DISSERTATION

CHARACTERIZATION OF *BRUCELLA* INFECTION IN RUMINANT HOSTS: DISEASE PATHOGENESIS, IMMUNOLOGY, AND EPIDEMIOLOGY

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CHARACTERIZATION OF BRUCELLA INFECTION IN RUMINANT HOSTS: DISEASE PATHOGENESIS, IMMUNOLOGY, AND EPIDEMIOLOGY

Brucellosis is one of the most common zoonotic diseases worldwide, with endemic disease areas in the Middle East, Mediterranean Basin, Central Asia, Africa, and Central and South America. Disease is caused by various species of the gram negative bacteria *Brucella*. Infection in humans results primarily from contact with infected livestock or consumption of contaminated livestock products; cattle, small ruminants, and swine are the primary reservoir hosts. Although the *Brucella* bacterium was discovered over a century ago, control of disease remains a major challenge in many areas worldwide. Research on this pathogen has mostly been conducted in mouse models, which are naturally resistant to infection. Little is known of the immune response of natural ruminant hosts to *Brucella* infection.

Here we report an epidemiological study of brucellosis in Mongolia, as well as an experimental infection study of pregnant goats with two strains of *B. melitensis* – 16M, a fully virulent strain, and Rev. 1, a reduced virulence vaccine strain. Design of the experimental infection study was influenced by findings from field research in an endemic disease region. The objectives of the experimental challenge study were to characterize clinical disease, shedding, and tissue burdens in infected animals. The cellular immune response was then compared in animals infected with the two *B. melitensis* strains with the aim of identifying components of the protective response induced by the Rev. 1 vaccine strain and deficits in the immune response elicited by infection with virulent *B. melitensis* 16M.

A fluorescence polarization assay was utilized to identify antibodies in milk samples and estimate the proportion of *Brucella* positive cattle, yak, and hybrids in three regions of Mongolia. Additionally, prevalence of brucellosis in herd owners was assessed via questionnaire. Information was also collected from herd owners regarding animal husbandry practices and herd health in order to identify individual- and herd-level characteristics that are predictive for brucellosis. The study indicates that brucellosis remains endemic in cattle, yak, and hybrids within Bulgan and Khuvsgul provinces of Mongolia despite a national control program. Herd level prevalence was
determined to be 10.4% in the 77 herds tested. High levels of human disease were also reported. Results of the study indicate that the Mongolian brucellosis control program must be critically evaluated if the national goal of obtaining brucellosis-free status by 2021 is to be realized.

In an experimental challenge study, pregnant does infected with *B. melitensis* 16M at midgestation had an 86% abortion rate, while no Rev. 1-infected does aborted. Fetal infection rate was 92% and 43% in kids of 16M- and Rev. 1-infected does, respectively. Widespread tissue colonization was noted in 100% of 16M-infected does, and all of these animals shed brucellae in milk and vaginal secretions. Infection in does inoculated with Rev. 1 was more variable with only one animal showing generalized infection and colonization at levels similar to that of 16M-infected animals. Other Rev. 1-inoculated animals showed low levels of focal infection and shedding. Here we report the first isolation of *B. melitensis* from muscle tissue of experimentally infected goats. Milk was also found to pose a significant public health risk with three 16M-infected animals consistently shedding brucellae at levels of $10^4$ – $10^7$ CFU/ml over the four days on which samples were collected postpartum.

Despite the clear differences in clinical disease resulting from infection with the two strains of *B. melitensis*, protective versus deficient components of the immune response elicited by these two strains remain undefined. A pro-inflammatory response characterized by increases in granulocytes, monocytes, and CD4+ lymphocytes was identified by flow cytometric analysis of blood from 16M-infected does. In comparison cell numbers remained consistent with pre-infection levels in Rev. 1-inoculated animals. Limited production of IFN-γ and low level expression of the CD25 activation marker indicate a potential anergic state of CD4+ T cells in *B. melitensis*-infected goats. Increased numbers of IFN-γ producing WC1+ gamma-delta T cells at 28 days post-infection in Rev. 1-inoculated goats in comparison to 16M-infected animals may suggest a role of this cell type in the protective response elicited by the Rev. 1 vaccine strain.

The research presented in this dissertation builds upon current knowledge of *Brucella* epidemiology, pathogenesis, and immunology in natural ruminant hosts. The work provides a strong framework from which further comparative investigations of immune response to virulent *B. melitensis* and the reduced virulence *B. melitensis* vaccine strain, Rev. 1, can be conducted with the ultimate goal of defining components of a protective
versus deficient response to *Brucella* in a natural host. This will ultimately aid in development of improved vaccines facilitating control of disease in endemic areas like Mongolia.
ACKNOWLEDGEMENTS

I would like to acknowledge my PhD advisors Richard Bowen and Mercedes Gonzalez-Juarrero for their guidance during this work. They were receptive of my ideas and research aspirations and willing to venture out on new research paths, offering their different areas of expertise along the way. I would also like to thank my current graduate committee members, Robert Callan and Pauline Nol, as well as Robert Jones who had to step down from my committee. This work would not have been possible without the support from members of the Bowen lab, including Danielle Adney, who assisted with all of the goat sampling, and Paul Gordy, who was always there to teach new laboratory techniques.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1) Introduction

Brucellosis is one of the most common zoonotic diseases worldwide (Pappas et al., 2006). Caused by gram negative bacteria of the genus *Brucella*, brucellosis is predominately a disease of domestic animals; however, it is highly transmissible to humans. Cattle, small ruminants, and pigs are among the primary hosts. Disease in these animals is characterized by abortion, orchitis, and chronic shedding of bacteria. Humans are typically infected by consumption of unpasteurized dairy products or through occupational exposure. Some species of brucellae are extremely infectious with as few as 10 organisms capable of causing disease in humans (Godfroid et al., 2011).

Also known as undulant or Malta fever, acute brucellosis in humans is characterized by recurrent febrile episodes, profuse sweating, anorexia, fatigue, and arthralgia. A disease of numerous manifestations, any organ system can be affected by hematogenous spread of bacteria, leading to the development of debilitating complications. Without proper treatment, chronic or latent infections can develop. Signs of disease nearly 30 years after infection have been documented (Ogredici et al., 2010). This together with the granulomatous nature of lesions has resulted in the comparison of the disease to tuberculosis (Pappas et al., 2005, Sanjuan-Jimenez et al., 2013).

An estimated 500,000 new cases of brucellosis are diagnosed in humans annually, making this disease the most common bacterial zoonosis worldwide (Pappas et al., 2006). Reported brucellosis incidence, however, represents a great underestimate of true disease burden with an average of only one case in 26 being reported (Araj, 1999). The scarcity of reported cases is likely explained by a lack of sufficient healthcare in many regions with endemic disease, as well as challenges associated with diagnosis of disease. Endemic in the Middle East, Mediterranean Basin, Central Asia, and parts of Africa and South and Central America, brucellosis affects the world’s poorest populations. Brucellosis has major health and socioeconomic effects worldwide, with significant impacts directly on human health as well as on the productivity of livestock that serve as these people’s main source of subsistence. In a study of over 75 diseases affecting livestock, brucellosis was determined to be one of the 10 most important in terms of impact on impoverished people (Perry, 2002).

By no means an emerging disease, studies have shown that people and animals have been affected by brucellosis since the beginning of early civilization. Bacteria resembling brucellae have been found in skeletal
remains and carbonized cheeses from the Roman era (Capasso, 2002), and lytic lesions discovered in vertebrae from an ancestral hominid, *Australopithecus*, are consistent with brucellosis (D’Anastasio et al., 2009). If the lesions in this 2.5 million year old ancestral human are indeed a result of *Brucella* infection, brucellosis would be the first recognized infectious disease in humans. Despite advances in veterinary and human healthcare, brucellosis remains an important disease worldwide. Brucellosis has not attracted the research and financial resources that other global diseases such as malaria, HIV, and tuberculosis have drawn. In this sense brucellosis has been classified as a “neglected disease” by the World Health Organization (WHO). Many impoverished nations lack the resources to establish control programs, and brucellosis remains endemic in much of the developing world. While some industrial countries have successfully eradicated the species *B. abortus*, whose primary host is cattle, control of *B. melitensis* infection in small ruminants has proven more difficult. Among the select group of countries officially free of bovine brucellosis are Canada, Australia, Japan, and nations of northern Europe (Corbel, 1997, European Commission, 2012). The threat of re-introduction of disease, however, is ever present. Political and military turmoil has led to re-emergence of brucellosis in many areas of the world including the Balkan Peninsula and Central Asia (Pappas, 2010). Disease outbreaks have also occurred due to global trade of livestock and infected animal products. In addition, the role of wildlife as a reservoir for disease in livestock and humans has been recognized. After a 75 year eradication program, the United States was declared free of bovine brucellosis in 2008 (U.S. Department of Agriculture, 2008). Several cases of *B. abortus* infection have subsequently occurred, however, due to transmission of brucellosis from infected elk (*Cervus canadensis*) to cattle in the Greater Yellowstone Area (GYA) states of Montana, Idaho, and Wyoming (Rhyain et al., 2013, Higgins et al., 2012). Feral pigs (*Sus scrofa*) are also a reservoir of disease in the U.S. posing a risk to hunters as well as livestock (Olsen, 2010).

Our understanding of the role of wildlife as reservoirs of *Brucella* infection is constantly evolving. While six classical *Brucella* species have been recognized since the 1960s, over the past decade the number of known *Brucella* species has almost doubled (Pappas, 2010). These novel species have been isolated from marine mammals, rodents, foxes, and baboons, as well as from diseased humans. The ecology and zoonotic threat of these new species are only beginning to be understood. Old species, as well, are posing new threats. While previously believed to be relatively host specific, species barriers to *Brucella* infection, or what we know of them, are breaking down.
Examples of disease transmission between wildlife and livestock are increasingly being recognized. Even fish have been found to be infected with *Brucella*, a result of consumption of diseased livestock carcasses discarded into a river (El-Tras *et al.*, 2010). Domestic animals are posing new threats to human health as well. Cattle infected with *B. melitensis* and *B. suis*, species typically restricted to small ruminant and swine hosts respectively, are emerging as a major veterinary and public health problem in many areas of the world (Godfroid *et al.*, 2011).

While progress has been made in our understanding of *Brucella* infection, many questions remain to be answered if control of this disease is to finally be achieved. Brucellosis prevalence and the *Brucella* species responsible for infection are unknown in many areas of the world. *Brucella* virulence mechanisms are still largely undefined. Host range and species barriers to infection are not well understood. Disease ecology is rapidly changing and must be studied as it is essential that opportunities for brucellae to jump host species be reduced. Effective vaccines for use in humans have not yet been developed, and disease prevention in the human population relies on control of disease in the animal reservoir. Veterinary vaccines, however, also must be improved since those in use are imperfect and not effective in certain domestic species and in wildlife. A prerequisite to the development of improved vaccines and treatment strategies is study of the immune response of different host species to *Brucella* infection. This literature review outlines current understanding of the disease so that a framework is set to explore emerging disease problems and our research objectives. *Brucella* taxonomy and disease ecology, epidemiology, host-pathogen interactions, diagnostic and treatment options, and disease control strategies will be discussed.

