THESIS

DEVELOPING A MURINE MODEL FOR Q FEVER

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ABSTRACT

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Coxiella burnetii is a gram-negative, intracellular bacterium that causes disease in humans and animals. The bacterium is commonly found in nature and humans and animals develop infections by inhaling infectious aerosols. Animal infections are generally asymptomatic, but the organism can induce abortion in pregnant sheep, goats, and cattle. Human infections, called Q fever, can induce mild to moderate disease, and lifelong infections may develop. Research to characterize this bacterium has been difficult due to its intracellular nature, and studying experimental infections in animal models has provided important information about the bacterial lifecycle and pathogenesis of the disease. The studies described here focused on evaluating a number of facets of *C. burnetii* infection in C57BL/6 inbred mice. Infections were determined through immunofluorescence detection, quantitative PCR assays, and histopathologic analysis. Mice developed similar histopathologic lesions as humans, specifically hepatitis, interstitial pneumonia, and myocarditis when infected by intranasal inoculation with the Nine Mile phase I strain of the bacterium. Detection of bacterial DNA in tissues and frequency of histopathologic lesions were highest two weeks after infection, with a significant decrease observed 42 and 59 days

after infection. Mouse age and chemically induced immunosuppression with dexamethasone or cyclophosphamide were evaluated in *C. burnetii* infected mice to determine if these factors exacerbated disease. These studies revealed that neither age, (nine-weeks versus nine-months), or chemically induced immunosuppression (dexamethasone versus cyclophosphamide) significantly enhanced disease manifestations in infected mice. Additionally, antimicrobial treatment with doxycycline was evaluated in treating *C. burnetii* infections in mice. Such treatment reduced splenic pathology but did not significantly reduce the frequency of other histopathologic lesions or the amount of bacterial DNA detected in tissues. Overall, C57BL/6 mice infected by intranasal inoculation develop histopathologic lesions similar in many respects to what is observed in infected humans. However, disease manifestations were not exacerbated by host age or the immunosuppressive treatments investigated. Additionally, the dose of doxycycline received by mice was only marginally effective in treating bacterial infection in mice.

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CHAPTER 1: LITERATURE REVIEW

History of Coxiella burnetii

Coxiella burnetii was discovered during a public health review of an illness plaguing slaughterhouse workers in Brisbane, Australia from 1933 to 1935 [1]. The most common symptom was fever, which lasted from seven to 24 days. Dr. Edward Derrick was the first person to determine that the febrile illness was caused by a novel infectious organism and gave the disease the name Q fever. Subsequently, he was able to illicit a febrile response in guinea pigs when he inoculated them with blood and urine from infected patients; however he was unable to isolate or identify the infectious organism [1]. He sent infected guinea pig liver samples to virologist Dr. Francis Macfarlane Burnet who was able to reproduce the febrile illness in mice and observed rickettsia-like particles in infected mouse spleens [2]. Dr. Burnet along with Dr. Mavis Freeman infected several animal species to determine the pathogenesis and virulence of the organism in different hosts. They determined that the guinea pig liver emulsion was infective for mice and monkeys, the organism was filterable, and the pathogen survived in the chorio-allantois of developing eggs without inducing lesions [2].

In 1938, Dr. Herald Cox isolated a similar small, pleomorphic; gram-negative pathogen from *Dermacentor andersoni* ticks near Nine Mile Creek, Montana and named it *Rickettsia diaporica* [3-5]. The Australian and *D. andersoni* isolates were compared for virulence and infectivity and determined to be the same species. Cox re-named the pathogen *Rickettsia burnetii* after discoveries made by Dr. Burnet. Dr. Cornelius Philip solicited that the pathogen be placed in a new genus, *Coxiella*, to honor Dr. Cox's discovery [6]. *Coxiella burnetii* was classified into the Proteobacteria phylum, alpha-1 class, Rickettsiales order, *Rickettsiaceae* family, in the *Rickettsia* tribe [7]. The pathogen was evaluated in a variety of comparison studies to other rickettsial organisms, specifically *Rickettsia prowazeki*, and did not have the same characteristics. Based on the comparison of 16S ribosomal RNA gene sequences, *Coxiella burnetii* was reclassified and remained in the Proteobacteria phylum, but was placed in the Gammaproteobacteria class, Legionellales order, in the *Coxiellaceae* family [8]. *Coxiella burnetii's* closest phylogenetic neighbors are Legionella and Francisella [8]. *Legionella pneumophila* is the closest related pathogen due to its genetic and phenotypic characteristics, specifically the fact that the bacterium undergoes intracellular growth within a membrane bound vacuole [8].

Bacteriology

C. burnetii is a small, pleomorphic, intracellular bacterium measuring 0.2 by 0.7 micrometers [9]. Much variability has been observed when the organism undergoes Gram-staining; however, electron microscopy has shown that the

outer membrane of the bacterium has characteristics typical of gram-negative bacteria [9, 10].

The complete genome of *C. burnetii* Nine Mile strain was sequenced by Seshadri *et al*. in 2003 [11]. *C. burnetii* has a single, circular chromosome two mega base pairs in length [12]. The Nine Mile strain is 1,995, 275 base pairs long and has a molecular G+C content of 42.6% [11, 13]. This strain harbors one 34.7 kilo base plasmid, QpH1, while other plasmids have been identified in other strains including, QpDG, QpDV, and QpRS, which allows for genetic typing between strains [13-16]. Plasmid type has been proposed to be related to virulence and disease severity in humans [14]. Insertion elements (IS) were commonly observed in the Nine Mile genome, with approximately 20 copies of the *IS1111* gene; the IS is not located in the QpH1 plasmid [11]. Additionally, the *C. burnetii* genome has components of type I, II, and IV secretion systems; the type IV secretion system is similar to the one observed in the Legionella genome [17, 18].

Davis and Cox were the first researchers to observe that the bacterium was distinctly pleomorphic [3]. In 1959, Dr. Nonna Kordova discovered that there was antigenic variability among the bacterium [19]. These findings led to additional studies evaluating the differences in the two bacterial life forms and the idea of a biphasic developmental cycle [20, 21]. In 1981, researchers McCaul and Williams proposed that *C. burnetii* had a biphasic developmental life cycle, which included a small, metabolically inert small cell variant (SCV) and a large, metabolically active large cell variant (LCV) [3, 10, 22]. The researchers

observed another cell variant in electron microscopy studies and identified it as a spore-like particle (SLP) [8, 23].

Small cell variants are rod shaped, have electron-dense condensed chromatin, and are 0.2 to 0.5 um long. LCVs are pleomorphic with dispersed chromatin, measuring greater than 1 um in length, and are metabolically active *in vitro* [10, 22]. The cell variants have contrasting structural components and resistance mechanisms [13, 23]. Unlike the fragile LCV, the SCV is resistant to lysis by pressure and other physical disruptions while retaining its morphologic features [23, 24]. During infection, SCVs are phagocytosed by eukaryotic cells, pulmonary macrophages and Kupffer cells, and sequestered in phagolysosomes. The acidic environment of the phagolysosome activates the transition from SCVs to metabolically active LCVs. The LCVs are able to transition back prior to lysis of the phagolysosome, resulting in the release of SCVs [22, 23]. McCaul and Williams proposed that the spore-like form is induced by eukaryotic cell changes during LCV replication, which leads to the development and release of spore-like particles [10]. The SLP form of the bacterium has not been fully elucidated since its discovery in 1981. Recently, it was theorized that SLPs differentiate to SCVs [13, 25]. However, these particles have not been isolated or purified to prove such hypotheses. Additionally, the endospore form is only occasionally observed and the *C. burnetii* genome lacks known sporulation genes [10, 11, 22]. The SCV has shown resistance to biochemical and physical agents, suggesting this variant replaces the need for a SLP [24, 26]. Definitive information is not available about the existence of a SLP *C. burnetii* variant in the developmental lifecycle.

However, research has proven that a biphasic developmental cycle exists for *C. burnetii* and the different resistance mechanisms and metabolic activities of the variants are essential for bacterial survival in the host.

Bacteria enter into host cells through the process of microfilament endocytosis in which the host cell, upon binding the bacterium, induces reorganization of the actin cytoskeleton leading to membrane protrusions at the site of bacterial attachment [27-30]. *C. burnetii* entry into host cells ends in the pathogen residing in a parasitophorous vacuole (PV) resembling a secondary lysosome [8, 13]. The PV is acidic, pH 4.7- 4.8, and the acidic nature of this vacuole activates *C. burnetii* SCVs to transform to LCVs [13, 31]. The *C. burnetii* growth cycle has been defined in cell culture and includes lag, log, and stationary phases [18]. In Vero cell culture, Coleman *et al*., observed a lag phase beginning two days post infection, with no increase in bacterial replication, characterized by SCVs transitioning to LCVs. This was followed by exponential growth in the PV dominated by LCVs four days after infection. Finally, the LCV transitions back to a SCV during a stationary phase that starts six days after infection just before cell lysis, which allows SCVs to infect other cells [18]. A similar system is believed to occur in the mammalian host, with SCVs infecting alveolar macrophages during inhalation of infectious organisms [24, 32].

In addition to the biphasic developmental cycle, *C. burnetii* has two phase variations, phase I and II, based on the lipopolysaccharide (LPS) structure [33]. These forms are indistinguishable under a microscope but have distinct virulence attributes [13]. Phase I is characterized by a full-length, smooth-type LPS and is

the more virulent form, while the phase II form has a truncated, rough-type LPS and is the avirulent form [33]. Phase II LPS lacks sugar groups virenose and dihydrodroxystreptose observed in phase I [34]. The antigenic change from the virulent to the avirulent form is often the result of a chromosomal deletion, affecting the biosynthetic genes in the O-antigen cluster of the LPS and genes involved in virenose synthesis [35, 36]. The LPS is theorized to be antigenic for *C. burnetii* and has been found to induce production of inflammatory cytokines in human and murine macrophages and stimulates antibody production [37, 38]. Phase I cells are not as readily ingested by host cells as phase II forms. Phase I requires $\alpha_v\beta_3$ integrin on host cells whereas phase II cells require $\alpha_v\beta_3$ integrin and CR3 on the host cell for entry [39]. Phase I cells can be isolated from infected hosts but phase II cells cannot [13]; the only time phase II cells can be obtained is following serial passages in tissue culture or eggs [40]. Phase I cells have several ways to evade identification by the host innate immune system, leading to phagocytosis of the organism. Virulent phase I organisms stimulate the activation of two src-related protein tyrosine kinases, Lyn and Hck, resulting in actin cytoskeletal rearrangement and impairment of phagocytosis by the host cell [27, 30]. Antibodies to surface proteins on *C. burnetii* are sterically inhibited to bind phase I organisms [41]. Phase I LPS masks Toll-like receptor (TLR) ligands to prevent host dendritic cells from binding, and inhibits CR3 interaction with host cells to prevent efficient binding, evading phagocytosis by the host cell [42]. Additionally, membrane ruffling induced by endocytosis blocks binding of phase I cells to CR3 on the host cell, decreasing efficiency of internalization [27].

Contrastingly, avirulent phase II organisms cannot prevent phagosome-lysosome fusion resulting in phagocytosis of the organism [43]. Phase II organisms have a lower carbohydrate content due to the truncation in their LPS, making the cell highly hydrophobic, which increases interactions with the receptors on the host cell plasma membrane [32]. The phase I cells are more virulent in the host due to their ability to evade identification and subsequent phagocytosis by host cells.

Epidemiology

C. burnetii is a ubiquitous environmental pathogen that has been reported throughout the U.S. [44] and in nearly every country except for New Zealand and the Antarctic [13, 45]. The bacterium is able to infect a wide range of reservoirs including, domestic and wild mammals, birds, and arthropods [13, 46]. Cattle, sheep, and goats are the primary reservoir hosts and are responsible for the majority of human infections [47]. The primary mode of transmission for humans and animals is inhalation of infectious organisms; the infectious dose for humans has been found to be fewer than 10 bacteria [48, 49]. Ingestion of infectious organisms and the bite of infected ticks are also routes of infection [32, 46, 50]. Several studies have determined arthropod reservoirs, particularly tick species, to be important in the spread of the bacteria but not essential in the bacterial lifecycle [47, 51]. The bacterium targets the reproductive organs of reservoir hosts, specifically the placenta, uterus, and mammary glands; infected placentas harboring greater than 10^9 microorganisms per gram of tissue have been documented [52-54]. Bacterial transmission primarily occurs during parturition when infected placental tissue and birthing contents become aerosolized [48, 55-

57]. Bacteria are also excreted in small amounts in the urine, feces, and milk of infected parturient reservoir hosts, and have been found in vaginal mucus of non-pregnant and pregnant ewes, representing additional modes of bacterial transmission [58, 59]. The bacterium can survive in the environment for long periods of time and infectious aerosols have been found two weeks after parturition [47]. Also, previous studies by Welsh *et al.* indicated that *C. burnetii* could survive in the soil for up to 150 days [57]. Research by Kersh *et al*. evaluated the presence of *C. burnetii* in environmental samples throughout the United States between 2006 and 2008 [44]. Kersh found that *C. burnetii* was found in a broad variety of environments, especially public locations (banks, schools, post offices) with no surrounding farms, ranches, or livestock [44]. The ability of the bacterium to survive in the environment for long periods of time, infect several reservoir hosts, and become aerosolized allows *C. burnetii* to contaminate or infect a wide range of habitats and species, which makes it a successful pathogen.

