouse from the DM treated group had organs that were contaminated with another bacteria which meant plates could not be read accurately.

<table>
<thead>
<tr>
<th>Organ/tissues tested for bacterial colonisation</th>
<th>DM for 9 days initiated pre challenge (cull D8)</th>
<th>Water for 9 days initiated pre challenge (cull D8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of positive mice (cfu/g)</td>
<td>SEM</td>
<td>N° of positive mice</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Lung (counts/g)</td>
<td>2.18 x10^3</td>
<td>7.05 x10^2</td>
</tr>
<tr>
<td>Spleen (counts/g)</td>
<td>&lt; 650</td>
<td>0/4</td>
</tr>
<tr>
<td>Brain (counts/g)</td>
<td>&lt; 140</td>
<td>0/4</td>
</tr>
<tr>
<td>Adipose (counts/g)</td>
<td>&lt; 200</td>
<td>0/4</td>
</tr>
<tr>
<td>Testes (counts/g)</td>
<td>&lt; 170</td>
<td>0/4</td>
</tr>
<tr>
<td>Blood (counts/ml)</td>
<td>&lt; 45</td>
<td>0/4</td>
</tr>
<tr>
<td>Bone Marrow (counts/g)</td>
<td>&lt; 90</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Table 3: Viable counts in each tissue at day 15 in mice treated with Doxycycline monohydrate (DM) or water for 16 days initiated 1 day pre challenge.

<table>
<thead>
<tr>
<th>Organ/tissues tested for bacterial colonisation</th>
<th>DM for 16 days initiated pre challenge (cull D15)</th>
<th>Water for 16 days initiated pre challenge (cull D15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of positive mice (cfu/g)</td>
<td>SEM</td>
<td>N° of positive mice</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Lung (counts/g)</td>
<td>3.31 x10^3</td>
<td>5.29 x10^2</td>
</tr>
<tr>
<td>Spleen (counts/g)</td>
<td>&lt; 650</td>
<td>0/5</td>
</tr>
<tr>
<td>Brain (counts/g)</td>
<td>&lt; 140</td>
<td>0/5</td>
</tr>
<tr>
<td>Adipose (counts/g)</td>
<td>&lt; 200</td>
<td>0/5</td>
</tr>
<tr>
<td>Testes (counts/g)</td>
<td>&lt; 170</td>
<td>0/5</td>
</tr>
<tr>
<td>Blood (counts/ml)</td>
<td>&lt; 45</td>
<td>0/5</td>
</tr>
<tr>
<td>Bone Marrow (counts/g)</td>
<td>&lt; 90</td>
<td>0/5</td>
</tr>
</tbody>
</table>
Table 4: Viable counts in each tissue at day 12 in mice treated with doxycycline monohydrate (DM) or water for 7 days initiated 5 days post challenge.

<table>
<thead>
<tr>
<th>Organ/tissues tested for bacterial colonisation</th>
<th>DM for 7 days initiated post challenge (cull D12)</th>
<th>Water for 7 days initiated post challenge (cull D12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean of positive mice (cfu/g)</td>
<td>SEM</td>
</tr>
<tr>
<td>Lung (counts/g)</td>
<td>1.22 x10^3</td>
<td>8.85 x10^2</td>
</tr>
<tr>
<td>Spleen (counts/g)</td>
<td>*1.38 x10^4</td>
<td>1/5</td>
</tr>
<tr>
<td>Brain (counts/g)</td>
<td>&lt; 140</td>
<td>0/5</td>
</tr>
<tr>
<td>Adipose (counts/g)</td>
<td>&lt; 200</td>
<td>0/5</td>
</tr>
<tr>
<td>Testes (counts/g)</td>
<td>&lt; 170</td>
<td>0/5</td>
</tr>
<tr>
<td>Blood (counts/ml)</td>
<td>&lt; 45</td>
<td>0/5</td>
</tr>
<tr>
<td>Bone Marrow (counts/g)</td>
<td>&lt; 90</td>
<td>0/5</td>
</tr>
</tbody>
</table>
Table 5: Viable counts in each tissue at day 19 in mice treated with doxycycline monohydrate (DM) or water for 14 days initiated 5 days post challenge. The splenic tissue for one mouse in the DM treated group was contaminated with another bacteria which meant plates could not be read accurately.