1.2) *Brucella* Taxonomy and Rapidly Evolving Disease Ecology

1.2a) The Six Classical *Brucella* Species

Bacteria of the genus *Brucella* were first isolated in 1887 by David Bruce. The isolates were cultured from British soldiers stationed on the island of Malta who died of a disease then known as undulant fever. The species isolated by Bruce was later named *B. melitensis*, and within two decades of its discovery the epidemiological link was drawn between disease in humans and consumption of milk from infected goats. This led to the recommendation that all milk be boiled prior to consumption, drastically reducing disease incidence among the British soldiers. The discovery of the second member of the genus *Brucella* was made in 1897 by Bernhard Bang.
This organism, later named *B. abortus*, was isolated from cattle suffering from contagious abortion. Thus, bovine brucellosis is also known as Bang’s disease today (Dalrymple-Champneys, 1950).

By the 1960’s six species of brucellae had been discovered: *Brucella melitensis, B. abortus, B. suis* (Huddleson, 1931), *B. ovis* (Buddle, 1956), *B. neotomae* (Stoenner & Lackman, 1957), and *B. canis* (Carmichael & Bruner, 1968). Although the genus is highly homogenous with identical 16S rRNA (Gee et al., 2004) and recA gene sequences (Scholz et al., 2008a), and displays greater than 95% homology based on DNA-DNA hybridization studies (Verger et al., 1985), differences in host preference and biochemical properties resulted in the division of the genus into the six classical *Brucella* species (Osterman & Moriyón, 2006). *Brucella melitensis, B. abortus, and B. suis* are further divided into biovars. Evidence is accumulating that host specificity may not be as stringent as previously believed. Nevertheless, the primary hosts of *B. melitensis* are sheep and goats, while *B. abortus* primarily infects cattle. *Brucella suis* has a broader host range. The different biovars of this species are known to infect swine, wild boar (*Sus scrofa*), European hare (*Lepus capensis*), reindeer (*Rangifer tarandus*), and rodents. *Brucella melitensis*, *B. abortus*, and *B. suis* are the most pathogenic in humans (Table 1.1).

**Table 1.1.** Currently described *Brucella* species, preferred hosts, and zoonotic potential.

<table>
<thead>
<tr>
<th>Species</th>
<th>Primary host(s)</th>
<th>Zoonotic Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Classical species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td>Cattle</td>
<td>High</td>
</tr>
<tr>
<td><em>Brucella melitensis</em></td>
<td>Sheep, goats</td>
<td>High</td>
</tr>
<tr>
<td><em>Brucella suis</em></td>
<td>Swine, hare, reindeer, rodents</td>
<td>High</td>
</tr>
<tr>
<td><em>Brucella ovis</em></td>
<td>Sheep</td>
<td>None</td>
</tr>
<tr>
<td><em>Brucella canis</em></td>
<td>Dogs</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>Brucella neotomae</em></td>
<td>Desert wood rat</td>
<td>None</td>
</tr>
<tr>
<td><strong>Newly described species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brucella ceti</em></td>
<td>Whales, dolphins, porpoises</td>
<td>Moderate?</td>
</tr>
<tr>
<td><em>Brucella pinnipedialis</em></td>
<td>Seals</td>
<td>Moderate?</td>
</tr>
<tr>
<td><em>Brucella microti</em></td>
<td>Voles</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Brucella inopinata</em></td>
<td>Unknown</td>
<td>Moderate?</td>
</tr>
<tr>
<td><em>Brucella papionis</em></td>
<td>Baboon</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

While all members of the genus *Brucella* are small, gram negative coccobacilli and facultative intracellular organisms, brucellae differ in their antigenic components. *Brucella* species are divided into smooth (S) and rough (R) strains. Natural virulent field strains of *B. melitensis, B. abortus, B. suis, and B. neotomae* are classified as
smooth strains, while *B. ovis* and *B. canis* are naturally rough strains. This distinction refers to the structure of the lipopolysaccharide (LPS) in the bacterial cell wall. While S-LPS consists of three components, lipid A, core oligosaccharide, and O-antigen, in R-LPS the O-antigen is either absent or reduced to only a few sugar residues (Corbel, 1997). Smooth strains are generally more pathogenic in humans (Rittig *et al.*, 2003). *Brucella canis* is typically considered to play a limited role in human disease, although evidence exists that the number of cases may be underestimated (Dentinger *et al.*, 2014, Lucero *et al.*, 2010). *Brucella ovis* infection has never been reported in humans.

### 1.2b) The Novel Species *B. ceti* and *B. pinnipedialis*

Recent findings of novel *Brucella* species in wildlife and human hosts have led to considerable changes in *Brucella* taxonomy over the past decade (Table 1.1). In 1994 brucellae were isolated from marine mammals, greatly expanding the genus’ ecological range. This was not an isolated report. In the year of its discovery in the aquatic ecosystem, brucellae were isolated from four different species across two oceans: a harbor seal (*Phoca vitulina*), harbor porpoise (*Phocoena phocoena*), and common dolphin (*Delphinus delphis*) in Scotland (Ross *et al.*, 1994) and a fetus of a captive bottlenose dolphin (*Tursiops truncatus*) in California, U.S.A. (Ewalt *et al.*, 1994). Brucellae have now been found in a diverse range of marine mammal species (Guzman-Verri *et al.*, 2012, Hernandez-Mora *et al.*, 2013) with some populations exhibiting greater than 75% seroprevalence (Van Bressem *et al.*, 2001). Disease manifestations in marine mammals include reproductive lesions (Ohishi *et al.*, 2003), meningoencephalitis (Hernandez-Mora *et al.*, 2008), pulmonary and other abscesses (Cassle *et al.*, 2013), and asymptomatic infections (Nymo *et al.*, 2011). The brucellae isolated from marine mammals were officially recognized in 2007 as two separate species, *B. ceti* and *B. pinnipedialis*, preferentially infecting cetaceans and pinnipeds, respectively (Foster *et al.*, 2007). The taxonomy of marine mammal *Brucella* species, however, is still tentative. Biotyping studies have revealed distinct groups within the marine mammal brucellae (Maquart *et al.*, 2009a, Cloeckaert *et al.*, 2011), and there are indications that they differ in pathogenicity to humans (Whatmore *et al.*, 2008, Maquart *et al.*, 2009b). Three cases of naturally-acquired *B. ceti* infection of humans have been documented (Sohn *et al.*, 2003, McDonald *et al.*, 2006). Patients presented with intracerebral granulomas and spinal osteomyelitis, severe disease manifestations not commonly seen in human infections with the classical brucellae.
1.2c) The Novel Species *B. microti*

Another novel *Brucella* species, *B. microti*, was recognized in 2008 (Scholz *et al.*, 2008c). This species was first isolated from the common vole (*Microtus arvalis*) during an epizootic affecting the wild vole population within a region of the Czech Republic in 1999-2003 (Hubalek *et al.*, 2007). The voles suffered from a systemic disease characterized by edema of the extremities, skin abscessation, arthritis, lymphadenitis, orchitis, and peritoneal granulomas. Although *B. suis* and *B. neotomae* infection of rodents has been previously reported, molecular analysis indicated that the vole isolate differed from all other known *Brucella* species.

*Brucella microti* has subsequently been isolated from red foxes (*Vulpes vulpes*) in Austria during routine screening of hunted animals for zoonotic disease (Scholz *et al.*, 2009). Voles are a common food source of fox, suggesting that these animals may have become exposed by ingestion of infected prey. The discovery of *B. microti* in fox occurred 7 years after its initial isolation from voles and at a site over 100 km distant. This finding expands the geographic range of *B. microti* and suggests persistence of the organism in the region. This persistence may be due not only to the presence of animal reservoirs but also long-term survival of *B. microti* in the environment, a characteristic atypical of brucellae. Soil samples collected from the Czech Republic years after the epizootic in voles ended were found positive for *B. microti* by both PCR and culture (Scholz *et al.*, 2008b).

While there are no reported cases of *B. microti* infection in humans, its zoonotic potential is unknown. The wide distribution of its host, the common vole, across Eurasia and the potential persistence of *B. microti* in soil also has raised the possibility of re-emergence of brucellosis in European livestock. While these questions remain unanswered, the severe systemic disease observed in voles (Hubalek *et al.*, 2007) as well as experimental cellular and murine infection studies demonstrate the significant pathogenicity of *B. microti* (Jimenez de Bagues *et al.*, 2010). In human macrophage-like THP-1 cells, levels of intracellular *B. microti* have been found to be 100-fold higher than that of *B. suis* 1330 24 hours after *in vitro* infection. Intraperitoneal inoculation of Balb/c mice with $10^5$ colony forming units (CFU) of *B. microti* resulted in an 82% mortality rate within 4-7 days of infection. No other *Brucella* species is known to cause mortality of wild type laboratory mice at this standard infection dose. The enhanced capacity for replication within macrophages and the rapid death of mice following *B. microti* infection may be explained by the high metabolic activity, rapid growth on standard culture media, and increased acid
tolerance of *B. microti* compared to the classical brucellae (Scholz *et al.*, 2008c, Jimenez de Bagues *et al.*, 2010). In mice that survive the acute phase of infection, however, *B. microti* is rapidly cleared. This suggests that mice infected with *B. microti*, in comparison to *B. suis*, either mount a stronger immune response or that *B. microti* is more susceptible to host defense mechanisms.

Whole genome sequencing and phylogenetic analysis indicate that *B. microti* is ancestral to the classical brucellae (Audic *et al.*, 2011). *Brucella microti* may serve as an evolutionary link between the closely related genus *Ochrobactrum*, which is primarily made up of soil- and other free-living bacteria, and the facultative intracellular brucellae. Perhaps as the classical species became adapted to livestock hosts rather than a free-living lifestyle, they lost the ability to cause acute death (as seen in murine infections with *B. microti*), since this is not a favorable characteristic for intracellular pathogens that depend on their hosts’ survival. As the classical brucellae diverged from a potential free-living ancestor, they also likely adapted defense mechanisms to withstand the host immune system, a quality that may be absent in *B. microti* based on macrophage and murine infection studies (Jimenez de Bagues *et al.*, 2010).

1.2d) The Novel Species *B. inopinata* and other *B. inopinata*-like Bacteria

In 2010 another *Brucella* species, *B. inopinata*, was added to the genus (Scholz *et al.*, 2010). Currently, *B. inopinata* is represented by a single isolate (strain BO1) and several “*B. inopinata*-like” bacteria that are yet to be officially classified. Strain BO1 was isolated from a breast implant infection of a woman from Portland, Oregon (De *et al.*, 2008). The patient had a 5-month history of reoccurring fever, lethargy, myalgias, and breast swelling but denied any common risk factors associated with brucellosis. Molecular analysis of bacteria cultured from the breast implant demonstrated that the isolate clearly belonged to the genus *Brucella* based on DNA-DNA hybridization results and 16S rRNA gene sequence similarities. *Brucella inopinata* is genetically distinct from all other brucellae, however. Other members of the genus have identical 16S rRNA and recA sequences, and strain BO1 is characterized by 16S and recA sequences differing in five and seven nucleotides, respectively, from the *Brucella* consensus sequences. In some ways *B. inopinata* strain BO1 is similar to *B. microti*, displaying a highly active metabolism and rapid growth on standard media. With these growth characteristics and no known animal reservoir, this raises the question of whether *B. inopinata* is a soil microbe and an opportunistic pathogen, potentially like *B.*
and the distantly related *Ochrobactrum* species. Experimental infection studies in mice and macrophage cell lines indicate that in terms of pathogenicity *B. inopinata* behaves more like the classical brucellae and *B. microti* than the opportunistic pathogens of the genus *Ochrobactrum*. In murine infection models, strain BO1 actually persists in the spleen and liver at higher levels than the classical brucellae, suggesting the ability to establish chronic infection (Jimenez de Bagues *et al.*, 2014). In terms of mortality, however, strain BO1 behaves more like *B. microti* with high mortality rates observed in C57BL/6 and CD1 mice.