Human Disease

Humans primarily become infected through the inhalation of infectious aerosols [48]. Several studies have attempted to determine the risks associated with *C. burnetii* contaminated dairy products. However, results to these studies are contradictory and the risk of infection by that route for humans remains inconclusive [25, 46, 60]. While infected ticks are important in the natural transmission cycle, they apparently do not transmit *C. burnetii* to humans [25, 50]. Human to human transmission is rare, but has been observed in cases of

sexual transmission [61, 62] and perinatal infections [63]. Human disease can be classified as either acute or chronic. Approximately 60% of infected people develop asymptomatic infection [25, 64]. Flu-like symptoms, fever, and severe headaches are clinical symptoms associated with acute disease, and pneumonia and hepatitis are common histopathologic lesions associated with acute infection [1, 65, 66]. Chronic disease develops in approximately 1-2% of acutely infected cases; symptoms may not develop until years after infection [64]. Chronic disease is often associated with culture-negative endocarditis, and rarely, osteomyelitis, osteoarthritis and Q fever fatigue syndrome [64, 67, 68]. Development of certain disease manifestations associated with *C. burnetii* infection have been linked to the phase and strain of the pathogen, the inoculation route, the dose received [14, 69], and host factors [70, 71]. Observational research by Dr. Thomas Marrie demonstrated that inhalation of infectious organisms induced pneumonia while intraperitoneal inoculation and ingestion of infectious organisms resulted in hepatitis [69]. Pathologies associated with *C. burnetii* infection have also been geographically linked. Q fever pneumonia is frequently observed in Canada and Spain while Q fever hepatitis is commonly observed in France and Australia [72]. Hepatitis is currently the most common presentation of acute Q fever worldwide [25], and can present as clinically asymptomatic and granulomatous hepatitis [25]. Atypical pneumonia is the second most frequently observed lesion associated with *C. burnetii* infection [25]. Pulmonary lesions are usually mild; however, the duration of symptoms can last from 10 to 90 days [66]. The most commonly observed

chronic disease manifestation associated with chronic Q fever is endocarditis, which may appear with pericardial effusion and/or pericarditis [25, 73, 74]. This lesion is almost exclusively seen in patients with pre-existing valvulopathies [46, 68, 74].

The limited understanding of the disease pathogenesis in humans is due in part to the difficulties associated with characterizing an intracellular pathogen and lack of available animal models developing similar disease to humans. Although little information is available on disease pathogenesis in humans, previous research has uncovered immune components necessary in preventing and clearing bacterial infection. Cell mediated immunity is essential in controlling Q fever, and deficiencies in this function result in chronic disease [13, 25, 75]. Previous research has determined that chronically infected people lack sufficient T-cell responses, preventing bacterial clearance [76, 77]. Additionally, people with chronic disease produce greater amounts of prostaglandin E2 and high levels of tumor necrosis factor, which cause immunosuppression in the host [76, 78, 79]. Chronic Q fever patients also have increased IL-10 secretion, which is believed to control the inflammatory response and limit the pathogenic effects induced by *C. burnetii* allowing for prolonged infection [75]. Dysregulation of immune components clearly modulates the severity of Q fever disease; however, the role of host immune function in controlling bacterial infection requires further exploration and research.

The antimicrobial therapies prescribed to treat Q fever are based on the disease state. Standard treatment for acute disease is 100 milligrams per

kilogram (mg/kg) of doxycycline for 14 days. The primary alternative to doxycycline is fluoroquinolone therapy [46]. Suggested treatment for acutely infected pregnant women is cotrimoxazole 800 mg/kg until delivery [80]. Obstetric complications were observed in 81% of women who did not receive cotrimoxazole therapy during pregnancy [81]. Treatment of chronic disease is complicated and is often dependent on the observed disease manifestation. Synergistic therapy with hydroxychloroquine is often used to prevent resistance. The current recommendation is 200 mg doxycycline along with 600 mg of hydroxychloroquine daily for a minimum of 18 months [25, 81].

Development of a human vaccine against *C. burnetii* has been investigated since the discovery of the bacterium. A live-attenuated vaccine produced and tested in Russia in the 1960's [82, 83] was later abandoned over concerns of long-term persistence and potential development of endocarditis in vaccinated individuals [84]. A chloroform-methanol residue extracted vaccine was developed in the U.S. in the 1990s but was very reactogenic in animals and was never tested in humans [85]. A whole-cell formalin-inactivated Q fever vaccine (Q-vax) was developed and licensed for use in Australia and is currently given to high-risk individuals. This vaccine was prepared from the phase I Henzerling strain [13] and has been shown to be 100% effective in slaughterhouse workers years after vaccination [86, 87]. There are still concerns over the side effects of this vaccine in that it can cause severe reactions at the inoculation site [13, 88, 89]. It has also been associated with complications in

sero-positive patients, such that a pre-vaccination skin test is required for all vaccine recipients.

Animal Disease

Coxiella burnetii is able to infect a wide variety of both domestic and wild animal species. However, little is known about bacterial infection and pathogenesis in animals. The primary modes of transmission in animals are thought to be inhalation of infectious organisms and ingestion of contaminated feed and bedding [90]. Infection typically occurs during lambing and calving season, as contaminated aerosols are produced [54, 57, 91]. Additionally, *C. burnetii*-infected ticks are thought to be important in bacterial transmission among animals [90, 92]. *C. burnetii* infections in animals are generally asymptomatic but can cause reproductive complications, specifically stillbirths and abortions. Bacterial infection may also result in the birth of weak lambs, kids, or calves [93]. In most cases, abortion occurs at the end of gestation, with no previous clinical signs [53]. Aborted fetuses appear normal; however, the infected placenta typically has intercotyledonary thickening and discoloration [53]. Post-parturient mammals can shed bacteria into the environment for long periods of time, while infected, non-pregnant animals do not regularly shed bacteria into the environment [52, 91]. *C. burnetii* is shed from the vaginal mucus, feces, and milk of infected goats, ewes, and cattle [58]. Shedding rates differ between species and route. *C. burnetii* has been detected in the vaginal mucus of infected ewes up to 71 days after parturition and cattle have been reported to shed

bacteria into milk up to 13 months after parturition [94, 95]. Additionally, cattle can shed the organism in their birth secretions for successive years and sheep have been documented shedding the bacterium in successive pregnancies [94, 96]. Infection appears to be persistent in pregnant animals and immunosuppression caused by pregnancy has been known to enhance multiplication of the organism [97]. Overall, *C. burnetii* infection can induce abortion in pregnant animals and cause prolonged shedding rates. In previously infected animals, the bacterium becomes dormant until pregnancy, at which time, a recrudescence of bacterial infection is observed. Information about bacterial infection in the reservoir host is necessary for the prevention of zoonotic disease.

Diagnostics

Diagnosing *C. burnetii* infection in cattle, sheep, and goats is essential in controlling the spread of infection to other animals and humans. As with other diseases, diagnosis of *C. burnetii* infection relies both on detecting the agent and host immune responses to the agent. Bacterial cultures are important diagnostic methods in determining presence of bacteria from environmental and host samples, but historically, *C. burnetii* has been impossible to culture *in vitro* due to the strict metabolic requirements and intracellular nature of the bacterium [1]. Recently, Omsland *et al.* developed a cell-free laboratory medium that meets the strict growth requirements necessary for *C. burnetii* growth [98]. Although this method is novel and interesting, it has not yet been adapted for real world diagnostic settings.

Infected animals are generally asymptomatic, but the presence of abortions may indicate *C. burnetii* infection within a flock or herd. Placental tissues from aborted animals can be stained for bacterial detection using the Gimenez or Machiavello staining methods [25, 53, 99]. Recent PCR and qPCR assays have been developed to determine the presence of bacteria in excreta, milk, and tissues. These assays amplify repetitive gene sequences found in the *C. burnetii* genome such as the *IS1111* insertion gene [59, 100]. PCR assays have higher sensitivity and specificity to bacterial detection in animal samples (excluding serum) than other diagnostic methods, and may be the reason for the increase in the number of identified cases since the last decade [53]. Limitations of these PCR assays include their cost and inability to process at site of collection. However, it is essential to detect bacterial infection in herds early to prevent further spread and environmental contamination leading to future problems. In conclusion, diagnosing infections in animals is difficult due to the lack of specific clinical signs and the high frequency of subclinical infections. Therefore, a combination of serological testing and PCR assays are necessary to determine animal infection.

Serological assays are the primary diagnostics used for detecting bacterial infection in humans. The OIE reference assay for detecting infection in animals is the complement fixation (CF) test. However, the CF test has low sensitivity and the antigen used often gives false-negative results [25, 53]. An alternative reference test used widely for serodiagnosis of Q fever is indirect immunofluorescence (IFA) [53, 72]. The IFA assay allows for the distinction

between acute and chronic disease by evaluating binding of patient antibodies to both *C. burnetii* phase I and II [101]. Cases in which the phase II antibody titer is higher than phase I are considered acutely infected, whereas high antibody titers to the phase I antigen signifies chronic disease [53]. Additional serodiagnostics include microagglutination, radioimmunoassay, and ELISA [47, 90, 101]. One constraint of all serodiagnostic assays is they cannot detect bacterial infection until seroconversion has occurred [53]. There is a considerable lag time where the host is infected but has not seroconverted, and serodiagnostic testing within that time will result in a false-negative diagnosis [53]. PCR assays are able to detect bacterial infection much earlier than serodiagnostic assays. However, serum cannot be used as a sample because it contains too few intracellular organisms [101]. Concurrent use of serodiagnostics, IFA and PCR, is necessary to determine infection in humans to ensure that bacteria can be detected at any stage of the infection.

Treatment and Control

Q fever is primarily spread to humans and animals by inhalation of infectious organisms. Therefore, specific measures must be taken to protect humans in contact with reservoir hosts, cattle, sheep, and goats. Suspect infected herds should be monitored with serodiagnostics and a variety of control measures have been advocated to minimize risk of infection. To prevent bacteria from contaminating the environment, contaminated birth products should ideally be incinerated [25]. Manure should not be spread during windy periods to prevent widespread contamination, and suspect infected manure should be treated with

lime or a 4% calcium cyanide solution [25, 53]. Tetracycline's are often used to reduce the amount of bacterial shedding during parturition [53, 102]. Routine monitoring after infection is important in evaluating the progression of infection in the herd. However, negative results do not correlate with complete clearance of the bacterium as it is known to survive and remain infectious in the environment for long periods of time. Therefore, the only way to completely ensure prevention is through herd vaccination.

Implementing herd vaccination protocols dramatically reduces environmental contamination and the incidence of human disease [25, 53]. The most effective vaccines for animals are made from inactivated phase I form of the bacterium [53, 103]. Souriau *et al.* evaluated the efficacy of vaccinating pregnant goats with phase I and II vaccines and challenging them with *C. burnetii*. In this study it was found that phase II vaccine was not protective against abortions and could not prevent bacterial shedding in the milk, feces, or vaginal secretions whereas the phase I vaccine reduced bacterial shedding from the placental tissue and excreted milk, but could not prevent shedding in the milk [104]. The authors determined that phase I vaccines were only protective for uninfected animals and failed to prevent shedding from previously infected animals [53, 104]. Another downfall of the phase I vaccine is that vaccinated animals are indistinguishable from infected animals, which makes it difficult to determine which animals to vaccinate [53]. In conclusion, prevention of environmental contamination is essential for decreasing infection rates of humans and animals. Since the bacterium can survive in the environment for long periods of time,

infection cycles within herds will not be eradicated with antibiotic treatment alone. Herd vaccination programs are effective in decreasing bacterial shedding from animals and environmental contamination.

Animal Models

The need for animal models for *C. burnetii* is two-fold; to elucidate bacterial pathogenesis in human and animal hosts, and to develop vaccines and drug therapies. Few studies have explored developing an animal model for animal disease. Experimental infection studies have been performed in cattle, sheep, and goats [32, 56, 58, 104-106]. These studies were essential in uncovering bacterial shedding rates and routes from post-parturient animals. Such studies also demonstrated the low incidence of bacterial shedding from non-pregnant animals. Infected, non-pregnant animals do not display clinical symptoms, but typically develop antibody titers after infection [32]. Additionally, histopathologic disease is minor to non-existent. Information about potential bacterial recrudescence in infected non-pregnant animals reveals that immunosuppression, as seen in pregnancy, can often lead to development of clinical disease [58, 103, 104]. These findings have also been shown in guinea pig and mouse experimental infections, where animals were immunosuppressed after inoculation, inducing bacterial infection [107]. Experimental infection studies in pregnant goats revealed bacterial shedding after multiple pregnancies, with bacterial latency occurring between pregnancies [58]. Similar findings have also been shown in experimental mouse infection studies where mice shed bacteria after successive pregnancies [108]. Bacteria appear to become latent in the

animal host, becoming reactivated during pregnancy-associated immunosuppression. The duration of this latency period has not been elucidated in the reservoir host. However, it is essential in understanding bacterial pathogenesis in small ruminants. Further experimental studies need to be conducted in pregnant and non-pregnant animal models to determine if bacterial latency occurs and where bacteria lay dormant until activation leading to clinical disease. Developing an animal model for animal disease is essential in understanding bacterial life cycle in the animal host in order to control infection and prevent transmission to other animals and humans. Additionally, animal models are necessary for developing vaccine and drug therapies that could be used to treat and prevent this latency period as well as shedding associated with immunosuppression.