<table>
<thead>
<tr>
<th>Organ/tissues tested for bacterial colonisation</th>
<th>DM for 14 days initiated post challenge (cull D19)</th>
<th>Water for 14 days initiated post challenge (cull D19)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean of positive mice (cfu/g) SEM N° of positive mice</td>
<td>Mean of positive mice (cfu/g) SEM N° of positive mice</td>
</tr>
<tr>
<td>Lung (counts/g)</td>
<td>9.35 x10^2 1.46 x10^2 2/5</td>
<td>5.80 x10^3 4.70 x10^3 4/5</td>
</tr>
<tr>
<td>Spleen (counts/g)</td>
<td>*1.53 x10^3 1/4</td>
<td>*1.89 x10^3 1/5</td>
</tr>
<tr>
<td>Brain (counts/g)</td>
<td>&lt; 140 0/5</td>
<td>&lt; 140 0/5</td>
</tr>
<tr>
<td>Adipose (counts/g)</td>
<td>&lt; 200 0/5</td>
<td>&lt; 200 0/5</td>
</tr>
<tr>
<td>Testes (counts/g)</td>
<td>&lt; 170 0/5</td>
<td>&lt; 170 0/5</td>
</tr>
<tr>
<td>Blood (counts/ml)</td>
<td>&lt; 45 0/5</td>
<td>&lt; 45 0/5</td>
</tr>
<tr>
<td>Bone Marrow (counts/g)</td>
<td>&lt; 90 0/5</td>
<td>&lt; 90 0/5</td>
</tr>
</tbody>
</table>

Table 6: The limit of detection for each tissue for mice euthanised at the end of the study (day 35).

<table>
<thead>
<tr>
<th>Limit of detection (LOD)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>164 cfu/g</td>
</tr>
<tr>
<td>Brain</td>
<td>33 cfu/g</td>
</tr>
<tr>
<td>Adipose</td>
<td>22 cfu/g</td>
</tr>
<tr>
<td>Testes</td>
<td>42 cfu/g</td>
</tr>
<tr>
<td>Blood</td>
<td>18 cfu/ml</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>90 cfu/g</td>
</tr>
</tbody>
</table>
**Table 7:** Viable counts in each tissue at day 35 in mice treated with doxycycline monohydrate (DM) for 9 or 16 days initiated 1 day pre challenge. The group treated for 16 days only contained 8 mice because one mouse was euthanised due to low weight and failure to thrive prior to the start of the study and one mouse had contamination of the lung and splenic tissue which meant plates could not be read accurately.

<table>
<thead>
<tr>
<th>Organ/tissues tested for bacterial colonisation</th>
<th>DM for 9 days initiated pre challenge (cull D35)</th>
<th></th>
<th>DM for 16 days initiated pre challenge (cull D35)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean of positive mice (cfu/g)</td>
<td>SEM</td>
<td>N° of positive mice</td>
<td>Mean of positive mice (cfu/g)</td>
</tr>
<tr>
<td>Lung (counts/g)</td>
<td>5.84 x 10^2</td>
<td>1.72 x 10^2</td>
<td>6/10</td>
<td>7.71 x 10^2</td>
</tr>
<tr>
<td>Spleen (counts/g)</td>
<td>&lt; 164</td>
<td>0/10</td>
<td></td>
<td>2.85 x 10^3</td>
</tr>
<tr>
<td>Brain (counts/g)</td>
<td>&lt; 33</td>
<td>0/5</td>
<td>&lt; 33</td>
<td></td>
</tr>
<tr>
<td>Adipose (counts/g)</td>
<td>&lt; 22</td>
<td>0/5</td>
<td>&lt; 22</td>
<td></td>
</tr>
<tr>
<td>Testes (counts/g)</td>
<td>&lt; 42</td>
<td>0/5</td>
<td>&lt; 42</td>
<td></td>
</tr>
<tr>
<td>Blood (counts/ml)</td>
<td>&lt; 18</td>
<td>0/5</td>
<td>&lt; 18</td>
<td></td>
</tr>
<tr>
<td>Bone Marrow (counts/g)</td>
<td>&lt; 90</td>
<td>0/10</td>
<td>&lt; 90</td>
<td></td>
</tr>
</tbody>
</table>

**Table 8:** Viable counts in each tissue at day 35 in mice treated with doxycycline monohydrate (DM) for 7 or 14 days initiated 5 days post challenge.