Another case of human disease has been attributed to a *B. inopinata*-like isolate. Strain BO2 was isolated from a lung biopsy of a 52-year-old man with chronic destructive pneumonia in Australia (Tiller *et al.*, 2010b). Again the patient denied any risk factors typically associated with *Brucella* infection. Of potential interest, however, the patient lived in Oregon as a young adult, at which time he suffered from liver failure and severe pneumonia of unknown etiology. While the patient’s symptoms were treated by multiple courses of antibiotic therapy, he reported two decades later in Australia with chronic destructive pneumonia, from which he again recovered with an extended course of antimicrobial treatment. Isolates taken from a lung biopsy on his most recent presentation were identified to belong to the genus *Brucella* based on biochemical profiles and molecular analysis. The isolate, strain BO2, was determined to have 100% sequence homology with the 16S rRNA sequence of *B. inopinata* strain BO1; however, the *recA* sequence of BO2 was more similar to the consensus sequence of the classical brucellae than to that of *B. inopinata* BO1. While the sources of infection remains undefined, the isolation of strains BO1 and BO2 demonstrate that novel *Brucella* strains are capable of causing significant disease in humans.

Sequence similarities between strains BO1 and BO2 and brucellae isolated from Australian rodents suggest that rodents may be reservoirs for *B. inopinata*-like organisms and a potential source of infection for humans. During the 1960s and 1980s, routine surveys of wild rodents in Australia resulted in the isolation of 13 *Brucella* strains, most of which were originally classified as atypical *B. suis*. Advances in molecular typing technologies allowed for reanalysis of these rodent isolates (Tiller *et al.*, 2010a). They were found to have a 16S rRNA nucleotide sequence distinct from the consensus sequence of the classical *Brucella* species indicating that they are more closely related to *B. inopinata* than *B. suis*. Similarities in sequences that encode outer membrane proteins *omp2a/2b*, as well as similarities in *IS711* insertion sequence profiles between the rodent and human strains, also
support classification of the Australian rodent isolates into a lineage of *B. inopinata*. Thus, three rodent-specific *Brucella* species have now been identified: *B. neotomae*, *B. microti*, and *B. inopinata*-like strains.

Most recently, isolates from frogs have been added to the rapidly expanding *B. inopinata* lineage (Eisenberg *et al.*, 2012, Fischer *et al.*, 2012). This represents one of the first descriptions of *Brucella* infection in non-mammalian hosts, expanding our current understanding of the ecological range of the genus *Brucella*. These novel amphibian *Brucella* strains were isolated from wild-caught African bullfrogs (*Pyxicephalus edulis*) found dead in the quarantine unit of a German zoo, as well as from a big-eyed tree frog (*Leptopelis vermiculatus*) with subcutaneous abscesses that was bought in a German pet shop. The 16S rRNA sequences of the isolates had 100% and 99.8% sequence homology with the 16S rRNA sequence of *B. inopinata*.

### 1.2e) The Novel Species *B. papionis*

The most recent organism to be added to the genus is *B. papionis* (Whatmore *et al.*, 2014), bringing the number of recognized *Brucella* species to eleven with five new species described in the past decade (Table 1.1). *Brucella papionis* infection has been associated with two cases of stillbirth and retained placenta in baboons (*Papio spp.*) (Schlabritz-Louisevitch *et al.*, 2009). The isolates were from cervical and uterine swabs from two baboons, one wild-caught, one colony-born, at a primate research center in Texas, USA following stillbirth in 2006. Phenotypic characterization of the isolates showed slow growth and limited metabolic reactivity, similar to the classical brucellae rather than recently described *B. microti* and *B. inopinata*. Sequencing of 16S rRNA demonstrated that the baboon isolates clearly belonged to the genus *Brucella*. The 16S rRNA sequence of the baboon isolates had a 2 bp difference from the consensus sequence shared by all brucellae except *B. inopinata*. The natural host of this new *Brucella* species and its zoonotic potential remain to be defined.

### 1.2f) The Classical Species in New Hosts: *B. melitensis* Infection of Cattle

With five new *Brucella* species officially recognized since 2007 and several additional isolates pending classification, what is known of *Brucella* disease ecology is rapidly changing. It is not just the novel species, however, that are contributing to our evolving knowledge of *Brucella* disease ecology. The classical *Brucella* species are being discovered in new host species, both domestic and wild, and are posing new threats to animal and
human health. Cattle, for example, are no longer exclusively considered a source of *B. abortus* infection. Cases of cross-infection with *B. melitensis* and *B. suis* are increasingly being documented.

In southern Europe, northern Africa, and the Middle East in particular, *B. melitensis* infection of cattle is an emerging veterinary and public health problem (Corbel, 1997, Godfroid *et al.*, 2011). Case reports of *B. melitensis* outbreaks in cattle have been reported in Spain (Alvarez *et al.*, 2011), France (Verger *et al.*, 1989, Mailles *et al.*, 2012), the United States (Kahler, 2000), Israel (Shimshony, 1997, Banai, 2002), Saudi Arabia (Radwan *et al.*, 1993), Egypt (Samaha *et al.*, 2008, Holt *et al.*, 2011, Wareth *et al.*, 2014b), Kenya (Muendo *et al.*, 2012), and China (Jiang *et al.*, 2013). In many countries of the Near East, *B. melitensis* is the most common species of *Brucella* isolated from all hosts, including small ruminants, cattle, buffalo, and camels (Refai, 2002). As more epidemiological typing studies are carried out, *B. melitensis* infection in cattle will likely be found to be commonplace in other regions of the world as well. Outbreaks are typically associated with the presence of infected small ruminants in the surrounding area. Although the routes of disease transmission between small ruminants and cattle and the dose necessary to establish infection in cattle are unknown, field reports suggest that *B. melitensis* is easily introduced into the cattle population. In regions in which animal movement is not well controlled or shared pastureland is present, cross-species infection of *B. melitensis* is widespread (Samaha *et al.*, 2008, Muendo *et al.*, 2012). These conditions are ubiquitous in many impoverished areas of the world and define nomadic herding practices. Outbreaks of *B. melitensis* in cattle are difficult to detect since abortion storms typically do not occur (Alvarez *et al.*, 2011, Banai, 2002). Control of outbreaks is also complicated by a lack of knowledge on the epidemiology of *B. melitensis* infection in this host and absence of an effective vaccine.

**1.2g) The Classical Species in New Hosts: *B. suis* Infection of Cattle and *B. abortus* Infection of Feral Swine**

*Brucella suis* infection in cattle is also increasingly becoming a public health concern (Godfroid *et al.*, 2011). In some areas within South America and Mexico, cattle, rather than pigs, are the predominant source of *B. suis* biovar 1 infection in humans (Corbel, 1997). Disease transmission between feral swine, wild boar, domestic pigs, and cattle is also complicating disease control efforts in the United States, Europe, and Australia. In the southern United States, for example, *B. suis* biovar 1 infection is prevalent in feral swine and these animals have transmitted infection not only to domestic pigs, but also to cattle (Ewalt *et al.*, 1997).
In a study that followed six cows naturally infected with *B. suis* biovar 1 (via potential contact with *Brucella* positive feral swine in Florida) and subsequently housed with uninfected, non-vaccinated pregnant controls, no transmission to calves or control cattle was observed (Ewalt *et al.*, 1997). This indicates that *B. suis* causes a noncontagious disease condition in cattle and, thus, cattle cannot serve as maintenance hosts for *B. suis*. Shedding of *B. suis* in milk of infected cows was observed, however, demonstrating the public health risk of this spill-over infection.

Brucellosis has been eradicated from domesticated swine and cattle in the U.S., and feral pigs threaten states’ disease free status. This problem will likely grow due to the expanding range of feral pigs across the United States. Resident feral pig populations are now reported in 38 states (Olsen, 2010), with brucellosis reported in feral swine in at least 13 states (Pedersen *et al.*, 2012). *Brucella suis*-infected feral swine are also a direct disease risk to hunters; three cases of human infection were reported in patients participating in the hunting and butchering of feral pigs in Florida (Center for Disease Control, 2009).

To further complicate the ecology of brucellosis infection among feral swine and cattle, a recent study suggests feral pigs may also be reservoirs of *B. abortus* infection. Widespread infection within a feral swine herd in South Carolina with *B. suis* and both field and vaccine strains of *B. abortus* was discovered, despite the lack of contact between cattle and these swine for over 30 years (Stoffregen *et al.*, 2007). *Brucella abortus* has also been isolated from a feral pig in southeastern Texas. This pig may be the source of an outbreak of *B. abortus* in a beef cattle herd in the same area of Texas in 2011 based on molecular analysis of cattle and swine isolates (Higgins *et al.*, 2012). It is also possible that disease transmission occurred in the opposite direction. Despite these recent findings, *B. abortus* infection of feral swine does not appear to be widespread. PCR and genotyping of *Brucella* isolated from 21 feral pigs across 7 states indicated that all isolates were *B. suis* (Pedersen *et al.*, 2014).

1.2h) *Brucella abortus* in Wildlife: Bison and Elk

Feral swine are not the only threat to the re-emergence of brucellosis in cattle in the United States. *B. abortus* infection is enzootic in bison (*Bison bison*) and elk within the Greater Yellowstone Area (GYA) of Montana, Wyoming, and Idaho. Since 2002, brucellosis has been detected in 17 beef cattle and ranched bison herds
in the GYA (Rhy an et al., 2013). All cases of brucellosis in livestock in this area have been attributed to transmission from elk based on molecular analyses of isolates (Rhy an et al., 2013, Higgins et al., 2012). Seroprevalence levels in bison are reported to be about 65% and have remained relatively constant over the past two decades (Scurlock & Edwards, 2010). Approximately half of the seropositive bison sampled in one study were culture positive; the discrepancy in test results likely being due to low and often undetectable tissue burden in chronically infected animals (Roffe et al., 1999). Bison function as maintenance hosts for \textit{B. abortus} in the GYA. Their gregarious herd structure naturally allows for transmission events to occur especially at the time of calving (Scurlock & Edwards, 2010). In contrast, \textit{B. abortus} infection in elk was not believed to be self-sustaining. Elk prefer to calve in seclusion, naturally limiting transmission events. Many elk herds within the GYA, however, are supplementally fed during the winter, leading to artificially high densities of elk during calving periods. This has allowed brucellosis to be maintained within these fed elk herds at a level of 20-30% (Cross et al., 2010, Scurlock & Edwards, 2010). Unfed elk within the GYA in comparison traditionally had seroprevalence levels of 2-3% (Cross et al., 2010, Scurlock & Edwards, 2010). Over the past decade, however, brucellosis has become more prevalent in these unfed herds approaching levels of 10-20% in some areas. This increase has been attributed to recovering elk populations and higher densities of elk congregating during calving season (Cross et al., 2010). The trend is worrisome since it suggests that free-ranging elk are now functioning as maintenance hosts for \textit{B. abortus}. Despite the higher prevalence of brucellosis in bison compared to elk, elk are more likely to share pastureland with livestock. The recent increase in \textit{Brucella} seroprevalence rates among elk coincides with an increase in transmission events from wildlife to livestock. From 1990-2002, no known cases of brucellosis occurred in livestock within the GYA (Rhy an et al., 2013). From 2002-2012, 17 livestock herds tested positive. Management strategies are limited given the challenges associated with keeping elk spatially apart from livestock and poor efficacy of brucellosis vaccines in elk.