Several rodent models have been developed for human Q fever; guinea pigs and mice are the most common models [73, 109-111]. Many rodent models mimic clinical and histopathologic disease associated with human acute Q fever when infected with the phase I form of *C. burnetii*; common symptoms include, fever, splenomegaly, and interstitial pneumonia [112]. Establishing rodent models for human chronic disease proves to be challenging. The primary hallmark of human disease is endocarditis, which is rarely observed in experimental infections in rodents, but has been observed in mice infected with large doses of phase I *C. burnetii* [111]. Recently, a mouse model that mimics human chronic Q fever was developed in transgenic-mice constitutively expressing IL-10 in macrophages [113]. Infection of these mice led to a persistent bacterial infection

in which macrophages were unable to kill *C. burnetii*, which is believed to be the situation in cases of chronic Q fever [113]. Development of this model was important in understanding bacterial persistence in the host. However, chronic histopathology (endocarditis) observed in human infection was not seen in infected transgenic mice. While no animal model truly mimics human acute and chronic disease, development of a mouse model for chronic disease revealed important information about bacterial persistence in the mouse host, which can be translated to human disease. In conclusion, continual efforts to develop an animal model for human infection are essential in understanding bacterial pathogenesis and developing effective vaccines for public health benefit.

Future Directions for Coxiella Research

Forgoing review of *C. burnetii* shines on two areas of research that are in desperate need of resolution, vaccine development and diagnostics. Uncovering information about bacterial pathogenesis and development of chronic infections in reservoir hosts is essential in vaccine developmental research. Developing animal models for *C. burnetii* infections provides insight about the elusive nature of this bacterium filling the missing gaps in current knowledge

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CHAPTER 2: EFFECT OF INOCULATION ROUTE ON BACTERIAL INFECTION OF TISSUES AND DEVELOPMENT OF HISTOPATHOLOGIC LESIONS IN MICE INFECTED WITH *COXIELLA BURNETII*

Introduction

Coxiella burnetii is a gram-negative, intracellular bacterium that is commonly found in the environment and causes a disease termed Q fever in humans and coxiellosis in animals. Approximately 60% of human cases are asymptomatic, but Q fever pneumonia and hepatitis are common pathologies observed in symptomatic cases [64, 114]. Infection can occur in several ways with inhalation being the most common, followed by ingestion, and the bite of infected vectors, primarily ticks [115].

Regardless of the many routes of natural exposure, the majority of experimental mouse infection studies have used an intraperitoneal (IP) route of infection. The IP inoculation route is able to reproduce lesions commonly induced by the bacterium (pneumonia and hepatitis) but is not a natural route of infection and has questionable significance in the study of disease. Aerosol exposure would best model natural exposure; however, this method increases biohazard risks due to the production of infectious fine particle aerosols.

Previous studies have evaluated *C. burnetii* infection in naturally susceptible as well as immunodeficient mouse strains including; A/J, SCID, and BALB/c strains. A study conducted in 1986 by George Scott *et al*., found that the A/J inbred mouse strain was most susceptible to *C. burnetii* infection, followed by the BALB/C strain; the most resistant mouse strain was C57BL/6 [112]. Infection, morbidity, and mortality rates in the susceptible mouse strains was much higher than the rates observed in the C57BL/6 mice [112].

This project was initiated in order to establish a mouse model of coxiellosis that could be used in subsequent vaccine and pathogenesis trials. The specific objectives of this study were to determine if C57BL/6 mice develop similar infection and pathological lesions observed in infected humans and to compare two routes of inoculation, intraperitoneal (IP) and intranasal (IN), to observe differences in the pathogenesis and pathology of infected mice. This information will be used to develop an easy and efficient inoculation route in mice that replicates the common pathological lesions observed in natural infections.

Two experiments were performed to assess disease and course of infection induced by *C. burnetii* in C57CL/6 mice. In Experiment 1, we evaluated route of inoculation, comparing intranasal versus intraperitoneal exposures, with the mindset that intranasal was a more natural route of exposure. For experiment 2, we infected a larger group of mice by the intranasal route and maintained them for two months in order to evaluate long-term effects of

infection; these mice were also used as controls for the studies described in Chapters 3 and 4.

Materials and Methods

Animals

Ten A/J (5 week-old female) and 50 C57BL/6 mice (30 five week-old females and 20 eight week-old females) were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed at the Colorado State University Infectious Disease Research Center in ventilated cages under HEPA-filtered barrier conditions. Water and food were provided *ad libitum*. Animal care and use procedures were carried out in accordance with university policies [116].

Microorganism

The Nine Mile Phase I strain (RSA493) of *Coxiella burnetii*, was obtained from the American Type Culture Collection (Manassas VA, USA). To prepare a working stock of the organism, 10 A/J mice were infected by IP inoculation (50 uL) of the organism [2, 117]. Five mice were euthanized at seven days post inoculation (DPI) and five mice were euthanized at 11 DPI. Necropsies were performed, spleens were collected and pooled by mouse euthanasia date (7 or 11 DPI) and stored at -80 degrees Celsius (℃). Spleens from five mice euthanized 7 DPI were thawed and homogenized with a Ten Broeck grinder, diluted to a final volume of 40 ml in phosphate buffered saline (PBS) without additives, and was stored in 1 ml aliquots at -80℃.

Mouse Infection Experiments

Mice were infected with 3000 C57BL/6 infectious dose 50% (ID_{50}) splenic stock homogenate. Briefly, C57BL/6 mice were intranasally inoculated with serial tenfold dilutions of splenic stock homogenate $10E^{0}$ to $10E^{-6}$, five mice in each dilution group. Seven days after infection, mice were euthanized and splenic tissue was collected for conventional PCR analysis. The number of PCR positive splenic samples in each dilution group determined subsequent ID50.

Thirty C57BL/6 mice were randomly allocated into two inoculation route groups (15 per group). Mice inoculated IN were anesthetized prior to challenge with an IP inoculation of ketamine and xylazine (100 and 10 mg/kg respectively). Three mice from each inoculation group were euthanized and characterized on days 7, 14, 21, 28, and 35 post-inoculation. In a second trial, 20 C57BL/6 mice received the same inoculation dose and route as listed above. Five mice were euthanized at 14 and 28 DPI; the remaining 10 mice were euthanized at 59 DPI. All mice were checked daily for mortality and morbidity.

Necropsies were performed at each time point and sections of the spleen, liver, lungs, heart, and kidney were collected in 10% neutral-buffered formalin. The size of the spleen was reviewed and recorded on a graded scale of 1 to 4, with 1 indicating a normal sized spleen and 4 being grossly enlarged (approximately ≥ four times the size of normal). Tissues were embedded in paraffin, and 5-micrometer sections were stained with hematoxylin and eosin for microscopic review, Dr. Colleen Duncan conducted all histopathologic evaluations. One half of the spleen from each mouse was aseptically collected

and stored at -80℃ for DNA extraction. Bone marrow samples were collected for the 20 intranasally inoculated mice in Experiment 2, by flushing the femoral cavity with 0.5 ml of PBS and storing those samples at -80℃ for quantitative PCR analysis.

Infection was characterized by conventional and quantitative polymerase chain reaction (PCR) and indirect immunofluorescence assays (IFA). Details of PCR assays are listed in Table 2. For quantitative PCR, a threshold cycle (Ct) less than 38 indicated bacterial DNA presence in the spleen.

Microbiological Assays

Immunofluorescence detection of bacteria. Approximately 10 mg of spleen was squashed between two microscope slides and fixed in 70% acetone for at least 12 hours. These splenic squash mounts were stained with a rabbit antiserum against the *C. burnetii* Nine Mile Phase I antigen (Critical Reagents Program, Frederick, MD, USA). This primary antibody was prepared as a dilution of 1:2500 with 1% equine serum. Slides were placed in a 37℃ humidity chamber for 30 minutes (min) washed in PBS for 10 min, and rinsed in distilled water (dH20) for 30 seconds (sec). Slides were air dried and then stained with DyLight 488-labeled goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:800 with PBS. Slides were again placed in the humidity chamber for 30 min, followed by washing as stated above. The slides were air-dried and mounted with Dako mounting media (Carpinteria, CA, USA). Slides were randomly labeled, and reviewed in a blinded manner under a total magnification of 400X (Olympus FSX-100, Center Valley, PA, USA).

The strength of the fluorescence signal was subjectively graded on a scale from 0-3, with 0 designated as negative fluorescence and 3 as a strong signal of fluorescence examples of graded images are shown in Figure 1.

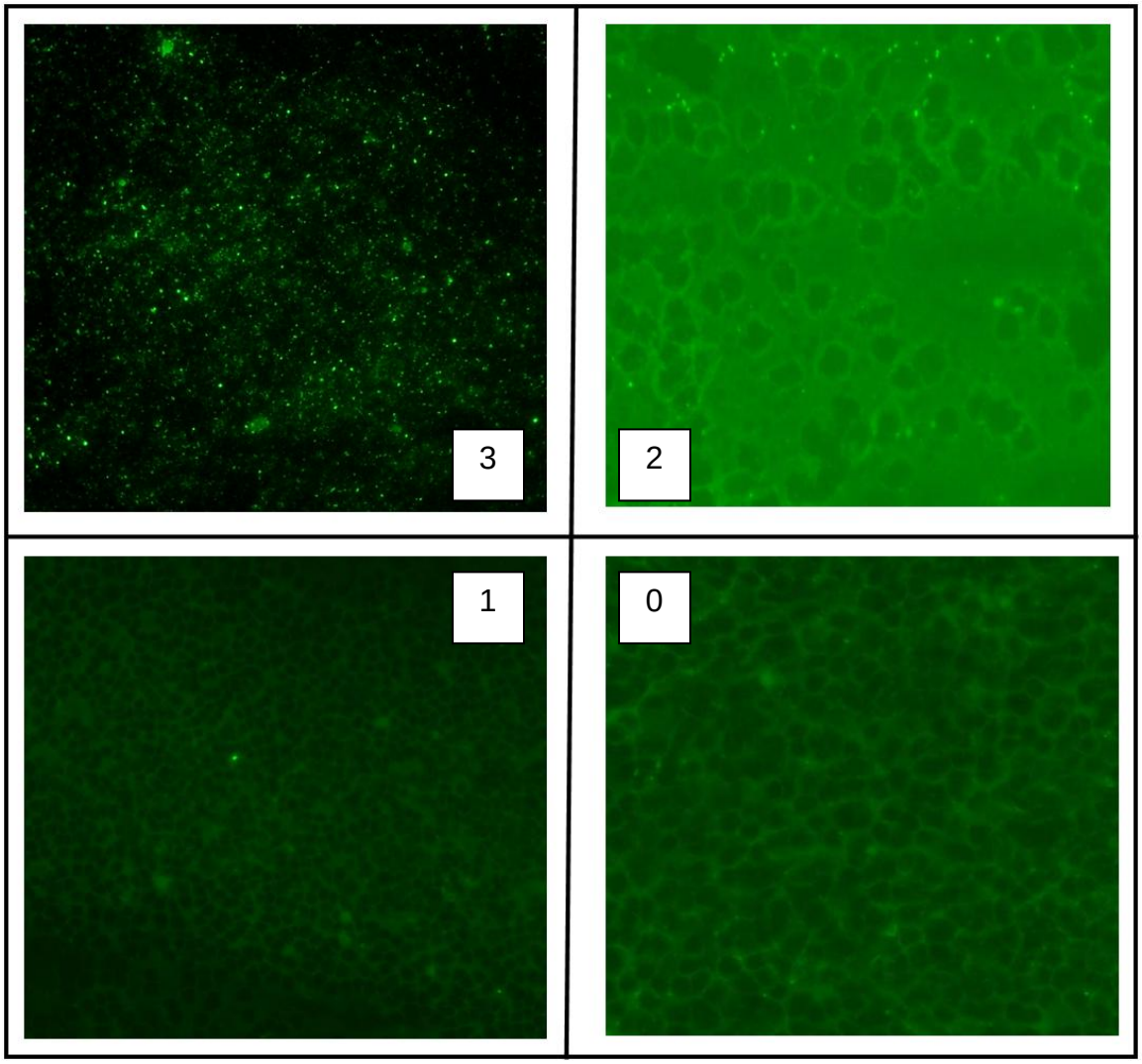

Figure 1. Examples of the IFA Grading Scheme.

A score of 3 indicates a strongly positive fluorescent signal 2, a moderate positive fluorescent signal, and 1 a very mild signal. Negative samples were given a score of zero.

PCR assays. Bacterial DNA extraction was performed on mouse splenic samples using a QIAGEN QIAamp DNA mini kit (Valencia, CA, USA). Ten milligrams of splenic tissue from each mouse was weighed and used in the extraction protocol according to manufacturer's instructions. Extracted DNA was precipitated in ethanol (EtOH). The precipitate was centrifuged at 14,000 x g for 15 min the pellet was washed in 1 ml of 70% EtOH then allowed to air dry. The DNA pellet was resuspended in 50 uL of TE (10 mM Tris, 1 mM EDTA, pH 8.0) and DNA concentration analyzed using a Thermo Scientific NanoDrop 1000 spectrophotometer (Rockford, IL, USA); OD 260/280 were evaluated to assess purity. All samples were diluted to a final concentration of 100 ng in 5 uL with TE.