<table>
<thead>
<tr>
<th>Organ/tissues tested for bacterial colonisation</th>
<th>DM for 7 days initiated post challenge (cull D35)</th>
<th></th>
<th>DM for 14 days initiated post challenge (cull D35)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean of positive mice (cfu/g)</td>
<td>SEM</td>
<td>N° of positive mice</td>
<td>Mean of positive mice (cfu/g)</td>
</tr>
<tr>
<td>Lung (counts/g)</td>
<td>2.20 x 10^2</td>
<td>40</td>
<td>2/10</td>
<td>1.41 x 10^2</td>
</tr>
<tr>
<td>Spleen (counts/g)</td>
<td>&lt; 164</td>
<td>0/10</td>
<td>&lt; 164</td>
<td></td>
</tr>
<tr>
<td>Brain (counts/g)</td>
<td>&lt; 33</td>
<td>0/5</td>
<td>&lt; 33</td>
<td></td>
</tr>
<tr>
<td>Adipose (counts/g)</td>
<td>&lt; 22</td>
<td>0/5</td>
<td>&lt; 22</td>
<td></td>
</tr>
<tr>
<td>Testes (counts/g)</td>
<td>&lt; 42</td>
<td>0/5</td>
<td>&lt; 42</td>
<td></td>
</tr>
<tr>
<td>Blood (counts/ml)</td>
<td>&lt; 18</td>
<td>0/5</td>
<td>&lt; 18</td>
<td></td>
</tr>
<tr>
<td>Bone Marrow (counts/g)</td>
<td>&lt; 90</td>
<td>0/10</td>
<td>&lt; 90</td>
<td></td>
</tr>
</tbody>
</table>
Table 9: Viable counts in each tissue at day 35 in mice treated with water for 14 days initiated 5 days post challenge.

<table>
<thead>
<tr>
<th>Organ/tissues tested for bacterial colonisation</th>
<th>Water for 14 days initiated post challenge (cull D35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean of positive mice (cfu/g)</td>
</tr>
<tr>
<td>Lung (counts/g)</td>
<td>$1.35 \times 10^3$</td>
</tr>
<tr>
<td>Spleen (counts/g)</td>
<td>&lt; 164</td>
</tr>
<tr>
<td>Brain (counts/g)</td>
<td>&lt; 33</td>
</tr>
<tr>
<td>Adipose (counts/g)</td>
<td>&lt; 22</td>
</tr>
<tr>
<td>Testes (counts/g)</td>
<td>&lt; 42</td>
</tr>
<tr>
<td>Blood (counts/ml)</td>
<td>&lt; 18</td>
</tr>
<tr>
<td>Bone Marrow (counts/g)</td>
<td>&lt; 90</td>
</tr>
</tbody>
</table>
B2. Cytokine levels in the blood of mice from AJ mouse efficacy study 3

Table 10: Levels of IFN-γ, TNF-α, IL1, IL2, IL4, IL6, IL8, IL10 and IL12 in AJ mice following an aerosol challenge with a mean retained dose of 6.7 x 10^4 cfu of C. burnetii. Mice were treated with 9 or 16 days of doxycycline monohydrate commenced 1 day pre challenge or 7 or 14 days of doxycycline monohydrate commenced 5 days post challenge. The serum was taken at completion of therapy.

<table>
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<th>Treatment groups</th>
<th>IL-1b</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IL-12</th>
<th>IFN-γ</th>
<th>TNF-α</th>
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<td>21.8</td>
<td>15.5</td>
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<td>10.5</td>
<td>100.5</td>
<td>12.5</td>
<td>31.3</td>
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<td>3</td>
<td>6.8</td>
<td>7.5</td>
<td>121</td>
<td>34.5</td>
<td>71.5</td>
<td>17.5</td>
<td>210.8</td>
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<td>143.5</td>
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<td>13.8</td>
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<td>24.5</td>
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<td>16.3</td>
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<td>1.5</td>
<td>-16.5</td>
<td>15.5</td>
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</table>
Table 11: Levels of IFN-, TNF-, IL1, IL2, IL4, IL6, IL8, IL10 and IL12 in AJ mice following
an aerosol challenge with a mean retained dose of 6.7 x 104 cfu of C. burnetii. Mice were
treated for 5 days with doxycycline monohydrate or water commenced on the day of
challenge. Two groups of mice were not challenged with C. burnetii but provided with
doxycycline monohydrate or water for 5 days as a comparison. The serum was taken at
completion of therapy.

Table 12: Levels of IFN-, TNF-, IL1, IL2, IL4, IL6, IL8, IL10 and IL12 in AJ mice following
an aerosol challenge with a mean retained dose of 6.7 x 104 cfu of C. burnetii. Mice were
treated with 9 or 16 days of doxycycline monohydrate commenced 1 day pre challenge or 7
or 14 days of doxycycline monohydrate commenced 5 days post challenge. The serum is
from the groups at the end of the study (day 35).

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Appendix C: List of publications, presentations and posters completed during the PhD research degree

C.1. Publications


C.2. Oral presentations


C.3. Posters


Clay KA, Hartley MG, Whelan A, Norville IH. Coxiella burnetii can be found in cells associated with seminal fluid but is not attached to the spermatozoa during acute infection in the A/J mouse model. Poster presented at: 16th Medical Biodefense Conference, Bunderswehr, Munich, Germany. 28-31st October 2018.
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capabilities on deployments – Rapidly Deployable Outbreak Investigation Team (RDOIT) investigates Q fever outbreak in Kosovo in April 2016]. Wehrmedizinische Monatsschrift. 2019; 60(9/10):289.


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