1.2i) Sustainable Versus Spill-Over Infection

An important consideration in regards to brucellosis in wildlife and non-traditional host species is whether the infection is sustainable or simply a spill-over infection (Godfroid et al., 2013). Feral pigs are reservoirs of \textit{B. suis} biovar 1, while bison and elk are reservoirs of \textit{B. abortus} in the United States. \textit{Brucella} infection is sustained in
these species in the absence of continuous spill-over from domestic pigs and cattle. Feral pigs may also be maintenance hosts of *B. abortus* infection (Stoffregen et al., 2007).

Further studies are necessary to determine whether cattle can serve as maintenance hosts for *Brucella* species other than *B. abortus*. *Brucella melitensis* infection of cattle seems to occur almost exclusively in regions of high disease prevalence in small ruminants; however, it is unknown whether the bacteria can be maintained and transmitted within cattle herds without continuous spill-over from goats and sheep. As described above, a pilot study performed by Ewalt (1997) suggests cattle are unable to serve as maintenance hosts of *B. suis*. Ewalt was unable to isolate *Brucella* from vaginal swabs, blood, or placenta of infected cows and did not observe transmission to non-infected co-housed controls over a two year period.

Establishing the role that host species play in *Brucella* ecology will greatly impact disease control strategies (Godfroid et al., 2005). The ability of an animal population to serve as a reservoir of infection depends not only on the host species’ inherent susceptibility to infection but also on ecological factors. As the current circumstances with free-ranging elk in the GYA illustrate, population density plays an important role in disease transmission especially during calving season when potentially infectious fetal material is present in the environment. Shrinking wildlife habitats, recovering wildlife populations, and certain management decisions, such as the creation of animal feeding grounds, are causing *Brucella* infection to become more prevalent in some wildlife populations.

1.2j) Other Wildlife Hosts

While feral pigs, elk, and bison are the primary wildlife reservoirs of brucellosis in the United States, brucellae are known to infect approximately 80 other wildlife species (Stoffregen et al., 2007). Brucellae belonging to the six classical species have been isolated from muskoxen (*Ovibos moschatus*), moose (*Alces alces*), reindeer, caribou (*Rangifer tarandus caribou*), white tailed deer (*Odoncoileus virginianus*), roe deer (*Capreolus capreolus*), bighorn sheep (*Ovis canadensis*), chamois (*Rupicapra rupicapra*), ibex (*Capra ibex*), takin (*Budorcas taxicolor*), African buffalo (*Syncerus caffer*), impala (*Aepyceros melampus*), waterbuck (*Kobus elipsiprymnus*), fox, raccoon (*Procyon lotor*), opossum (*Didelphis virginiana*), European hare, and capybara (*Hydrochaeris hydrochaeris*) (Godfroid, 2002, Nymo et al., 2011, Kreeger et al., 2004). Many other species have been found seropositive. The
presence of anti-Brucella antibodies, however, only suggests exposure to brucellae; antibodies may be a result of infection and subsequent self-limiting disease. Some carnivores, for example, have shown transient titers apparently due to consumption of infected prey (Tessaro & Forbes, 2004). These animals are likely only dead-end hosts in areas where brucellosis is enzootic and probably do not play a role in the spread of disease.

Brucella abortus and B. suis are the classical Brucella species most commonly isolated from wildlife hosts. Sustainable reservoirs of infection for B. abortus include bison, elk, and potentially feral pigs in North America (Stoffregen et al., 2007) and buffalo in Africa (Madsen & Anderson, 1995). Maintenance hosts of B. suis include feral pigs and wild boar in North America, Europe, and Australia, hare in Europe, and potentially armadillos (Chaetophractus villosus) in South America (Kin et al., 2014). Surprisingly, despite its high virulence in humans, B. melitensis is not known to have a wide ecological range in wildlife. Brucella melitensis has been isolated from ibex (Ferroglio et al., 1998, Mick et al., 2014) and chamois (Garin-Bastuji et al., 1990) in France and Italy, Iberian wild goat in Spain (Capra pyrenaica) (Munoz et al., 2010), Arabian oryx (Oryx leucoryx) in Saudi Arabia (Ostrowski et al., 2002), and takin in China (Luo et al., 2012). Significant disease, including anorexia, blindness, polyarthritis, and orchitis, has been reported in several cases. While most of these infections are likely due to spill-over from livestock, there is recent evidence that ibex may be a reservoir of B. melitensis in southern France (Mick et al., 2014). The ibex population in this region has a 45% seroprevalence rate despite the absence of an infectious source in domestic ruminants for the past 15 years. Molecular analyses indicate that the B. melitensis biovar 3 strain currently circulating in the ibex population is similar to historic livestock isolates as well as isolates from cattle and humans in a 2012 outbreak that occurred in the area. This represents the first report of B. melitensis spillover from wildlife to livestock. In regards to the other classical brucellae, the naturally occurring rough species are more host specific. There have been isolated reports of B. canis infection in asymptomatic cattle (Baek et al., 2012), and farmed red deer (Cervus elaphus) in New Zealand are sustainable reservoirs of B. ovis (Ridler et al., 2000). There is some evidence that B. ovis may also infect bighorn sheep (McCollum et al., 2013).

1.2k) Brucellosis in Non-Mammalian Hosts

An additional wildlife species has recently been found to be infected with B. melitensis; high levels of B. melitensis infection were discovered in catfish (Clarias gariepinus) in Egypt (El-Tras et al., 2010). Carcasses of
infected cattle and small ruminants illegally discarded into a river are believed to be the source of infection. This finding expands the host range of the classical *Brucella* species to that of non-mammalian species and identifies a potential new source of zoonotic infection. As described above, atypical brucellae in the *B. inopinata* lineage have also been isolated from non-mammalian hosts (Eisenberg *et al*., 2012, Fischer *et al*., 2012).

Our knowledge of *Brucella* disease ecology is thus rapidly evolving. With each novel *Brucella* species discovered, the global distribution and host range of infection expands. The classical species are meanwhile rapidly adapting to a changing environment, challenging the characteristic host range restriction of this genus. New domestic and wildlife hosts are increasingly being identified in both the terrestrial and aquatic environments.

1.3) **Global Distribution of Brucellosis in Humans and Livestock**

Brucellosis is the most common zoonotic infection worldwide with more than 500,000 people diagnosed each year. In livestock, the global disease burden is also immense. Disease is endemic in the Middle East, the Balkan Peninsula, Central Asia, and regions of Africa and Latin America (Table 1.2). The global distribution of brucellosis in humans has most recently been reviewed by Racloz *et al*. (2013), Dean *et al*. (2012), and Pappas *et al*. (2006). Official reports of human cases, as well as cases in livestock, are also available from the World Organization for Animal Health (OIE) in a database searchable by country (http://www.oie.int/hs2/). Unfortunately the OIE statistics currently available are from 2004, and since primarily passively acquired national data are reported, true disease burden is greatly underestimated in most cases. This underestimation is due to a lack of access to quality healthcare in many endemic areas, the non-specific nature of brucellosis in humans, and frequent misdiagnosis. In Africa, for example, brucellosis is frequently misdiagnosed as malaria (Crump *et al*., 2013). Studies incorporating active surveillance of acute febrile illness and follow-up serology generally provide the most accurate determination of human disease prevalence. An analysis by Dean *et al*. (2012) illustrates that caution must be taken while interpreting disease incidence data; in a comparison of various studies of human brucellosis in Egypt, an active surveillance study estimated disease incidence at 70 cases per 100,000 people. Only 5% of these cases were identified by hospital-based surveillance and less than 0.5% were officially reported by the Ministry of Health. Unfortunately major gaps exist worldwide in the data available from active surveillance studies in humans and livestock. The epidemiology of human brucellosis has changed considerably over the past 20 years and this must
also be taken into account when interpreting older studies. Implementation of national disease control programs, changing socioeconomic conditions, and political unrest have all impacted brucellosis prevalence in recent years (Pappas et al., 2006). Lastly, most studies published on human and animal disease prevalence are based entirely on the results of serological assays. Since serological tests are unable to differentiate between *Brucella* species, the lack of culture- and molecular- based studies has resulted in an inability to determine the origin of infection in humans and animals and subsequently the failure to plan appropriate disease control strategies (Godfroid et al., 2013). These limitations must all be taken into account when considering the epidemiological data reviewed below.

**Table 1.2.** Examples of incidence rates of human brucellosis reported in various countries. Countries with incidence rates above 10/100,000 are considered endemic disease areas.

<table>
<thead>
<tr>
<th>Incidence per 100,000 population</th>
<th>Countries</th>
</tr>
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<tbody>
<tr>
<td>&lt; 1</td>
<td>USA, Canada, UK, Germany, France, Israel (Jewish population), Australia</td>
</tr>
<tr>
<td>1 - 10</td>
<td>Israel (Arab population)</td>
</tr>
<tr>
<td>10 - 50</td>
<td>Polynesia, Greece*, Chad*</td>
</tr>
<tr>
<td>50 - 100</td>
<td>Egypt*, Kyrgyzstan</td>
</tr>
<tr>
<td>100 - 200</td>
<td>Iran*, Syria</td>
</tr>
<tr>
<td>200 - 300</td>
<td>Iraq*</td>
</tr>
</tbody>
</table>

*Incidence data are sub-national.

**1.3a) North America**

In the U.S. brucellosis eradication efforts began in 1934 at which time seroprevalence in cattle was 11.5% (Ragan, 2002). Brucellosis has since been eradicated from cattle and domestic pigs in the country, although occasional cases of *B. abortus* and *B. suis* infection still occur due to spillover from wildlife reservoirs (U.S. Department of Agriculture, 2008, Rhyan et al., 2013). Canada has also eradicated bovine brucellosis, and although brucellosis persists in bison reservoirs, the situation differs from that in the U.S. The infected bison inhabit northern Alberta and the Northwest Territories, and due to spatial separation from livestock, transmission events to cattle have not been reported to date (Pare et al., 2012).