Primers were designed using the primer-basic local alignment search tool (Primer-BLAST) software (National Center for Biotechnology Information, Bethesda, MD, USA) to amplify an 861 base-pair fragment of the insertion sequence gene *IS1111* found in approximately 20 copies in the Nine Mile phase I genome [11]. Conventional PCR was performed on the splenic DNA samples. Primer sequences and cycling conditions are listed in Table 1.

The PCR amplicon was separated by electrophoresis on a 1% agarose gel prepared with ethidium bromide, and was purified using a QIAquick PCR purification kit for band extraction and analysis (QIAGEN Inc., Valencia, CA, USA). The extracted amplicon was sent to the University of California Davis proteomics laboratory for nucleotide sequencing.

Quantitative PCR (qPCR) assays were conducted using primers and probe based on previously published assays to amplify the Nine Mile *IS1111*

sequence [51, 118]. Primers, fluorescent probes, and cycling conditions, are listed in Table 1. Serial ten-fold dilutions of the amplicon obtained by conventional PCR were prepared with TE and used as a standard curve for each qPCR assay. In order to compare variability between assays, the threshold for each run was set at 1000.

Table 1.

PCR Primers and Amplification Conditions.

Primer Name Sequence of Primer or Probe (5'- 3')	**Concentration nM**	**Cycling Conditions**	**Amplicon Size (bp)**
Standard PCR		95℃ (20 sec), 60℃ (60 sec), 72℃ (2 min) x 40 cycles	
IS1111F3 GCGAGCGAAGCGGTGGGATT	500		861
IS1111R22 AGCCCGTATGCAGCGAAGCG	500		
Quantitative PCR		95℃ (10 min) x 1 cycle 95℃ (15 sec), 60℃ (60 sec) x 40 cycles	
IS1111F CCGATCATTTGGGCGCT	1600		63
IS1111R CGGCGGTGTTTAGGC	800		
IS1111P* TTAACACGCCAAGAAACGTATC -GCTGTG	200		

*Fluorescent oligonucleotide probe labeled with 5' FAM and 3' fluorescence quencher (BHQ).
BP: base pair length of amplicon.

Statistical Analyses

Mean splenic genome equivalent (GE) values for IP and IN inoculation routes and euthanasia groups “early” and “late” were compared using analysis of variance (ANOVA) test. For the IN control inoculated mice in experiment 2, mean splenic and bone marrow GE values were compared between mice euthanized “early” days 14 and 28 post inoculation and “late” mice euthanized 59 DPI using ANOVA. A GE value less than 5 was converted to a value of 1 for statistical analysis. Categorical variables including IFA score, DPI, and inoculation route for IP and IN inoculation groups were compared using the Pearson’s Chi-square test and odds ratios (where applicable). The Fischer’s exact test (FET) was used to evaluate groups with categorical frequencies less than five. IFA scores were dichotomously grouped; negative to weakly positive spleen samples (IFA scores 0 and 1) were considered negative, while mild to moderately positive samples (IFA scores 2 and 3) were considered positive. Mice in the inoculation route study were dichotomously grouped by their euthanasia date; “early,” days seven, 14, and 21 after infection, and “late,” days 28 and 35 post inoculation. The frequency of splenomegaly, histopathologic lesions, and IFA scores were evaluated between “early” and “late” mouse groups. Infected control mice were also dichotomously grouped by their euthanasia dates. Infected control mice in the “early” group were euthanized on days 14 and 28 post inoculation; while mice in the “late” group were euthanized 59 DPI. Statistics were performed using SPSS software (IBM, version 19, Somers, NY), p-values ≤ 0.05 were considered significant for all tests.

Results

Experiment 1: Evaluation of IP versus IN Inoculation Route

There were no observable clinical signs of disease or mortality in any mouse throughout the 35-day time course of infection. Splenic enlargement evident at necropsy was observed in 3 of 15 IN and 6 of 15 IP inoculated mice (pooled across euthanasia time points), a difference that was not statistically significant (p=0.4, FET). Splenomegaly was observed in 6 of 18 mice euthanized 7, 14, and 21 DPI and 3 of 12 mice euthanized 28 and 35 DPI this was not a statistically significant difference (p=0.5, FET).

Hepatitis was characterized by aggregates of lymphocytes and histiocytic cells randomly distributed throughout the parenchyma (Figure 2A). Larger foci were associated with hepatocyte degeneration and loss. Hepatitis was observed in 10 of 15 IP and 8 of 15 IN inoculated mice, which was not a statistically significant difference (OR: 1.8, 95% CI: 0.4-7.7). Hepatitis was observed in 5 of 9 mice euthanized at both the “early” (7, 14, and 21 DPI) and “late” (28, 35 DPI) time points, and was not a statistically significant difference (p=0.7 FET).

Bronchopneumonia characterized by a focus of suppurative inflammation centered around large airways (Figure 2B) was observed in 4 of 9 IN inoculated mice at days 7 and 14 post infection, but was not observed in IP inoculated mice at any time point, this difference was not statistically significant (p=0.1, FET). Mild interstitial pneumonia, characterized as a patchy expansion of interlobular septae by lymphocytes and plasma cells (Figure 2C) was observed in 9 of 15 IP and 10 of 15 IN inoculated mice, and was not a statistical difference between

inoculation routes (OR: 0.8, 95% CI: 0.17-3.3). Ten mice were from the “early” group (euthanized 7, 14, and 21 DPI), and 9 mice were from the “late” group (euthanized 28, 35 DPI). There was no statistically significant difference in the frequency of interstitial pneumonia observed between these mouse groups (p=1.0, FET).

Histologic lesions were also observed in the heart and kidney of infected mice. Myocarditis, characterized as small aggregates of lymphocytes and plasma cells distributed throughout the myocardium (Figure 2D), was observed in 10 of 30 mice, five from each inoculation group, a difference that was not statistically different between inoculation routes (OR: 1, 95% CI: 0.22-4.6). There was no difference in the frequency of myocarditis between “early” and “late” mouse groups (p=1.0, FET). Rare mononuclear interstitial nephritis was observed in 4 of 15 IP and 2 of 15 IN inoculated mice. There was no difference in the frequency of nephritis between inoculation routes or between “early” and “late” mouse groups (p=0.7, FET). Splenic lymphoid hyperplasia was observed in 1 IN inoculated mouse euthanized at 14 DPI, but was not seen in any other mouse throughout the infection time course.

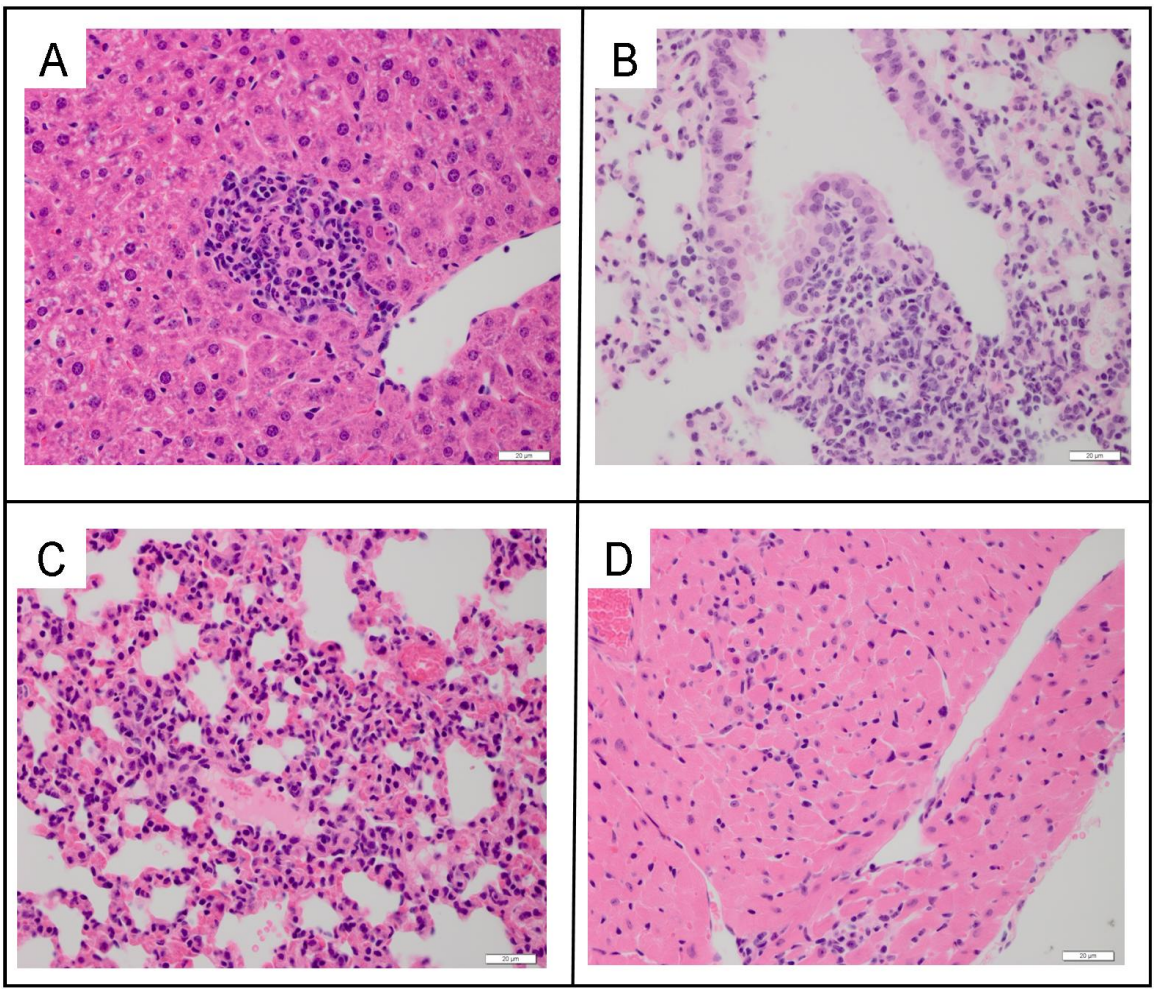

Figure 2. A-D) Histopathologic lesions associated with *C. burnetii* infection.

A) Hepatitis, B) bronchopneumonia, C) interstitial pneumonia, D) myocarditis.

Table 2.

Comparison of *C. burnetii* Infection in Mice Inoculated by the Intraperitoneal and Intranasal Routes.

Inoculation Route	DPI of Necropsy	Number of Mice	Splenic PCR GE* value (mean ± SD)
IP	7	3	79.8 ± 136.5
IN	7	3	8.5± 7.5
IP	14	3	82.2 ± 19.4
IN	14	3	113.7 ± 132.7
IP	21	3	3.5 ± 4.4
IN	21	3	7.7 ± 6.8
IP	28	3	3.4 ± 4.0
IN	28	3	1.0 ± 0.0
IP	35	3	3.4 ± 4.2
IN	35	3	11.9 ± 18.9
IP	All	15	34.5 ± 65.4
IN	All	15	28.6 ± 65.3

*GE: genome equivalent, values <5 were converted to a value of 1 for statistical analysis.
IP: intraperitoneal inoculation
IN: intranasal inoculation
DPI: days post inoculation.
SD: standard deviation.
IFA: indirect immunofluorescence assay.

The mean GE values for mouse splenic tissue, inoculation route, and euthanasia DPI are listed in Table 2. Comparison of mean GE values revealed no significant difference in the amount of bacteria in the spleen between IP and IN infected mice or between mice in “early” and “late” groups (F: 1.2, DF: 3, p=0.3). The IFA scores assigned to splenic squash mounts ranged from negative to moderately positive. There was no statistically significant difference in the frequency of positive IFA scores between inoculation routes (OR: 1, 95% CI: 0.22-4.6).

Experiment 2: Long-term Infection Following Intranasal Inoculation of *C. burnetii.*

There were no observable clinical signs in any of the 20 IN inoculated control mice throughout the 59-day infection time course. One mouse did not recover from anesthesia after the inoculation, blood was collected and a necropsy was performed. Splenomegaly was observed in 15 mice from experiment 2; 5 from the “early” group (mice euthanized 14 and 28 DPI) and 10 from the “late” group (mice euthanized 42 and 59 DPI). Mice in the “late” group were two times more likely to have splenomegaly than mice in the “early” group (95% CI; 0.27-0.93).

The same histopathologic lesions described for inoculated mice in Experiment 1 were also observed in the mice in this experiment. Hepatitis was observed in 9 of 10 mice from the “early” group and 8 of 10 mice from the “late” group, and was not statistically significant between mouse groups (p=0.5, FET). Interstitial pneumonia was observed in 8 of 10 mice from the “early” group and 1 of 10 mice from the “late” group, and was a statistically significant difference between mouse groups (p=0.001, FET). Mild myocarditis was observed in 4 of 10 mice from the “early” group and 1 of 10 from the “late” group, a difference that was not statistically significantly (p=0.3, FET). Interstitial nephritis was observed in 3 of 10 mice from the “early” mouse group, and 0 of 10 mice from the “late” group; the difference between mouse groups was not statistically significant (p=0.9, FET). Mild splenic lymphoid hyperplasia was observed in 8 of 10 from the

“early” and 10 of 10 from the “late” group; the difference between mouse groups was not statistically significant (*p*=0.5, FET).