Brucellosis in sheep due to *B. ovis* infection is likely endemic in the U.S. A study of rams in Colorado, Utah, and Wyoming performed from 2000-2007 found seroprevalence rates of 10% (Van Metre et al., 2012). 

*Brucella melitensis* infection of small ruminants has never been endemic in the country.
Human brucellosis cases in the U.S. have been reduced from over 6000 in 1945 (Troy et al., 2005) to 116 in 2004 (OIE, http://www.oie.int/hs2/). The current incidence rate is 0.02-0.09 per 100,000 (Dean et al., 2012) although it is estimated that only 3.5-10% of infections in the U.S. are detected and reported (Troy et al., 2005). California and Texas account for 50-60% of the cases with risk factors for infection including Hispanic ethnicity and ingestion of unpasteurized dairy products, namely soft cheeses imported from Mexico where brucellosis is endemic.

While recent epidemiological studies are lacking, *B. melitensis* has traditionally been the primary cause of human infection in Mexico (Lucero et al., 2008). A relative increase in *B. abortus* cases in the Hispanic population of San Diego, California, however, may suggest increased circulation of this *Brucella* species in Mexico (Troy et al., 2005). Passively collected surveillance data indicate that 2000 people tested positive for brucellosis within Mexico in 2008 (Oseguera Montiel et al., 2013). Actual disease incidence is likely considerably higher (Dean et al., 2012). Although a caprine brucellosis control program has been in place in Mexico since 1971, disease prevalence in goats remains high. A recent cross-sectional surveillance study in goats within two Mexican states found individual and flock level prevalences of 19% and 71%, respectively (Oseguera Montiel et al., 2013).

Within Central America, *B. melitensis* is prevalent only in Guatemala; however, bovine and swine brucellosis are widespread throughout the region (Moreno, 2002). No data are available on prevalence rates in swine, goats, sheep, or dogs. Data are also limited on cases in humans. Prevalence in cattle is estimated at 4-8% with 10-25% of herds infected. Sporadic control programs in Central American countries have been unsuccessful at reducing the prevalence of brucellosis.

### 1.3b) South America

Brucellosis remains a major zoonosis in South America, although select countries such as Uruguay and Chile have nearly achieved disease-free status. Both countries successfully eradicated *B. melitensis* and *B. suis*, and prevalence in cattle is well below 1% (Aznar et al., 2014). Brucellosis is endemic in Argentina with 2.1% of individual cattle and 12.4% of beef herds infected. While prevalence in swine is poorly understood, *B. suis* is more commonly isolated from human patients in Argentina than *B. abortus* (Aznar et al., 2014, Lucero et al., 2008). Cattle may also be a source of *B. suis* infection (Corbel, 1997). In Brazil, *B. abortus* is more prevalent than *B. suis,*
with B. ovis and B. canis also present. *Brucella melitensis* has never been isolated in Brazil. Prevalence of bovine brucellosis is quite variable, with 0.3% of herds infected in the south and 41% of herds seropositive in central Brazil (Aznar et al., 2014, Borba et al., 2013). In Ecuador, two recent active surveillance studies of human, bovine, and caprine brucellosis indicate seroprevalence rates of 1.9% in humans (4.8% in slaughterhouse workers), 7.2% in cattle, and 17.8% in goats (8-9% of goats culture positive) (Ron-Roman et al., 2014, Poulsen et al., 2014).

### 1.3c) Europe

Brucellosis is declining in the European Union (EU) with 352 human cases reported by member states in 2011 (European Food Safety Authority, 2013). Of the officially reported cases in 2011, 80% were from Greece, Italy, Portugal, and Spain where brucellosis remains endemic. *Brucella melitensis* is of greatest concern in these countries. The number of officially reported cases in the EU is likely a great underestimation of actual disease burden. For example, the European Food and Safety Authority (2013) reported the annual incidence of disease in Greece in 2011 at 0.81 cases per 100,000 people. Sub-national data collected in Central Greece from 2003 to 2005 indicate a disease incidence of 32.5 per 100,000 (Minas et al., 2007).

Approximately half of EU member states are officially *B. abortus* and *B. melitensis* free. Among the brucellosis free countries are the Scandinavian countries and Germany, with human cases in these countries occasionally diagnosed in immigrants from Turkey and other brucellosis endemic areas (Pappas et al., 2006, European Commission, 2012). Northern Ireland will be the most recent country to be declared free of bovine brucellosis, making the entire United Kingdom disease free (2015). France achieved eradication of *B. abortus* and *B. melitensis* in 2003, although a localized outbreak in cattle and humans occurred in 2012 as a result of spill-over from wildlife (Garin-Bastuji et al., 1990). Spain, Portugal, and Italy continue to make progress toward eradication of brucellosis in cattle and small ruminants (European Food Safety Authority, 2013, Mancini et al., 2014). In addition to *B. abortus* and *B. melitensis*, *B. suis* biovar 2 occasionally infects livestock in the European Union. Wild boars and hare serve as reservoirs of infection with rare spill-over to commercial swine and cattle (Szulowski et al., 2013, Godfroid & Kasbohrer, 2002). *Brucella suis* biovar 2 is not zoonotic, however, in contrast to *B. suis* biovar 1, which is prevalent in feral swine within North and South America and Australia.
While brucellosis is declining in the EU, it remains a major zoonosis in other regions of Europe including the Balkan Peninsula and Turkey. Brucellosis prevalence is related to socioeconomic status within Europe. Southeastern Europe has suffered from the Balkan war, the fall of communism, and economic recession (Hotez & Gurwith, 2011). This has led to the re-emergence of brucellosis within Macedonia, Serbia, Albania, Bosnia and Herzegovina, and Bulgaria among other countries in the region (Pappas et al., 2006, Hotez & Gurwith, 2011, Bosilkovski et al., 2010, Cekanac et al., 2010, Russo et al., 2009). Bulgaria, for example, had been declared brucellosis-free in 1958. However, from 2005-2007 105 human cases and 635 cases in livestock were recorded. The re-emergence of disease was attributed to shortcomings in a public health system still recovering after the fall of the Soviet Union, as well as economic instability forcing increased importation of animals from brucellosis endemic regions like Greece and Turkey (Russo et al., 2009).

1.3d) Asia

The countries with the highest disease incidence rates worldwide are located within Asia; however, major gaps exist in prevalence data (Dean et al., 2012, Pappas et al., 2006). The disease is endemic in the Middle East, republics of the former Soviet Union, Mongolia, India, and regions of Southeast Asia. While declining trends in brucellosis have been reported in Russia, an increase in cases has been reported in South Korea and China. According to the most recent national-level data available, Syria has the highest disease incidence worldwide with 160 cases per 100,000 inhabitants (Pappas et al., 2006). There is evidence from a sub-national study that prevalence in regions of Iraq may be even higher with 27% or 268 per 100,000 inhabitants infected (Dean et al., 2012, Yacoub et al., 2006). A study in Iran reported 142 cases per 100,000 people and an apparent association between disease in humans and consumption of dairy products from cattle (Haghdoost et al., 2007). This is unique among Middle Eastern countries, which primarily report human disease caused by B. melitensis. It is unknown whether the cattle in Iran are infected with B. melitensis or B. abortus. In many countries in the region, B. melitensis is commonly isolated from cattle, camels, and equids in addition to sheep and goats (Refai, 2002). For example, in Jordan 8.5% of donkeys are seropositive for brucellosis (Abo-Shehada, 2009). In Israel B. melitensis outbreaks often occur in cattle kept in the proximity of small ruminants (Shimshony, 1997, Banai, 2002). While Israel has made progress in controlling brucellosis over the past three decades, disease remains endemic in rural,
primarily Arab communities, with an incidence rate of 7.0 per 100,000 in the Arab population compared to 0.2 per 100,000 among the Jewish population (Anis et al., 2011).

Outside of the Middle East, brucellosis is also endemic in India. Prevalence appears to be increasing in the country with *B. melitensis*, *B. abortus*, *B. ovis*, *B. suis*, and *B. canis* all present (Renukaradhya et al., 2002). Data from India are sparse, but with the largest livestock population in the world and no brucellosis control program in place, millions of *Brucella* positive animals are likely present (Kumar, 2010). The prevalence of brucellosis in India’s 185 million cattle is estimated to be 5-26.5% (Renukaradhya et al., 2002, Chand & Chhabra, 2013). Prevalence in sheep and goats is 7.9% and 2.2% respectively, with *B. melitensis* causing the majority of human infections for which there is no data on disease incidence (Renukaradhya et al., 2002, Pappas et al., 2006).

Brucellosis has also had a significant impact in Mongolia. Considered to have the second highest incidence of human brucellosis in the world a decade ago (Pappas et al., 2006), knowledge of current disease incidence and epidemiology in Mongolia and all of Central Asia is incomplete (Dean et al., 2012). Brucellosis is a re-emerging disease in countries of the former Soviet Union and former Eastern Bloc satellite states, such as Mongolia, Kyrgyzstan, and Tajikistan (Pappas et al., 2006, Bonfoh et al., 2012, Tsend et al., 2014, Zolzaya et al., 2014). Economic instability following the collapse of the Soviet Union in 1990 led to a lapse in veterinary care and disease control measures (Pappas et al., 2006, Foggin et al., 2000). Regional estimates for seroprevalence of brucellosis in Mongolia are 2.3-27.3% in humans, 6.2% in sheep, 5.2% in goats, 16.0% in cattle, 2.5% in camels, 8.3% in horses, and 36.4% in dogs (Tsend et al., 2014, Zolzaya et al., 2014). These numbers are considerably higher than officially reported data. In Kyrgyzstan 77.5 cases per 100,000 people were reported in 2007 (Bonfoh et al., 2012).

While high levels of animal and human brucellosis persist in the republics of the former Soviet Union, the Russian Federation has reported a sharp decline in bovine brucellosis. In 2008, only 68 *Brucella* positive cattle herds existed nationwide (Ivanov et al., 2011). Reports in the English language are scarce, however, and brucellosis prevalence in small ruminants and humans is unknown.
China has reported changing patterns of brucellosis in recent years. Brucellosis was endemic in the 1950s-1970s, but by the mid 1990s levels of disease in livestock fell below 1% (Deqiu et al., 2002, Liu et al., 2014). Over the past decade, however, brucellosis has rapidly re-emerged, and the geographic distribution of the disease has shifted from pastoral to periurban and urban areas. Traditionally, brucellosis endemic regions were limited to rural regions of western and northern China (Deqiu et al., 2002). Recently, developed areas of southern China including the Guangdong Province have reported drastic increases in human brucellosis. In Guangdong, only 51 confirmed cases of brucellosis were reported from 1955-2004, while in the subsequent 5 years a total of 112 cases were reported. While human brucellosis is most commonly associated with B. melitensis and occasionally B. abortus in China, B. suis has also been isolated from recent cases in Guangdong suggesting a shift in the circulating species of Brucella (Chen et al., 2013, Jiang et al., 2013).

In Southeast Asia, brucellosis is poorly studied, however, there is evidence that rates of infection are increasing among cattle. In Malaysia, for example, despite an eradication program, a recent study reported 2.5% seroprevalence rates and 21.8% herd prevalence rates in cattle (Anka et al., 2013). Similar levels of disease have been reported in Indonesia and Thailand.

1.3e) Africa

Brucellosis is considered endemic in North Africa (Pappas et al., 2006) with several studies recently published on disease prevalence in humans and livestock in Egypt (Holt et al., 2011, Samaha et al., 2008, Wareth et al., 2014b, Wareth et al., 2014a, Samaha et al., 2009). The predominant Brucella species circulating in Egypt is B. melitensis with small ruminants, cattle, buffalo, and camels all infected with this species. Estimates of disease incidence in humans vary widely from 0.28-70.0 cases per 100,000 (Dean et al., 2012). A seroprevalence study conducted on 100 Egyptians who worked at dairy farms or abattoirs found evidence of anti-Brucella antibodies in 5-8% of the people sampled (Samaha et al., 2009). Foodborne brucellosis is also a concern in Egypt; PCR analysis of cow and buffalo milk sold for human consumption detected Brucella DNA in 8% of samples (Wareth et al., 2014b). Estimates of the prevalence of brucellosis in Egyptian livestock vary widely (Wareth et al., 2014a). Samaha et al. (2008) determined prevalence to be 3.6-5.4% in goats, sheep, buffalo, and cattle sampled in 2007 with all
identifiable isolates determined to be *B. melitensis* biovar 3. Holt *et al.* (2011) reported 11% prevalence rates in cattle and buffalo.

Less is known about the prevalence of brucellosis in sub-Saharan Africa although it is believed to be endemic in most countries (Racloz *et al.*, 2013, Pappas *et al.*, 2006). Brucellosis has been reported in West Africa (Sanogo *et al.*, 2013, Boukary *et al.*, 2013), Central Africa (Dean *et al.*, 2012), and East Africa (Megersa *et al.*, 2011, Muendo *et al.*, 2012, Crump *et al.*, 2013, Kunda *et al.*, 2007), with the only organized brucellosis eradication programs existing in southern Africa (McDermott & Arimi, 2002). On a global scale sub-Saharan Africa thus may have the largest concentration of human and animal brucellosis, a consequence of extensive disease burden and sheer number of people and animals on the continent (Racloz *et al.*, 2013).

In sub-Saharan Africa brucellosis prevalence is best understood in cattle with a limited number of studies conducted in small ruminants. *Brucella suis* is believed to be prevalent in some areas of Africa but its epidemiology is poorly understood (McDermott & Arimi, 2002). Even less is known about brucellosis burden in the human population. The disease is largely ignored, and most cases go undiagnosed and untreated. Many brucellosis cases are incorrectly diagnosed as malaria or typhoid fever. In a study of patients presenting with fever in Tanzania, diagnostic tests revealed that more patients actually had brucellosis than malaria (Crump *et al.*, 2013). Reported disease rates include: 35 cases annually per 100,000 in a nomadic community in Chad (Dean *et al.*, 2012) and 2-6% prevalence in Tanzanian hospital patients with fever (Crump *et al.*, 2013, Kunda *et al.*, 2007). In livestock, brucellosis prevalence has been reported to be 10.6%, 2.2%, and 1.9% in cattle, camel, and goats in Ethiopia (Megersa *et al.*, 2011) and 3.8%, 2.3%, and less than 0.5% in cattle, sheep, and goats in Niger (Boukary *et al.*, 2013).

Identifying the species of *Brucella* responsible for disease is critical for implementation of control measures. Although there are many gaps in published biotyping studies and no reports on the sources of human disease in sub-Saharan Africa, there are indications that the distribution of the *Brucella* species differs between the various regions of Africa. In North Africa, *B. melitensis* predominates even in cattle and buffalo (Wareth *et al.*, 2014b, Samaha *et al.*, 2008). Both *B. melitensis* and *B. abortus* have been isolated from cattle in Kenya (Muendo *et
al., 2012), while in West Africa and southern Africa B. abortus infection of cattle seems to predominate (Sanogo et al., 2013, Matope et al., 2009).

Resources for disease control are extremely limited in sub-Saharan African and other infectious diseases are present that have higher mortality rates than brucellosis; thus disease control priorities in livestock and humans must be carefully considered in this region (McDermott & Arimi, 2002, Pappas et al., 2006, Racloz et al., 2013). The importance of brucellosis control, however, was underscored by findings of a study carried out by a group in Kenya. Of over 75 diseases affecting livestock, brucellosis was determined to be one of the 10 most important in terms of impact on impoverished people (Perry, 2002).

1.3f) Oceania

Australia and New Zealand are free of bovine brucellosis, with both countries reporting their last cases in 1989 (http://www.oie.int/hs2/). Brucellosis in small ruminants due to B. melitensis has never been reported in Australia or New Zealand; however, B. ovis is a major concern. In New Zealand B. ovis infects farmed red deer in addition to sheep (Ridler et al., 2000). While brucellae of zoonotic concern are absent in New Zealand, 20-50 cases of B. suis infection occur annually in people within Australia (http://www.oie.int/hs2/). Feral swine are sustainable reservoirs of B. suis biovar 1 in the country with a seroprevalence rate of 10.5% (Pearson et al., 2014), and these animals pose significant risk to feral pig hunters. While B. suis is not endemic in commercial piggeries in Australia, domestic swine within Polynesia have high rates of infection. An estimated 20% of swine herds in Polynesia are seropositive contributing to an average annual rate of human infection of 19 cases per 100,000 inhabitants. Bovine and small ruminant brucellosis are not reported in Polynesia (Guerrier et al., 2011).

1.4) Transmission

1.4a) Within Livestock Populations

The process of transmission of B. abortus between cattle is well described in the literature (Crawford et al., 1990), and information on this subject has changed little over recent years. In order for transmission to occur between cattle, an infected animal must be excreting brucellae. Excretion is almost entirely limited to the time period immediately following abortion or full-term parturition with high numbers of B. abortus present in uterine
fluid and within the placenta. Three factors determine the likelihood of transmission to susceptible animals: the number of brucellae excreted during parturition, the survival of these bacteria in the environment, and the probability of a susceptible animal being exposed to enough brucellae to establish infection. It is estimated that $10^5$ CFU of *B. abortus* are sufficient to induce infection in 78% of naïve cattle via conjunctival exposure (Manthei, 1968). Slightly more bacteria are likely necessary to infect animals via an oral route of exposure. One gram of placental tissue from an infected cow is estimated to contain 20-360 infectious doses (Olsen & Johnson, 2011). The level of shedding may vary depending on a number of factors. Shedding typically decreases with each subsequent parturition following infection (Crawford *et al.*, 1990). Persistence of *B. abortus* in the environment, and thus the duration of time during which naïve animals can be exposed to brucellae following a parturition event, is dependent on environmental conditions. Survival of *Brucella* is enhanced by the presence of moisture and lower temperatures. *Brucella abortus* can survive an estimated 180 days within a fetus in the shade, 5-150 days in water, 8-240 days in manure, and 151-185 days in cold, moist soil. Survival in direct sunlight is only 4.5 hours (Crawford *et al.*, 1990). The final factor determining transmission to susceptible animals, probability of exposure, depends largely on husbandry practices. If allowed the opportunity, cattle will often investigate, smell, or ingest placental material left on the pasture or barn floor. The oral route is considered the primary means by which susceptible animals are exposed, although in intensively farmed cattle housed indoors, the conjunctival route is also likely important. Vertical transmission is also a common route of infection. An estimated 20% of surviving calves are infected *in utero*. *Brucella*-contaminated milk is another potential source of infection for calves (Crawford *et al.*, 1990, Nicoletti, 1980).

*Brucella* infection in bulls can manifest as orchitis, epididymitis, and seminal vesiculitis, and localization of brucellae in these organs typically results in shedding in the semen. However, when used for natural mating, infected bulls are considered a negligible source of infection for naïve cows. *Brucella*-contaminated semen is of considerable risk to naïve cows bred by artificial insemination (Crawford *et al.*, 1990). The difference in risk may be due to the different locations in which semen are deposited. It is possible that brucellae are unable to survive within the cervix due to the presence of antimicrobial factors (Nicoletti, 1980).
Although a minor source of exposure overall, dogs may play important roles in *B. abortus* epidemiology in some areas. Dogs are often kept on farms and if given the opportunity will consume bovine placental material. High levels of *B. abortus* infection have been documented in dogs in some locations; for example in regions of Mongolia 36% of dogs are seropositive (Zolzaya *et al.*, 2014). *Brucella abortus* can cause abortions in pregnant dogs, and the resulting environmental contamination has been reported to cause infection of cattle kept in close proximity (Crawford *et al.*, 1990, Nicoletti, 1980).

In small ruminants transmission of *B. melitensis* is believed to occur via the same routes as *B. abortus* infection in cattle. The primary route of exposure is oral, with *in utero* transmission also playing a role. While venereal transmission is considered to be of limited importance in the spread of *B. melitensis*, *B. ovis* is primarily acquired via this route. Ewes can maintain *B. ovis* in the vagina for months and serve as mechanical vectors of infection for rams. Direct ram-to-ram transmission also occurs likely via homosexual activity or oral contact with infected urine (Center for Food Security and Public Health, 2007, Hartley *et al.*, 1955).

In swine, transmission of *B. suis* frequently occurs via both consumption of birth or abortion products and venereal transmission (World Organization for Animal Health, 2009c). Similar routes of transmission are observed in dogs with *B. canis* infection. High numbers of brucellae are shed in semen and urine of infected male dogs (Carmichael & Joubert, 1988). In infected bitches abortion can result in contamination of the environment with $10^{10}$ brucellae/ml of uterine discharge, an amount equating to 500 oral infectious doses/ml. Infection of pups can also occur *in utero* (Wanke, 2004, Hollett, 2006).

**1.4b) Zoonotic Transmission**

Brucellosis is not considered a contagious disease in humans, although rare reports of sexual transmission, *in utero* infection, and nosocomial infection exist (Meltzer *et al.*, 2010, Mesner *et al.*, 2007). Except in these rare circumstances, brucellosis is contracted via contact with infected animals or animal products. Most cases are caused by *B. melitensis* and *B. abortus*, with *B. suis* also highly zoonotic but less widespread. Disease in humans is occasionally caused by *B. canis* and infection by this species may be underreported (Dentinger *et al.*, 2014, Lucero
et al., 2010). Infection with marine mammal strains of brucellae has been diagnosed in four individuals (Sohn et al., 2003, McDonald et al., 2006).

Brucellosis is typically of foodborne or occupational origin. Unpasteurized cow, small ruminant, and camel milk or milk products are most commonly associated with foodborne brucellosis. Brucellae persist in soft cheeses, butter, and ice cream to a greater extent than hard cheeses and yogurt due to the low pH of the later products. If sufficiently cooked, muscle and organ meat from infected animals is not a source of human infection. In some cultures, raw or partially cooked liver, spleen, and fetuses are consumed, however, and these can be heavily contaminated with brucellae (Godfroid et al., 2005). Foodborne exposure is the most common route of infection in travelers as well as in people of endemic countries where milk is not traditionally pasteurized or boiled before consumption. In other endemic areas unpasteurized products are not commonly consumed, and in these cases infection is primarily occupational. Herders/farmers, abattoir workers, leather makers, veterinarians, hunters, and laboratory personnel can be exposed to high levels of brucellae. Infection often occurs via inhalation or through skin lesions.