Mean GE values, including standard deviations, for splenic and bone marrow samples are listed in Table 3. There was no statistically significant difference in the amount of bacterial DNA detected in the splenic tissue of mice from “early” and “late” groups (F: 1.4, DF: 1, *p*=0.3), or in the amount of bacterial DNA detected in the bone marrow (F: 3.3, DF: 1, *p*=0.1). IFA results were significantly different between “early” and “late” mouse groups; 4 of 9 “early” mice had IFA positive samples compared to 0 of 10 samples from the “late” group (*p*=0.02).

Table 3.

Comparison of the Amount of Bacterial DNA Detected in *C. burnetii* Infected Tissues and the Duration of Infection.

Mouse Group	Necropsy DPI	Number of Mice	Splenic qPCR GE* (Mean ± SD)	Bone Marrow qPCR GE* (Mean ± SD)
“Early”	14, 28	4,5	13.5 ± 4.5	10.4 ± 6.0
“Late”	59	10	0.2 ± 0.1	1.0 ± 0.0
Total	14, 28, 59	19	8.3 ± 12.3	2.6 ± 5.4

*GE: genome equivalent, values <5 were converted to 1 for statistical analysis.
DPI: days post inoculation.
SD: standard deviation.

Discussion

This study demonstrated that the C57BL/6 mouse strain is readily infected with the Nine Mile phase I strain of *Coxiella burnetii* and failed to develop clinical disease, but did manifest a number of the lesions associated with coxiellosis in humans and other animals. Hepatitis was the most common pathology, seen in

84% of all infected mice. Hepatic lesions were not observed in any IN inoculated mice until 14 days after infection, but were observed in all IP inoculated mice throughout the infection time course. These results suggested that the IP inoculation route may have induced a faster systemic immune response and hepatitis was seen in IP mice earlier than IN inoculated mice, or that the IP route provided a more direct access for the bacteria to infect mouse liver tissue. The similar frequency of hepatic lesions observed between IP and IN inoculated mice at later time points suggested that the inoculation route was a factor at early time points but is not a confounding factor at later time points.

Slight variations in pulmonary lesions were evident between inoculation routes. Four IN infected mice developed bronchopneumonia after inoculation, which was not observed in any IP infected mice. Bronchopneumonia, inflammation of the lung originating from the major airways and is most commonly caused by inhalation of an airborne pathogen or foreign material. As such, the pattern of inflammation in the lung of the IN inoculated mice euthanized at early infection time points was interpreted as secondary to the inoculation procedure and not necessarily the agent. Further, the lesion was not seen in mice after 14 days post inoculation suggesting that bronchopneumonia resolved over time. Mild interstitial inflammation was seen with equal frequency in both inoculation groups and is consistent with hematogenous dissemination, which has been previously described [119]. In the IN inoculated control mice, interstitial pneumonia was seen at a higher frequency in mice euthanized at the early

infection time points than mice euthanized at 59 DPI; suggested resolution of bacterial infection from the lung tissues over time.

Myocarditis is a severe manifestation associated with *C. burnetii* infection. This lesion developed independent of inoculation route and was observed in the same frequency in both IN and IP infected mice. In the IN inoculated control mice, myocarditis was observed in only one mouse 59 DPI, indicating bacterial resolution over time. Myocarditis has not been previously described in any inbred mouse strain following intranasal inoculation with the Nine Mile Phase I strain of *C. burnetii*. However, previous studies have observed myocarditis in mice and guinea pigs infected with *C. burnetii* by the intraperitoneal and aerosol routes [73, 93].

Lymphoid hyperplasia, described as a non-specific inflammatory lesion associated with the spleen was observed in the majority of infected mice. Development of this lesion was most likely induced by up-regulation of the immune system in relation to bacterial infection, and has been described in previous mouse infection studies [1, 2, 4]. A second non-specific inflammation was observed in the kidneys of infected mice. Rare interstitial nephritis was observed in mice from each inoculation group, but was not observed in any mouse 59 DPI. This lesion appeared to be inflammatory in nature, which suggested that the tissue was reacting to the bacterial infection by stimulating production of immune cells. Observations of histopathologic lesions induced by *C. burnetii* infection in the IN inoculated control mice revealed that lesions associated with the lungs, kidneys, and heart were less common approximately

two months after infection. However, lesions associated with the liver were still observed at high frequencies 59 days after infection. This information demonstrated that the bacterium might have induced a long-term infection in the liver tissue of infected mice.

Bacterial infection of mouse tissues was similar between IP and IN infected mice, demonstrating that dissemination and infection are independent of inoculation route. In the IN inoculated control mice, a high frequency of bacterial DNA was detected in the splenic tissue of infected mice 28 days after infection, which decreased significantly at 59 DPI. In both studies, the highest frequency of IFA positive samples was observed between 14 and 21 DPI, and no positive samples were observed from the splenic tissue of mice euthanized at 59 DPI. Together, these results demonstrated that bacterial DNA detection in the spleen was highest early during the infection and declined approximately one month after infection. Conversely, bacterial DNA detection in the bone marrow of IN inoculated control mice euthanized at 59 DPI was higher than mice euthanized at 14 and 28 DPI. Collectively, these data support the concept that persistence of *C. burnetii* varies among tissues, which may have important implications for chronic and recrudescent disease. An increase in bacteria in the bone marrow demonstrated a potential bacterial sequestering and possible development of chronic infection, as has been described in human cases of Q fever [120, 121].

The most common pathology associated with human acute Q fever includes hepatitis and pneumonia [122]. In contrast, chronic disease in humans is typically seen as endocarditis, and less common, osteomyelitis [120]. In humans,

splenomegaly associated with non-culturable endocarditis, is clinically suggestive of Q fever [73, 74]. This study found that the most common lesions observed in infected mice were hepatitis and interstitial pneumonia, followed by, myocarditis, nephritis, and splenic lymphoid hyperplasia. Development of these lesions was independent of the route of infection. The lesions associated with the heart, lung, and kidneys observed in infected mice were less common two-months after infection. Pathologies related to the spleen and liver, were still observed 59 days after infection, which demonstrated that intranasal infection with *C .burnetii* induces pathologic lesions and bacterial infection in mouse tissues that remain two months after initial infection. Also, that the C57BL/6 inbred mouse strain develops similar pathology to humans when infected with *C. burnetii*, and the route of exposure did not confound this finding.

Given the frequency of inhalation as a means of exposure in naturally acquired *Coxiella burnetii* human infections [76], it is important to replicate this exposure route and pathology development in an animal model. By being able to study infection in an animal model, pathogenesis of the organism can be elucidated. The present study demonstrated that the IP and IN inoculation routes do not differ in their ability to infect or induce pathologic lesions in the mouse; however, there are subtle differences in the pathology present at early time points that researchers should be aware of. In this study, the C57BL/6 inbred mouse strain developed similar histopathology observed in naturally infected humans. The retention of pathologies of the liver two months after initial infection provides insight about long-term infection after natural exposure. These results

suggest that the intranasal route is an acceptable route of inoculation for future studies as it mimics natural pathogenesis of infection, results in similar quantitative microbiological findings as both IP and aerosol inoculation studies, and is a safer and more efficient than other inoculation methods.

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CHAPTER 3: EFFECT OF AGE AND CHEMICALLY INDUCED IMMUNOSUPPRESSION IN MICE

Introduction

The intracellular bacterium *Coxiella burnetii* is the causative agent of the human disease Q fever. Based on serum antibody titers to *C. burnetii* phase I or II antigens and the development of histopathologic lesions, disease is classified as either acute or chronic. Acute Q fever is generally asymptomatic but clinical signs may include flu-like symptoms, pneumonia, hepatitis, and menigoencephalitis [70]. Chronic Q fever occurs in approximately 1-2% of acutely infected cases [64]. Chronic disease commonly manifests as endocarditis, typically observed in patients with previous valvulopathies [70, 123], and less often as osteomyelitis and glomerulonephritis [70, 124].

There are many theories regarding why certain disease manifestations develop in *C. burnetii* infections, and studies have shown trends in specific pathologies related to genotype and geography and the route of exposure. Cases of Q fever pneumonia are more common in Spain and Canada, while cases of hepatitis are more commonly observed in France and Australia [70]. Q fever pneumonia has been more frequently described in cases of inhalation, while hepatitis has been more frequently described in cases of infection by ingestion [70]. Additional factors that may determine severity of *C. burnetii* infection include

inoculation dose, bacterial molecular characteristics (phase and strain), and the relationship between the bacterium and the host [32, 69, 125].

Host factors are also important in disease development and may determine the severity of pathological lesions associated with *C. burnetii* infection. Age appears to be an important host attribute, with the majority of Q fever cases observed in humans over the age of 15 years [71]. Q fever is often associated with occupation and most often infects people in close contact with reservoir hosts including, ranchers, abattoir workers, and veterinarians [126]. Immune status also appears to be an important factor during *C. burnetii* infection. Disease manifestations are typically seen in patients with immune disorders in which the cell mediated immune response is insufficient [32]. Chronic Q fever has been described in patients with acquired immunodeficiency syndrome, cancer, and leukemia [70], suggesting that host immune status may have an association in the severity of disease.

The following experiments were designed to evaluate the effects of age and immunosuppression on the development of clinical and pathological disease in mice infected with *C. burnetii*. This information is important for the development of a mouse model that will mimic disease manifestations observed in humans for the future goal of drug and vaccine development.

To evaluate the effect of age, two different age groups of mice were attained, 9 months and 9 weeks. In order to evaluate immunosuppression, mice were treated with either dexamethasone or cyclophosphamide with doses that have been previously described [127, 128]. Dexamethasone is a glucocorticoid

that suppresses the cell mediated immune response of the host by inhibiting genes that encode for production of cytokines, which reduces T cell proliferation essential in resolving *C. burnetii* infection [129]. Dexamethasone also affects the humoral immune response of the host by inducing B cells to produce smaller amounts of IL-2 receptors and cytokine important in the proliferation of B and T lymphocytes [130]. In addition to its effects on cell mediated and humoral immune responses of the host, dexamethasone down-regulates Fc receptors on macrophages which causes less efficient phagocytosis of opsonized cells [131]. Overall, this study is attempting to induce prolonged *C. burnetii* infection in mice with exacerbated pathologic disease observed in chronic Q fever cases by treating mice with dexamethasone.

Cyclophosphamide is a cytostatic alkylating agent that can induce immunosuppression when given at high doses [132]. The following study evaluated cyclophosphamide-induced immunosuppression in mice infected with *C. burnetii*. Cyclophosphamide suppresses proliferation of T lymphocytes via a nitric oxide (NO) pathway, which works by priming immunosuppressive myeloid progenitors for iNOS protein synthesis [133]. Additionally, cyclophosphamide reduces the number and the suppressive ability of regulatory T cells (T_{REGs}), which are essential in regulating the host immune response [132]. Cyclophosphamide can induce immunosuppression by modulating and promoting changes in the host immune cells. This experimental mouse study will reveal if treatment with cyclophosphamide will exacerbate *C. burnetii* disease.

Overall, the following mouse experimental infection studies will determine if mouse age or treatment with dexamethasone or cyclophosphamide results in prolonged coxiellosis and exacerbated clinical and histopathologic lesions associated with infection. The first study examines age and dexamethasone-treatment while the second study determines if cyclophosphamide treatment before or after infection alters disease progression in mice.

Materials and Methods

Animals

Ten A/J (5 week-old female), and 62 C57BL/6 (39 nine-week-old and 24 nine-month-old female) mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed at the Colorado State University Infectious Disease Research Center in ventilated cages under HEPA-filtered barrier conditions. Water and food were provided *ad libitum*. Animal care and use procedures were carried out in accordance with university policies [116].

Microorganism

The Nine Mile Phase I strain (RSA493) of *C. burnetii* was obtained from the American Type Culture Collection (Manassas VA, USA). Stocks were prepared as homogenates of pooled spleens from A/J mice inoculated intraperitoneally 7 days previously (see Chapter 2) and stored in aliquots at 80^{o}C [2, 117].

Mouse experimental infections

Sixty-two C57BL/6 mice were separated into groups (A-G) based on the planned euthanasia day and treatment received, as summarized in Table 1. All

mice were inoculated IN with 3000 C57BL/6 ID_{50} splenic stock homogenate diluted with phosphate buffered saline (PBS) described in Chapter 2. Mice were anesthetized prior to inoculation with a mixture of ketamine and xylazine (100 and 10 mg/kg respectively) injected intraperitoneally.

In addition to the two mouse ages examined, two immunosuppressive protocols were evaluated. First, dexamethasone sodium phosphate (SP) (Bimeda-MTC Animal Health Inc., Ontario, Canada) was added to drinking water at a concentration of 8 ug/ml, a modified dose from a previous study [127]. The water was changed and fresh solution of drug was provided every other day for the duration of the study. Second, 15 mice were immunosuppressed by IP treatments with cyclophosphamide (Mead Johnson, Evansville, IN, USA) at a dose of 150 mg/kg, as previously described [128]. Cyclophosphamide treatment was initiated prior to infection for some mice and after infection for others. Mice in groups G and H were described in Experiment 2, Chapter 2. Four mice were euthanized 14 DPI and five mice were euthanized 28 DPI, from group G. All mice from group H were euthanized at 59 DPI.