1.5) Clinical Presentation of Brucellosis

1.5a) Bovine Brucellosis

The most common clinical outcome of *B. abortus* infection in cattle is late-term abortion or full-term birth of weak offspring (Olsen & Tatum, 2010). Abortion typically occurs only during the first parturition following infection, with subsequent births often normal, although sometimes accompanied by bacterial shedding. One study found 20% of cows to shed brucellae in uterine secretions at the second parturition post-infection. Of the cows with negative uterine fluid cultures, however, 88% still shed brucellae in colostrum (Manthei & Carter, 1950). Infection acquired by calves at birth may be temporary or develop into latent infection. Heifer calves that develop latent disease remain asymptomatic and serologically negative until first parturition at which time abortion and seroconversion are frequently observed (Wilesmith, 1978, Nicoletti, 1980). While shedding of bacteria in milk is an obvious sequela of infection, overt signs of mastitis are not typically present (Morgan, 1960). Quality of the milk remains high in terms of absence of visible particles and a low leukocyte count (Emminger & Schlam, 1943).
Osteoarticular lesions are occasionally associated with *Brucella* infection in cattle. There is evidence suggesting that differences exist in the frequency of this disease presentation between different geographic locations, potentially due to the presence of different cattle breeds or *B. abortus* biotypes. For example, in western Sudan osteoarticular lesions are more commonly associated with *Brucella* infection; 92% of Zebu cattle (*Bos taurus indicus*) with hygromas and 62% of Zebu cattle with arthritis were found to be seropositive for *Brucella* (Musa *et al*., 1990).

In bulls, orchitis is the most common disease manifestation often with an associated seminal vesiculitis and epididymitis. Many bulls will remain asymptomatic, and infertility is not typically observed (Eaglesome & Garcia, 1992, Carvalho Neta *et al*., 2010). *Brucella abortus* infection of other livestock including buffalo, bison, yak, and elk resembles infection in cattle (Olsen & Johnson, 2011, Kreeger *et al*., 2000, Jackson *et al*., 2014, Nicoletti, 1980).

1.5b) Caprine Brucellosis

*Brucella melitensis* infection in goats has been reported to closely resemble disease in cattle infected with *B. abortus*. Sheep are more resistant to infection, and there is great variation in susceptibility between breeds (Alton, 1990a). *Brucella melitensis* is associated with late-term abortion during the first parturition post-infection. Pregnancy can also go full-term with the birth of weak kids, heavily infected but healthy kids, or kids that escaped infection. Infection of kids may be temporary, as development of latent infection seems to be rare (Alton, 1970). Following abortion or normal birth large numbers of brucellae are excreted; in goats shedding in uterine discharge can last 2-3 months and resume at subsequent parturitions (Alton, 1990a). Sheep are less likely to abort, although breed differences likely exist. In sheep shedding in uterine fluid is of shorter duration and rarely reoccurs during succeeding pregnancies. However, shedding in milk over succeeding pregnancies has been observed in sheep (Tittarelli *et al*., 2005). In male animals, especially in goats, orchitis appears to be a common manifestation of *B. melitensis* infection.

1.5c) Ovine Brucellosis

*Brucella ovis* causes epididymitis and impaired fertility in male sheep (Buddle, 1956). Although experimental infection of goats is possible, it has not been reported to naturally occur (Burgess *et al*., 1985, Ridler *et
al., 2000). Among rams, only 30-50% of serologically or bacteriologically positive animals will have palpable lesions (Poester et al., 2006, Van Metre et al., 2012). Shedding of brucellae in semen still occurs in asymptomatic rams, however, and these silent carriers disseminate infection throughout the herd. Fertility of asymptomatic animals may be normal or reduced. Infection is less common in ewes, but abortion or birth of weak lambs can occur (Poester et al., 2006, Hartley et al., 1955).

1.5d) Swine Brucellosis

*Brucella suis* infection of swine is unique among the brucellosis infections in livestock, in that a more systemic infection is frequently observed. Bacteremia may persist for several months, and osteoarticular lesions are more common than in other livestock. Infection in swine, however, may also be asymptomatic. The typical reproductive signs are frequently observed including abortion, weak piglets, orchitis, and epididymitis. Abortions in sows can occur at any time during pregnancy, and early fetal loss often leads to signs of irregular estrus or infertility (Poester et al., 2006).

1.5e) Canine Brucellosis

The typical reproductive lesions associated with *Brucella* infection of animals are also observed in dogs. In bitches the most common manifestation is late term abortion although early embryonic death occasionally occurs. Vaginal discharge can persist for weeks following abortion and contain high numbers of brucellae. In males, orchitis and epididymitis are observed, which can cause infertility. Prolonged bacteremia is observed as noted in swine. Diskospondylitis, meningoencephalitis, and uveitis are also occasionally observed in dogs with *B. canis* infection (Hollett, 2006, Wanke, 2004).

1.5f) Brucellosis in Other Livestock

Camels are susceptible to *Brucella* infection, exhibiting high seroprevalence rates in areas where *B. melitensis* or *B. abortus* are endemic. Disease in camels is reportedly more mild than in cattle, but orchitis, epididymitis, abortion, arthritis, hygromas, and shedding in uterine discharge and milk have all been recorded (Gwida et al., 2012).
*Brucella abortus*, *B. suis*, and *B. melitensis* can cause disease in equids. Although high seroprevalence rates have been reported in equids in *Brucella* endemic areas (Zolzaya *et al.*, 2014, Abo-Shehada, 2009), equids are generally disregarded in discussions of brucellosis in livestock. Poll evil (suppurative atlantal bursitis) and fistulous withers (suppurative supraspinous bursitis) are the most common clinical signs associated with *Brucella* infection in the horse, although the majority of infected horses remain asymptomatic. Predilection for the joints, muscles, tendons, and bursae in horses appears to be more common than predilection for the reproductive tissues, although abortion has been observed (Denny, 1973).

1.5g) Human Brucellosis

By definition brucellosis in humans is a disease of “protean manifestations,” significantly complicating diagnosis (Pappas *et al.*, 2005). Alternative names for human brucellosis include Malta fever and undulant fever, and flu-like illness is the classical presentation of acute disease. An estimated 53-100% of patients present with fever (Franco *et al.*, 2007). Although rarely fatal, brucellosis in humans can be a debilitating, chronic disease affecting any organ system. Clinical features of brucellosis depend on the stage of disease, the species of *Brucella* responsible for infection, and the organ systems involved. Table 1.3 presents the most common clinical findings in patients with brucellosis (Troy *et al.*, 2005, Franco *et al.*, 2007, Pappas *et al.*, 2005). Symptoms generally appear within 2-6 weeks of exposure, but the incubation period can be up to 3 months (Sauret & Vilissova, 2002, Reguera *et al.*, 2003). Fever, joint pain, night sweats, and constitutional symptoms including malaise, anorexia, weakness and weight loss are the most common symptoms. Complications occur in 10-30% of patients, with skeletal infection being the most common (Araj, 1999, Reguera *et al.*, 2003). Three distinct forms of osteoarticular disease are reported in patients with brucellosis: peripheral arthritis, sacroiliitis, and spondylitis (Pappas *et al.*, 2005). Of the osteoarticular complications, spondylitis is the most difficult to treat and can be associated with neurological complications. The reproductive system is the second most common site of focal complication with epididymoorchitis and spontaneous abortion reported. Endocarditis is the primary cause of mortality from *Brucella* infection, although cardiac complications are observed in only 2% of patients. The aortic valve is most commonly involved and surgical valve replacement is typically required in addition to an extended course of antibiotic therapy (Reguera *et al.*, 2003).
Table 1.3. Most common clinical signs and complications associated with brucellosis in humans.

<table>
<thead>
<tr>
<th>Clinical Presentation of Human Brucellosis</th>
<th>Percent of Cases</th>
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<tbody>
<tr>
<td>Signs and Symptoms</td>
<td></td>
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<tr>
<td>Fever or chills</td>
<td>53-100</td>
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<tr>
<td>Joint Pain</td>
<td>20-87</td>
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<tr>
<td>Sweating</td>
<td>4-96</td>
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<tr>
<td>Constitutive Symptoms</td>
<td>1-97</td>
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<tr>
<td>Hepatomegaly</td>
<td>6-75</td>
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<tr>
<td>Splenomegaly</td>
<td>6-51</td>
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<tr>
<td>Focal Complications</td>
<td></td>
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<tr>
<td>Osteoarticular</td>
<td>25-44</td>
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<tr>
<td>Genitourinary</td>
<td>10</td>
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<tr>
<td>Neurological</td>
<td>3-8</td>
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<tr>
<td>Cardiac</td>
<td>2</td>
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<tr>
<td>* &lt; 5% mortality rate*</td>
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While *B. melitensis* and *B. suis* are commonly described in the literature as causing more severe disease in humans than *B. abortus*, few studies have directly compared disease manifestations between species. A retrospective report of patients with *B. melitensis* and *B. abortus* infection in California, USA indicates that there are significant differences in presenting signs, degree of focal complication, and laboratory abnormalities of patients infected with these two species of *Brucella* (Troy et al., 2005). Patients infected with *B. melitensis* presented with a more “toxic disease” characterized by fever, abdominal tenderness, hepatosplenomegaly, thrombocytopenia, and liver enzyme abnormalities. In comparison, patients with *B. abortus* infection presented primarily with focal complications including osteomyelitis, arthritis, or complications with pregnancy.

Relapses occur in about 10% of brucellosis patients, generally within a year of initial infection, and are often due to inadequate duration of antibiotic treatment (Pappas et al., 2005). Relapse of disease 28 years after initial presentation has been reported, however (Ogredici et al., 2010). There is recent evidence that brucellae persist intracellularly years after diagnosis of brucellosis, completion of antibiotic treatment regiments, and a successful clinical cure. *Brucella* DNA has been detected in the blood of subjects with a previous history of *Brucella* infection that are currently asymptomatic as well as subjects with either chronic focal disease or complaints of chronic vague non-focal illness (Vrioni et al., 2008, Castano & Solera, 2009). Chronic persistence of bacteria after antibiotic treatment and clinical cure has been reported in patients with other infectious diseases such as tuberculosis (Young et al., 2002). Although further studies are needed, these preliminary findings suggest that while
a clinical cure is typically achieved after antibiotic treatment, the complete eradication of brucellae from the body may be unattainable in many cases.