On the scheduled day of euthanasia, mice were anesthetized with ketamine-xylazine and a terminal blood sample was collected by cardiac puncture. The mice were then euthanized and samples of spleen, liver, lung, heart, and kidney were collected into 10% neutral-buffered formalin. Those tissue sections were paraffin embedded, cut in 5-micrometer sections, and stained with hematoxylin and eosin for microscopic review; Dr. Colleen Duncan conducted all histopathologic evaluations. Splenic enlargement was graded on a scale from 0-

5; with 0 indicating a smaller than average spleen size, 1 an average sized spleen, and 5 a grossly enlarged spleen (approximately ≥ 5 times larger than average). Half of each spleen was stored at -80℃ for D NA extraction. Bone marrow samples were collected by washing femur cavities with 0.5 ml of PBS and stored frozen at -80℃ until assay.

Table 1.

Age and Immunosuppression Study Experimental Design.

Group	9 Week Mice (n)	9 Month Mice (n)	Immunosuppression Treatment	Day of Euthanasia (DPI)
A	7	8	None	14
B	8	8	DEX, DPI 28 to 42	42
C	8	8	None	42
D	5	0	CY initiated on -3 DPI	14
E	5	0	CY initiated on -3 DPI	28
F	5	0	CY initiated on DPI 28	42
G**	10	0	None	14, 28
H**	10	0	None	59

** Mice in groups G and H were part of the studies described in Chapter 2, Experiment 2.
DEX: dexamethasone in drinking water.
CY: cyclophosphamide delivered by IP injection.

Assays for Detection of *C. burnetii*

Indirect Immunofluorescence Assay (IFA). Approximately 10 milligrams of spleen were used to prepare splenic squash mounts. Slides were fixed in 70% acetone for at least 12 hours, then immunostained and evaluated as described in Chapter 2. Fluorescence signal was subjectively graded on a scale from 0-3, with 0 designated as negative fluorescence, and 3 designated as a strong signal of fluorescence.

Polymerase Chain Reaction (PCR). Bacterial DNA extraction was performed on mouse splenic and bone marrow tissue samples and both conventional and quantitative PCR assays were conducted to detect the *C. burnetii* insertion sequence *IS1111* gene as described in Chapter 2.

Statistical Analyses

Histopathology scores and tissue bacterial DNA infection (qPCR) results for group B (dexamethasone-treated) mice were compared to groups A and C (infected control mice). Histopathology and bacterial infection for groups D, E, and F (cyclophosphamide-treated) were compared to control mice in groups G and H (from previous experiment 2 in Chapter 2). Mean splenic and bone marrow bacterial genome equivalent (GE) values between mouse groups were compared using analysis of variance (ANOVA). GE values less than 5 were converted to a value of 1 for analysis. Mouse samples that fell below the detectable qPCR threshold value of 1000 were converted to missing values in SPSS to ensure accuracy when statistically evaluating quantitative data. The Pearson's Chi-square test and odds ratio (OR), when appropriate, were used to compare frequencies of categorical data. Fischer's exact test (FET) was reported for categorical variables in which the frequency was less than five. The presence of splenomegaly and IFA scores were dichotomously categorized into positive and negative for analysis. Spleens graded 2-5, and IFA scores 2-3 were considered positive. When interaction between variables was identified, stratum specific measures of effect were reported. The Mantel-Haenszel test was used to assess confounding among categorical variables. A p-value ≤ 0.05 was considered

significant for all tests. All statistical analyses were performed using SPSS software (IBM, version 19, Somers, NY).

Results

Clinical Signs and Gross Pathology

Morbidity was not observed in any mice throughout the 42-day time course of infection. One 9-week-old mouse from group A and one mouse from group G died following anesthesia and inoculation; necropsies were not performed.

Splenomegaly was observed in 16 of 42 mice from groups A and C, but was not observed in any dexamethasone treated mouse from group B. This difference was statistically significant (p=0.02, FET). There was no difference in incidence of splenomegaly between mice euthanized at different time points; enlarged spleens were observed in 12 of 15 mice from group A, euthanized 14 DPI, compared to 4 of 32 mice from groups B and C, euthanized 42 DPI (p=0.4, FET). Splenomegaly was seen in 15 of 20 mice from control mice in groups G and H, and 4 of 15 mice from cyclophosphamide-treated groups D, E, and F. Mice in groups G and H were 10.3 times more likely to have splenomegaly than cyclophosphamide mice in groups D, E, and F (95% CI: 2.2-47.3). Splenomegaly was seen in 3 mice from groups D, E, and G (euthanized 14 and 28 DPI) compared to 1 mouse from group F (euthanized 42 DPI) and was not a statistically significant difference (p=0.8, FET). However, duration of infection was a significant interaction term in the comparison of splenomegaly and cyclophosphamide treatment. There was no significant difference in

splenomegaly between groups D, E, and G, euthanized 14 and 28 DPI respectively, (p=0.4, FET), but there was a significant difference in incidence of splenomegaly between mouse groups F and H, euthanized 42 and 59 DPI (p=0.004, FET). Also, there was no statistically significant difference in splenomegaly between mice that began cyclophosphamide-treatment three days before infection (groups D and E) and mice that began cyclophosphamide treatment 28 DPI (group F; p=0.08, FET).

Histopathology

Hepatic lesions were seen in 47 of 62 infected mice and characterized as either inflammatory or degenerative. Inflammatory changes were lymphohistiocytic in nature with many small microgranulomas randomly distributed throughout the hepatic parenchyma (Figure 1A). In two mice, central coagulative necrosis with inflammatory foci and a rim of peripheral lymphocytes was observed. The second histologic pattern of liver disease was vacuolar degeneration of hepatocyte cytoplasm. This lesion was observed in all 16 dexamethasone-treated mice from group B (Figure 1B) and was not seen in any mice from groups A and C, ascribing to the drug treatment rather than infection. Hepatic lesions were observed in 12 of 23 young and 21 of 24 aged mice from mouse groups A, B, and C. There was a significant difference in the incidence of hepatitis associated with mouse age; aged mice were 6.4 times more likely to develop hepatitis compared to young mice (95% CI: 1.6-25.8). Duration of infection (14 versus 42 DPI) was a significant interaction term when evaluating frequency of hepatic lesions between young and aged mice. Hepatitis was seen

in 14 of 16 mice from group A (p=0.5, FET) and 19 of 32 mice from groups B and C (p=0.03, FET). Hepatic lesions were seen in 8 of 16 dexamethasone-treated mice from group B and 25 of 31 mice from groups A and C. Mice in groups A and C were 4.2 times more likely to have hepatic lesions than mice in group B (95% CI: 1.1-15.7). Seven of 20 mice from mouse groups D, E, and G (euthanized 14 and 28 DPI) had hepatic lesions compared to 5 of 15 mice from groups F and H (euthanized 42 and 59 DPI), and was not a statistically significant difference (p=0.6, FET). There was no statistically significant difference in hepatitis observed in mice treated with cyclophosphamide starting three days before infection (groups D and E) compared to mice that started treatment 28 DPI (group F). Hepatitis was seen in 9 of 10 mice from groups D and E and all 5 mice from group F (p=0.34, FET).

Mild interstitial pneumonia (Figure 1C), characterized by increased numbers of lymphocytes, plasma cells and rare neutrophils expanding the pulmonary interstitium, was the most common lung lesion and was observed in 18 of 23 young and 18 of 24 aged mice, from mice in groups A, B, and C. There was no statistically significant difference in the frequency of interstitial pneumonia between young and aged mice from groups A, B, and C (OR: 1.2, 95% CI: 0.3-4.6). Interstitial pneumonia was observed in 13 of 16 dexamethasone-treated mice from group B compared to 23 of 32 mice from groups A and C this difference was not statistically significant (p=0.7, FET). Mouse age was an interaction term in the comparison of the frequency of interstitial pneumonia between dexamethasone-treated mice in group B. Interstitial pneumonia was

observed in all 8 young mice from group A (p=0.12) and 5 of 8 aged mice from group B (p=0.36). Interstitial pneumonia was seen in 9 of 15 mice from cyclophosphamide-treated mice in groups D, E, and F and nine mice from control mice in groups G and H, and was not a statistically significant difference between mouse groups (OR: 1.7, 95% CI: 0.4-6.6). Duration of infection was an interaction variable when comparing interstitial pneumonia between cyclophosphamide-treated groups D, E, and F. Six of 20 mice from groups D, E, and G (euthanized 14 and 28 DPI) had interstitial pneumonia (p=0.3, FET) compared to 3 of 15 mice from groups F and H (euthanized 42 and 59 DPI) (p=0.07, FET). Interstitial pneumonia was seen in 4 of 10 mice that received cyclophosphamide treatment three days before infection (groups D and E) and all 5 mice from group F that received cyclophosphamide treatment 28 DPI, and this difference was not statistically significant difference (p=0.6, FET).

Small foci of lymphocytic inflammation within the heart (Figure 1D), were observed in 2 young and 3 aged mice from groups A, B, and C. Myocarditis was not seen in any dexamethasone-treated mice from group B, which was not a significant difference in comparison to myocarditis in groups A and C (p=0.15, FET). Myocarditis was seen in 7 of 15 cyclophosphamide treated mice from groups D, E, and F and 5 of 20 control mice from groups G and H, a difference that was not statistically significant (OR: 2.3, 95% CI: 0.53-9.7). Duration of infection was an interaction variable when comparing myocarditis between groups D, E, F, G, and H. Seven of 20 mice from groups D, E, and G (euthanized 14 and 28 DPI) had myocardial lesions (p=0.6, FET). However, myocarditis was

not seen in any mice from groups F and H (euthanized 42 and 59 DPI). Myocardial lesions were observed in 4 of 10 mice that began cyclophosphamide treatment three days before infection (groups D and E) compared to 3 of 5 mice from group F (euthanized 59 DPI), this difference was not statistically significant (p=0.1, FET).

Lymphoid hyperplasia was the most commonly observed splenic lesion and was observed in 6 of 23 young and 6 of 24 aged mice from mouse groups A, B, and C. There was no statistically significant difference in splenic hyperplasia between mouse ages (OR: 0.94, 95% CI: 0.25-3.6). Splenic lymphoid hyperplasia was not observed in any dexamethasone-treated mice from group B, this was a statistically significant different compared to hyperplasia observed in mice from groups A and C (p=0.02, FET). Splenic lymphoid hyperplasia was seen in all 15 mice from cyclophosphamide treated groups D, E, and F and 18 of 20 control mice from groups G and H.

Interstitial nephritis, aggregates of lymphocytes and plasma cells expanding the interstitium. Nephritis was seen in 4 of 23 young and 8 of 24 aged mice from groups A and C and was a statistically significant difference between these groups (p=0.02, FET). Nephritis was not observed in any dexamethasone-treated mouse from group B, which was a statistically significant difference compared to groups A and C (p=0.004). Nephritis was observed in 3 of 20 control mice from groups G and H, and was not seen in any mouse from cyclophosphamide-treated groups D, E, and F, this difference was not statistically significant (p=0.2, FET).

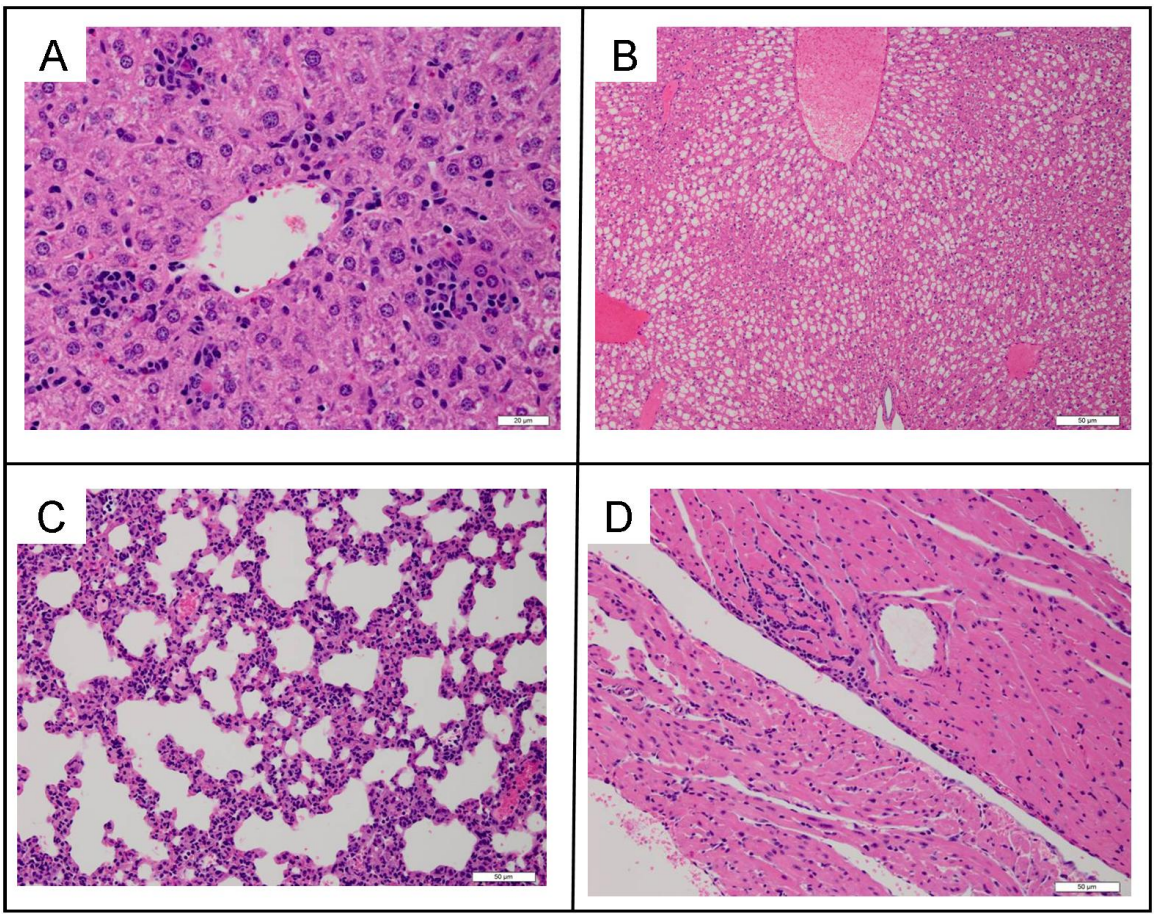

Figure 1: A-D) Histopathologic Lesions Associated with *C. burnetii.*

A) Hepatitis, B) steroid hepatopathy, C) interstitial pneumonia, and D) myocarditis.

Bacterial Infection in Mouse Tissues

Mean splenic and bone marrow bacterial GE values derived from the qPCR *IS1111* assay for each mouse group are listed in Table 2.

Table 2.

Effect of Age and Chemically Induced Immunosuppression on *C. burnetii* Infection in Mouse Tissues.

Mouse Group	**N 9 wks, N 9 mos**	**Treatment**	**Euthanasia DPI**	**Spleen GE* ± SD**	**Bone Marrow GE* ± SD**
A	7, 8	None	14	25.6 ± 22.7	10.7 ± 30.4
B	8, 8	DEX	42	1.3 ± 1.1	1.1 ± 0.25
C	8, 8	None	42	1.0 ± 0.0	1.0 ± 0.0
D	5, 0	CY -3	14	18.5 ± 21.7	1.4 ± 0.6
E	5, 0	CY -3	28	1.1 ± 0.1	1.0 ± 0.0
F	5, 0	CY 28 DPI	42	1.0 ± 0.0	1.0 ± 0.0
G	10, 0	None	14, 28	13.5 ± 4.5	10.4 ± 6.0
H	10, 0	None	59	0.2 ± 0.1	1.0 ± 0.0

* GE: genome equivalents, values <5 were converted to a value of 1 for data analysis.
N: number of mice in each group.
CY: cyclophosphamide beginning three days before infection or 28 days after infection.
DEX: dexamethasone
DPI: days post inoculation.
SD: standard deviation.

There was no statistically significant difference in the mean bacterial GE values between young and aged mice from groups A, B, and C (F: 1.3, DF: 1,

p=0.3). There was a statistically significant difference in the mean splenic bacterial GE values between dexamethasone-treated mice from group B compared to groups A and C (F: 9.1, DF: 1, *p*=0.006). There was no statistically significant difference in the mean splenic bacterial GE value from cyclophosphamide-treated groups D, E, and F compared to control mice in groups G and H (F: 0.15, DF: 1, *p*=0.7). There was no statistically significant difference in mean splenic bacterial GE values from mice that started cyclophosphamide treatment three days before infection (groups D and E) compared to mice that started cyclophosphamide treatment 28 DPI group F (F: 0.6, DF: 3, *p*=0.7).

There was no statistical difference in the frequency of IFA positive splenic samples between mouse ages, mouse groups A, B, and C, (OR: 1.5, 95% CI: 0.4-5.6). Additionally, no difference was observed in the frequency of positive IFA scores between dexamethasone-treated mice, in group B and mice in groups A and C (*p*=0.2, FET). Lastly, there was no difference in the frequency of IFA positive spleen samples between cyclophosphamide-treated mice in groups D, E, and F, and control mice in groups G and H (*p*=0.7, FET).

Mouse age had no effect on the amount of bacterial DNA detected in the tissues from mice in groups A, B, and C. There was no significant difference in the mean bone marrow bacterial GE values between young and aged mice from groups A, B, and C (F: 0.3, DF: 1, *p*=0.6). Treatment with dexamethasone had no significant effect on the amount of bacteria detected in the bone marrow of mice from group B. There was no statistically significant difference in mean bone

marrow GE values from mice in group B compared to groups A and C (F: 0.3, DF: 1, *p*=0.6). Cyclophosphamide treatment had no effect on the amount of bacterial DNA detected in the bone marrow of infected mice. There was no statistically significant difference in the mean GE value of the bone marrow from cyclophosphamide mice in groups D, E, and F compared to control mice in groups G and H (F: 2.2, DF: 1, *p*=0.2). Also, there was no statistically significant difference in mean bone marrow GE values from mice in groups D, E, and G (euthanized 14 and 28 DPI) compared to groups F and H (euthanized 42 and 59 DPI) (F; 2.2, DF: 3, *p*=0.1). Mean bone marrow bacterial GE values from mice that started cyclophosphamide treatment three days before infection (groups D and E) were not significantly different from mice that began cyclophosphamide treatment 28 DPI (group F) (F: 2.5, DF: 1, *p*=0.1).

Discussion

There are several disease manifestations that have been observed in human Q fever including, flu-like symptoms, pneumonia, hepatitis, and endocarditis [25, 114]. It is unclear why certain pathologies develop after infection, but disease variability has been linked to host factors [69, 70]. Age and host immune status were examined in mice to determine if these factors influence *C. burnetii* infection.

This study found that there was no exacerbation of histopathologic lesions or increase in the amount of bacterial DNA detected in mouse tissues between 9-week-old and 9-month-old C57BL/6 mice after *C. burnetii* infection. A previous mouse study by Leone *et al.* determined a significant difference in *C. burnetii*

bacterial burden of the spleen of 14-month old mice compared to 1-month-old mice disease, and the authors suggested that disease was exacerbated in the aged mice [134]. It is possible that our study did not observe a significant difference in *C. burnetii* infection between 9-week and 9-month-old mice because a more extreme age difference was necessary. Additionally, due to the constraints of pilot studies it is possible that low power prevented a significant difference from being observed.

Treatment with dexamethasone did not exacerbate *C. burnetii* disease in mice. Further, there was no interaction between age and dexamethasone treatment and disease development. As stated previously, dexamethasone can decrease the functionality of Fc receptors on macrophages [131]. In this study dexamethasone treatment may have induced changes in the macrophage receptors disrupting the infectious process of the bacterium. Fewer infected cells ascribes to fewer histopathologic lesions observed in infected mice. Steroid hepatopathy was observed in all dexamethasone treated mice. This hepatic pathology was caused by the dexamethasone treatment as similar pathologies have been observed in animals receiving long-term steroid therapy [135]. Treatment with dexamethasone did not exacerbate bacterial infection of mouse tissues or histopathologic lesions in mice infected with *C. burnetii*. Although the dosage of dexamethasone was derived from a previous study, the dose received by the mice may not have down-regulated the cell - mediated immune response, as anticipated and chronic disease did not develop. No information is available about the interaction of dexamethasone and *C. burnetii*. Overall, more studies

are necessary to evaluate the effect of dexamethasone-induced immunosuppression on *C. burnetii* infection in mice.

Cyclophosphamide did not significantly exacerbate *C. burnetii* disease in mice. However, grossly cyclophosphamide-treated mice had significantly less splenomegaly than untreated mice. Previous studies have determined that cyclophosphamide treatment preferentially depletes lymphocytes from lymphoid organs in mice [136]. Therefore, less incidence of splenomegaly could have been the result of cyclophosphamide induced lymphocytic depletion. As with the dexamethasone treatment, cyclophosphamide did not exacerbate *C. burnetii* disease in mice. While this dose has been used to induce lymphocyte depletion in mice, the interaction of *C. burnetii* and cyclophosphamide has not been previously evaluated. Therefore, studies need to be conducted on the efficacy of cyclophosphamide inducing immunosuppression in *C. burnetii* infected mice to determine if this treatment affects disease development.

The duration of infection was an interaction term in the evaluation of splenomegaly and interstitial pneumonia among cyclophosphamide-treated and untreated mice. In both comparisons, there was significantly less splenomegaly and interstitial pneumonia seen in mice euthanized late in the infection (42 and 59 DPI). However, there was no difference in the incidence of splenomegaly and interstitial pneumonia in mice euthanized early in the infection (14 and 28 DPI). Additionally, more bacterial DNA was detected in the spleen and bone marrow of infected mice early in the infection compared to mice that were euthanized later. Bacterial infection in mouse tissues was highest approximately two weeks after

infection after which point, the amount of bacterial DNA detected in the tissues and frequency of histopathologic lesions significantly decreased. These results demonstrated that bacterial dissemination induced tissue infection early in the infectious process leading to the development of histopathologic lesions. Significantly fewer bacteria were detected in infected tissues and fewer histopathologic lesions six weeks after infection due in part to resolution by the host immune response.

Histopathologic lesions observed in infected mice mimic lesions observed in human cases of Q fever specifically, interstitial pneumonia, hepatitis, and myocarditis [66]. Interstitial pneumonia and myocarditis were observed in all mouse groups and development of these lesions was unaffected by host age or chemical treatment. Therefore, age may not be an important factor in development of cardiac and lung pathologies in human Q fever.

This study demonstrated that mouse age did not have a significant impact on *C. burnetii* infection. Additionally, treatment with dexamethasone and cyclophosphamide did not significantly exacerbate *C. burnetii* disease in mice. Overall, this study determined that these attributes are not risk factors for enhanced *C. burnetii* disease in mice as they are determined to be for humans.

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CHAPTER 4: EVALUATION OF DOXYCYCLINE THERAPY IN TREATING *C. BURNETII* INFECTION IN MICE

Introduction

The intracellular bacterium *Coxiella burnetii* induces a spectrum of disorders in infected humans, ranging from subclinical to severe disease. In some untreated patients, infections can persist throughout life. Examples of chronic infections include cases of *C. burnetii* begin maintained for over 20 years in the bone marrow of infected humans and sequela such as endocarditis associated with long-term infections [120, 137]. The primary treatment for acute Q fever is a two-week course of doxycycline therapy [64]. In the majority of cases, this treatment clears infection; however, latent and chronic infections have been reported after doxycycline-treatment [120]. Chronic infections require long-term therapies to ensure the host maintains immunity and eventually clears the infection. Cases of chronic Q fever endocarditis require extensive antimicrobial therapy, with multi-drug therapies given for the life of the host [138]. Additionally, several cases of endocarditis have required valvular graft replacement [139]. There are many theories about why acute disease becomes chronic in 1-2% infected cases [64]. In order to prevent the conversion from acute to chronic disease, effective antimicrobial therapies must be given shortly after infection to

control and clear bacterial infection in the host. The objective of this study was to evaluate the effectiveness of doxycycline treatment on *C. burnetii* infected mice to provide a baseline assessment to which future antimicrobial trials could be compared.

Doxycycline is a tetracycline derivative that is effective in treating gram-positive and gram-negative aerobic and anaerobic pathogens and has been determined to be the best antimicrobial to treat *C. burnetii* infections [140, 141]. Doxycycline is bacteriostatic and works by penetrating the bacterial cell wall interfering with protein biosynthesis, primarily on the 70S ribosome [140]. Doxycycline also binds to the 30S ribosomal subunit preventing the binding of bacterial mRNA to tRNA and doxycycline inhibits mitochondrial protein synthesis [140]. Chronic Q fever is much more difficult to treat and adjunctive therapy with hydroxychloroquine is used as an alkalizing agent to disrupt the acidic environment of the parasitophorous vacuole [141]. The following experimental mouse infection study evaluates the effectiveness of doxycycline, administered in mouse drinking water, in preventing *C. burnetii* protein synthesis and subsequent infection.

Materials and Methods

Animals

Thirty-five C57BL/6 (8 week-old female) mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed at the Colorado State University Infectious Disease Research Center in ventilated cages under HEPA-filtered barrier conditions. Water and food were provided *ad*

libitum. Animal care and use procedures were carried out in accordance with university policies [116]. Clinical signs and water consumption were recorded daily, and mouse body weights were recorded twice weekly.

Microorganism

The Nine Mile Phase I strain (RSA493) of *C. burnetii* was originally obtained from the American Type Culture Collection (Manassas VA, USA). Laboratory stocks of the bacteria were prepared from the spleens of infected A/J mice, stored in aliquots at -80^{o}C and titrated by intranasal inoculation in C57BL/6 mice (see Chapter 2).

Mouse Experimental Infections

Mice were anesthetized prior to inoculation using an IP injection of a mixture of ketamine and xylazine (100 and 10 mg/kg respectively). Mice were inoculated IN with 3000 ID_{50} of *C. burnetii* diluted with phosphate buffered saline (PBS) described in Chapter 2. Mouse treatment groups are listed in Table 1; Groups A and B were treated with doxycycline seven days after infection; Group C began doxycycline-treatment 28 days post infection (DPI). Mice in groups D, E, and F (previously described in Experiment 2, Chapter 2) served as infected, non-treated controls, five mice from group D were euthanized 14 DPI and five were euthanized 28 DPI.

Doxycycline hyclate powder (CSU Veterinary Teaching Hospital pharmacy) was added to mouse drinking water at a final dose of 200 ug/ml as

previously described [142]. Medicated drinking water was changed every other day until the mice were euthanized

Table 1.

Doxycycline and *C. burnetii* Infection Study Design.

Group	Number of Mice	Days of Doxycycline Treatment	Day of Euthanasia
A	5	7 – 14	14 DPI
B	5	7 – 28	28 DPI
C	5	28 – 42	42 DPI
D	5	None	14 DPI
E	5	None	28 DPI
F	10	None	59 DPI

DPI: days post inoculation.

Blood samples were obtained under ketamine-xylazine anesthesia by intracardiac puncture, immediately prior to euthanasia. Necropsies were performed and samples of the spleen, liver, lungs, heart, and kidney were collected in 10% neutral-buffered formalin. Tissue sections were embedded in paraffin, and sections stained with hematoxylin and eosin for microscopic review, Dr. Colleen Duncan conducted all histopathologic evaluations. Splenic enlargement was graded on a scale from 0-5; with 0 indicating a smaller than average spleen size, 1 an average sized spleen, and 5 a grossly enlarged spleen (approximately ≥ 5 times larger than average). One half of each spleen was stored frozen at -80℃ for DNA extraction. Bone marro w washes were performed by injecting 0.5 ml of PBS into the broken femur cavity, and stored at -80℃.

Assays for Detection of *C. burnetii*

Indirect Immunofluorescence Assay (IFA). Approximately 10 milligrams of spleen were used to prepare splenic squash mounts. Slides were fixed in 70%

acetone for at least 12 hours, then immunostained and evaluated as described in Chapter 2. Fluorescence signal was subjectively graded on a scale from 0-3, with 0 designated as negative fluorescence, and 3 designated as a strong signal of fluorescence.

Polymerase Chain Reaction (PCR). Bacterial DNA extraction was performed on mouse splenic and bone marrow tissue samples, quantitative PCR assays were conducted to detect the *C. burnetii* insertion sequence *IS1111* gene as described in Chapter 2.

Statistical Analyses

Mean genome equivalent (GE) values from qPCR assays of spleen and bone marrow were compared between doxycycline-treated and non-treated, infected control mice using analysis of variance (ANOVA). GE values less than 5 were converted to a value of 1 for analysis. Mouse samples that fell below the detectable qPCR threshold value of 1000 were converted to missing values in SPSS to ensure accuracy when statistically evaluating quantitative data. Differences between doxycycline treatment groups were determined using the Tukey's post-hoc test. The Pearson's Chi-square test and odds ratios, when appropriate, were used to compare frequencies of categorical data. The Fischer's exact test (FET) was used for categorical frequencies less than five. For all statistical tests, a p-value ≤ 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software (IBM, version 19, Somers, NY).

Results

Clinical Disease and Gross Pathology

Morbidity was not observed in any of the mice over the 42-day infection time course. One infected control mouse died shortly after inoculation; a necropsy was not performed. Splenomegaly was the only significant gross lesion observed during necropsy; this was seen in two doxycycline-treated mice from mouse groups A and B and in 15 of 20 mice in groups D, E, and F. Mice that were not treated with doxycycline were 24 times more likely to have enlarged spleens than mice in groups A, B, and C (95% CI: 4.6, 129.8). There was not a significant difference in splenomegaly between mice that started doxycycline treatment seven days after infection (groups A and B) compared to those that started treatment 28 DPI (group C) (p=0.5, FET).

Histopathology

Hepatic lesions were seen in 30 of 34 infected mice and were characterized as mild to moderate inflammation, lymphohistiocytic in nature with many small microgranulomas randomly distributed throughout the hepatic parenchyma. Hepatitis was seen in 13 of 15 doxycycline treated mice from the mice groups A, B, and C, and 17 of 20 control mice from groups D, E, and F, which was not a significant difference (p=1.0, FET). Additionally, hepatitis was observed in 8 of 10 mice from groups A and B (euthanized 14 and 28 DPI) compared to 5 mice from group C (euthanized 42 DPI), this difference also was not significant (p=0.5, FET).

Mild, lymphoplasmacytic, interstitial pneumonia was the most commonly observed lung lesion. Interstitial pneumonia was observed in 4 of 15 doxycycline-treated from groups A, B, and C and 9 of 20 control mice from groups D, E, and F, which was not a significant difference (OR: 2.5, 95% CI: 0.6-10.7). Duration of infection was evaluated as an interaction term in the comparison of interstitial pneumonia and doxycycline treatment. Four mice from groups A, B, D, and E (euthanized 14 and 28 DPI) had pulmonary lesions (p=0.06, FET), and pneumonia was not observed in any mice from groups C and F (euthanized 42 and 59 DPI) (p=1.0, FET). There was no significant difference in the incidence of interstitial pneumonia between mice that received doxycycline-treatment seven DPI (groups A and B) compared to group C mice that started treatment 28 DPI (p=0.2, FET).

In addition to the major histopathologic lesions observed in the liver and lungs, lesions were seen in the spleen, heart, and kidney of infected mice. Splenic lymphoid hyperplasia was seen in 5 doxycycline-treated mice from group C, and 18 control mice from groups D, E, and F; this was a statistically significant difference between mouse groups (p=0.00, FET). Mild lymphocytic myocarditis was observed in one doxycycline treated mouse from group A and 5 control mice from groups E and F; this difference was not significant (p=0.2, FET). Lymphoplasmacytic interstitial nephritis was seen in four doxycycline-treated mice from groups A and B, and three control mice from groups D, E, and F, this difference was not statistically significant (p=0.7, FET). Interstitial nephritis was not seen in any dexamethasone mice that began treatment 28 DPI (group C)

compared to four mice that began doxycycline treatment 7 DPI (groups A and B) this difference was not statistically significant (*p*=0.2, FET).

Bacterial Burden in Tissues

Mean splenic and bone marrow bacterial GE values derived from the qPCR *IS1111* assay for each mouse group are listed in Table 2.

Table 2.

Detection of *C. burnetii* DNA in Tissues after Treatment with Doxycycline.

Group	Number of Mice	Treatment, Start Day	Day of Euthanasia	Spleen GE* ± SD	Bone Marrow GE* ± SD
A	5	Doxy, 7	14	7.7 ± 4.1	3.1 ± 4.2
B	5	Doxy, 7	28	1.0 ± 0.0	1.0 ± 0.0
C	5	Doxy, 28	42	0 ± 0	1.0 ± 0.0
D	5	None	14	21.2 ± 14.4	10.5 ± 12.2
E	5	None	28	2.3 ± 2.9	1.0 ± 0.0
F	10	None	59	1.1 ± 0.2	1.0 ± 0.0

* GE: genome equivalent, values <5 were changed to a value of 1 for statistical analysis.
Doxy: doxycycline treatment added to mouse drinking water.
SD: standard deviation.

Doxycycline-treated mice from groups A, B, and C and untreated mice from groups D, E, and F had similar quantities of bacterial DNA in their splenic tissue as determined by qPCR (F: 2.2, DF: 1, *p*=0.1). There was no statistically significant difference in the mean splenic GE values for mice in groups A, B, D, and E (euthanized 14 and 28 DPI) compared to mice in groups C and F (euthanized 42 and 59 DPI) (F: 1.1, DF: 1, *p*=0.3).

The amount of bacterial DNA detected in the bone marrow of doxycycline-treated mice in groups A, B, and C compared to control mice in groups D, E, and F was similar (F: 1.8, DF: 1, *p*=0.2). There was no difference in the amount of

bacterial DNA detected in the bone marrow of mice from euthanized 14 and 28 DPI (groups A, B, D, and E) compared to mice euthanized 42 and 59 DPI (groups C and F) (F: 2.3, DF: 1, p=0.1). Also, there was no difference in mean bone marrow GE values between doxycycline treated mice in groups A and B compared to group C (F: 0.4, DF: 1, p=0.5).

There was a significant difference in the quantity of splenic bacteria as determined by IFA analysis between doxycycline-treated mice in groups A, B, and C and non-treated groups D, E, and F. Eight mice from doxycycline treated mice in groups A, B, and C had IFA positive splenic samples compared to 3 control mice from groups D and F (p=0.03, FET). Seven mice from group A and B had positive IFA samples compared to one mouse from group C; this difference was not significant (p=0.1, FET).

Discussion

Doxycycline therapy is the most commonly prescribed antibiotic to treat human Q fever [64]. Results of this study suggested that doxycycline treatment did not significantly affect *C. burnetii* disease development in mice. Mice received doxycycline treatment beginning seven or 28 days after infection and there was no difference in the ability of doxycycline to resolve bacterial infection at either treatment start day. The incidence of splenomegaly and splenic hyperplasia were significantly decreased in doxycycline treated mice compared to infected control mice. These results suggested that doxycycline was effective in preventing *C. burnetii* from inducing an inflammatory reaction in the spleen.

As was observed in the previous chapters, detection of bacterial DNA in mouse tissues and incidence of histopathologic lesions were highest early in the course of infection and significantly decreased 14 days after infection. Duration of infection was an interaction variable in the evaluation of histopathologic lesions and doxycycline treatment. Splenomegaly was not seen in doxycycline-treated mice later in the infection (42 DPI) compared to mice euthanized early in the infection (14 and 28 DPI). In doxycycline-treated mice, cardiac and pulmonary lesions were more severe early in the course of infection, and these lesions were not seen in mice euthanized 42 days after infection. We did not have a group of untreated mice euthanized at day 42, but the untreated mice euthanized at day 59 showed a similar resolution of lesions, making it difficult to ascertain the efficacy of doxycycline treatment.

The frequency of hepatitis and nephritis were similar between doxycycline-treated mice and infected controls. Similar frequencies of nephritis were seen in both doxycycline and infected control mice early in the infection with complete resolution of by 42 and 59 DPI. These results suggest that this non-specific, inflammatory lesion completely resolves late in the infection with or without doxycycline treatment. Hepatitis was still evident in doxycycline-treated and infected controls late in the infection. These findings suggest that the liver becomes chronically infected in mice, and doxycycline treatment as dosed in this study was not sufficient to clear infection by 42 DPI.

These findings indicate that at the dosage of doxycycline utilized in this experiment was effective in mitigating splenomegaly and splenic histopathology, but did not appear to have high efficacy in accelerating resolution of histopathologic lesions induced by *C. burnetii* infection in mice

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Chapter 5: CONCLUSIONS FROM *C. BURNETII* MOUSE INFECTION STUDIES

Results from these mouse experimental inoculation studies demonstrate that the C57BL/6 mouse strain develops infection when intranasally inoculated with the *Coxiella burnetii* Nine Mile phase I strain. Previous studies have concluded that C57BL/6 mice are resistant to *C. burnetii* infection [112]. However, the mouse infection studies prove that C57BL/6 mice develop similar histopathologic disease observed in human Q fever. Humans develop disease through inhalation of infectious aerosols; our experiments mimic the natural route of exposure by infecting mice intranasally. Hepatitis and interstitial pneumonia were the most commonly observed histopathologic lesions in infected mice, and are the two primary disease manifestations observed in acute human disease [64]. Chronic Q fever is commonly associated with cardiac lesions, similarly, myocarditis was observed in intranasally infected mice, and has not been previously described with this inoculation route. Additionally, splenic lymphoid hyperplasia, interstitial nephritis, and myocarditis were also observed in infected mice. Hepatic lesions and splenic lymphoid hyperplasia were observed two months after infection and demonstrate that these tissues may develop chronic infection.

Development of human disease has been ascribed to many factors including host age and immune competency. Human disease is almost exclusively described in people over 15 years of age, and is most commonly seen in people over 50 years [70, 71]. However, these mouse experiments demonstrate that age did not exacerbate *C. burnetii* disease in mice.

Dexamethasone and cyclophosphamide drugs were given to mice to induce immunosuppression. The doses received by infected mice [128, 143, 144] did not induce exacerbate disease in mice, and more information is needed to understand the relationship of dexamethasone and cyclophosphamide with *C. burnetii*. Doxycycline treatment was evaluated in resolution of *C. burnetii* infection in infected mice. Treatment with doxycycline was effective in decreasing the incidence of splenic infection in mice. However, it was not effective in significantly decreasing the amount of bacterial DNA in infected tissues or development of histopathologic lesions. Our results demonstrated that the dose of doxycycline used was marginally effective in treating *C. burnetii* infection in mice and further studies need to evaluate the efficacy of higher doses.

Due to the intracellular nature of this bacterium, animal models are essential in the development of vaccine and drug therapies and uncovering pathogenic mechanisms of *C. burnetii*. These experimental infection studies found that intranasal infection induces histopathologic disease and tissue infections in C57BL/6 mice, and more severe disease is seen in aged mice. The liver and bone marrow of infected mice becomes chronically infected with bacterial detection and histopathologic lesions evident two months after infection,

and cannot be effectively treated with doxycycline. In conclusion, these studies reveal important findings about *C. burnetii* essential for understanding bacterial pathogenesis in the mouse host.

Experimental Research Constraints

Determining the number of animals in an infection study is surrounded with ethical concerns and costs. These experimental infection studies were conducted as pilot studies in an attempt to understand *C. burnetii* infection in mice. Therefore, low numbers of mice were maintained for each study. Unfortunately, the low power of these treatment groups may have prevented the observation of significant effects.

Smaller pilot immunosuppression and treatment studies should have been performed before infection studies in order to determine the appropriate dose for C57BL/6 mice. This may have helped solidify results about the effect of immunosuppression and doxycycline treatment on *C. burnetii* infections in mice.

Future Directions

The effect of chemically induced immunosuppression in *C. burnetii* infection in mice needs to be assessed. Experimental inoculation studies evaluating immunosuppression before infection will reveal differences in disease development compared to infected control mice. Also, immunosuppression after infection should be evaluated to determine if infection would recrudesce in tissues where lesions have resolved.

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