1.6) Pathogenesis

1.6a) Limitations of the Mouse Model

The pathogenesis of brucellosis infection is an active area of research. Many recent reviews describe *Brucella* virulence factors, mechanisms of cellular entry and survival, and trafficking within the host (de Figueiredo et al., 2015, Gorvel, 2008, de Jong et al., 2010, Atluri et al., 2011, Jimenez de Bagues et al., 2005). Information on this topic must be carefully interpreted, however, as most findings originate from *in vitro* experiments or murine studies and are then extrapolated to human and livestock hosts. *Brucella* infection of mouse models is distinct from that of natural hosts; mice are highly resistant to brucellosis (Grillo et al., 2012). Balb/c mice are among the most susceptible strains and are most commonly used in brucellosis studies. The increased susceptibility of Balb/c mice is due to an imbalance in T helper cell type 1 (Th1) versus Th2 immunity (Watanabe et al., 2004). T lymphocytes of Balb/c mice preferentially produce Th2 cytokines, and as a result, macrophages in these mice have impaired bactericidal activity. Since Th1 immunity is a critical component of the immune response to *Brucella* infection in natural hosts, the Balb/c mouse is an imperfect model for the study of *Brucella* immunology. The guinea pig (or a natural host) is a better model for the study of immune response; however, these species are rarely utilized. Recent progress in the development of mouse models for *Brucella*-induced abortion and osteoarticular disease demonstrate improved utility of the murine model for the study of these aspects of *Brucella* pathogenesis (Magnani et al., 2013, Wang et al., 2014).

The limitations of the murine model for the study of *Brucella* immunology in part stimulated the present dissertation. Objectives of the studies later described in chapters 3 and 4 include gaining a better understanding of *B. melitensis* pathogenesis in the goat and the resulting cell-mediated immune response instigated by infection. The next sections of the literature review will outline what is currently known of *Brucella* pathogenesis and host immune response from recent studies in the mouse model and limited studies in ruminants and humans.
1.6b) *Brucella* Entry and Intracellular Trafficking

Infection begins with entry of sufficient numbers of brucellae into the host. Ingested bacteria likely enter the host either via the oral mucosa or the intestinal Peyer’s patch (Ackermann *et al.*, 1988, Rossetti *et al.*, 2013). Inhaled bacteria likewise enter via the nasal mucosa or the pulmonary epithelium. Translocation of the epithelium is an active process likely involving various receptor molecules, which remain poorly defined. Putative receptors include extracellular matrix proteins, sialic acid residue containing proteins, the cellular prion protein, syndecans, and integrins. Interestingly, an integrin binding sialoprotein found to be upregulated in the Peyer’s patch during *Brucella* invasion, is also highly expressed in bone matrix and trophoblast, potentially explaining *Brucella*’s preference for these host sites (Rossetti *et al.*, 2013). Transcriptional analysis of Peyer’s patch cells during *Brucella* infection has also suggested that brucellae interfere with mucosal barrier function. Although a paracellular route of mucosal entry has not been previously described, brucellae trigger downregulation of the tight junction pathway and trefoil factors that promote mucosal healing within the intestine (Rossetti *et al.*, 2013). Regardless of the specific mechanism, brucellae are able to rapidly cross the mucosal barrier with minimal activation of the host immune system. Toll-like receptor (TLR) signaling pathways are subverted indicating that brucellae reduce or hide the pathogen-associated molecular patterns (PAMPs) that typically trigger immune system activation. Furthermore, the downregulation of cytokine expression within the intestine shortly after infection suggests brucellae are able to actively manipulate the host’s immune response for its benefit (Rossetti *et al.*, 2013).

Once past the mucosal barrier, brucellae enter mucosal neutrophils, macrophages, and dendritic cells. While neutrophils typically play a vital role in the innate immune response to bacterial pathogens, brucellae stimulate only limited neutrophil activation and have the ability to resist their killing mechanisms. Recent evidence also indicates that *B. abortus* actively induces cell death of human neutrophils, further limiting the capacity of neutrophils to kill invading brucellae and stimulate an appropriate immune response. This is consistent with the neutropenia observed in some human patients (Barquero-Calvo *et al.*, 2015).

Macrophages and dendritic cells are among the preferred replicative niches of brucellae; however, less than 10% of phagocytized brucellae survive an initial adaption period (de Figueiredo *et al.*, 2015). This initial mortality may be due to bactericidal activity of the macrophage or simply the bacteria’s failure to adapt to an intracellular
niche characterized by nutrient limitation and low oxygen tension. Brucellae enter macrophages via lipid raft domains, and once internalized, reside within a *Brucella*-containing vacuole (BCV). Intracellular trafficking of the BCV has been recently reviewed by Celli (2015). Brucellae actively modify trafficking and maturation of the BCV to promote intracellular survival and replication. Following phagocytosis, early maturation of the BCV involves interaction with endosomes and transient fusion with lysosomes. The extent of association with lysosomes has been a subject of some debate (Celli, 2015). Lysosomal-induced acidification of the BCV, however, is a necessary signal for induction of *Brucella*’s primary virulence factor, the type IV secretion system (T4SS). Regulation of vacuolar trafficking and subsequent survival and replication of brucellae within macrophages is dependent on a functional T4SS (de Jong & Tsolis, 2012). Laboratory strains of brucellae with mutations in the T4SS are unable to ultimately exclude lysosomal markers and survive within the macrophage (Celli *et al.*, 2003).

The identity and function of effector molecules secreted across the bacterial envelope by the T4SS remain poorly defined. Activation of the T4SS system, however, leads to association of the BCV with the endoplasmic reticulum (ER). Localization of brucellae in the ER was first discovered by electron microscopy of the goat uterus following experimental infection (Anderson *et al.*, 1986a). At this point in the maturation process, the BCV becomes a favorable niche for *Brucella* survival and replication. Surviving brucellae recover expression of genes encoding for key metabolic processes and begin to express additional virulence genes, the products of which function to inhibit the host immune response. There is evidence that brucellae interfere with the secretory pathway of the ER (Celli, 2015). In theory this could be a means by which brucellae interfere with MHC Class I presentation or the secretion of pro-inflammatory cytokines.

The process by which brucellae complete their intracellular cycle and exit the cell is poorly described. Although brucellae remain almost entirely intracellular while in the host, they must spread from cell-to-cell. During the final stages of the vacuolar maturation process, the BCV loses ER markers and gains features consistent with an autophagosome. It is from these autophagocytic vacuoles that brucellae exit the cell.
1.6c) Dissemination and Localization of *Brucella* in Host Tissue

*Brucella* infection can be divided into three phases: an incubation phase, an acute phase, and a chronic phase (de Figueiredo *et al.*, 2015). The steps described above comprise the incubation phase during which clinical signs are not yet apparent. During this phase brucellae enter the host, translocate across a mucosal barrier, and invade mucosa-associated macrophages and dendritic cells. Surviving brucellae establish a replicative niche within the host phagocytes. At this point infection is localized to the site of mucosal barrier penetration. If these steps of the infection process are successful, the acute phase of disease begins during which brucellae disseminate in host tissues. A cell-associated bacteremia occurs, allowing brucellae to remain concealed from the host immune system within the macrophages and dendritic cells that they have corrupted. Dissemination occurs rapidly, at least in natural hosts. In calves in which *B. melitensis* was inoculated directly into ileal loops, bacteremia occurred within 30 min post-infection. After 12 hours both the mesenteric lymph nodes and liver contained detectable brucellae (Rosetti *et al.*, 2013).

The signals determining localization of brucellae within host tissues are poorly understood. Preference for intracellular residence within macrophages and dendritic cells leads to colonization of reticuloendothelial organs including the spleen, liver, and lymph nodes. Brucellae can also replicate in epithelial cells, endothelial cells, fibroblasts, and microglia. Signals for localization to these cell types and colonization of the skeletal, nervous, and cardiovascular system in humans, for example, remain unknown. In ruminants, preference for male and female reproductive organs has been attributed to the presence of erythritol, a four-carbon sugar used by brucellae (Keppie *et al.*, 1965, Petersen *et al.*, 2013). High levels of erythritol are present in the placenta and male genitalia of natural hosts (cattle, sheep, goats, and swine), but are absent in other species which do not show a preponderance of reproductive lesions (humans, rats, rabbits, and guinea pigs) (Keppie *et al.*, 1965). Further support for the effect of erythritol on *Brucella* localization is provided by a recent study in which brucellae were found to localize to an erythritol-containing gel injected into the back of experimentally infected mice (Petersen *et al.*, 2013). Novel roles for erythritol have also recently been elucidated. In addition to its role as an energy source, erythritol functions to upregulate expression of certain virulence traits including *Brucella*’s T4SS. Erythritol also appears to encourage extracellular growth of brucellae. While brucellae primarily maintain an intracellular lifestyle, high numbers of extracellular bacteria are present in the placentas of goats and cows infected with *B. abortus* (Carvalho Neta *et al.*, 2013).
2008, Meador et al., 1989). Taken together these results indicate that erythritol functions to induce localization of brucllæ to the pregnant uterus, inhibit intracellular growth within trophoblast cells, and stimulate massive extracellular replication. The presence of high numbers of extracellular bacteria induces placentitis and eventual abortion. Whether the induction of virulence factors by erythritol serves to promote bacterial replication and local inflammation or to prime the bacteria for transmission to a new host remains to be elucidated.

1.6d) Pathobiology

The mechanism by which Brucella causes abortion and why abortion typically occurs only during the first parturition post-infection are poorly understood. Brucellæ preferentially replicate within trophoblast cells, metabolically active cells of the placenta that play a vital role in providing nutrients to the fetus as well as physically interacting with the endometrium. The placentitis that develops secondary to Brucella colonization likely hinders the delivery of nutrients to the developing fetus, thereby contributing to fetal stress and death. Recent microarray data indicate that brucellæ may also actively inhibit host production of certain chemokines and other gene products that play a role in fetal development and protection against hypoxia (Mol et al., 2014). In addition, a Brucella-induced hormonal shift may contribute to abortion. Brucellæ cause a decrease in progesterone production and an increase in estrogen levels. This shift triggers the endometrium to produce prostaglandins resulting in premature delivery (Poester et al., 2006).

The mammary gland and associated lymph nodes are also important sites for Brucella persistence and transmission in ruminant hosts. Macrophages transport brucellæ to the mammary glands where the organism replicates in alveoli and ducts (Meador et al., 1989). The uterus becomes a less favorable niche for brucellæ after parturition. With the expulsion of the placenta, the erythritol producing trophoblast is no longer present, and brucellæ preferentially reside in the mammary gland and associated lymph nodes between pregnancies. Following subsequent parturitions, replication is again stimulated and brucellæ are shed in the milk.

In human hosts, the pathogenic mechanisms underlying focal complications of brucellosis infection including skeletal, cardiovascular, and neurological disease are poorly understood. Neurologic disease has only been documented in humans and cetaceans with brucellosis and cannot be studied in a mouse model. Recent
discovery of osteoarticular infection and skeletal pathology in Balb/c mice infected with \textit{B. melitensis} will aid in the elucidation of the pathogenesis of disease in humans (Magnani \textit{et al.}, 2013). Experimental infection studies of human osteoblastic cell lines have also been performed (Delpino \textit{et al.}, 2009). \textit{Brucella abortus}, \textit{B. melitensis}, and \textit{B. suis} are able to replicate within human osteoblast cells, while wild type \textit{B. canis} and a mutant \textit{B. abortus} strain deficient in the T4SS are unable to survive within these cells. The \textit{Brucella}-infected osteoblasts secrete pro-inflammatory cytokines and the presence of monocytes has a synergistic effect on this immune response. The results suggest that the chronic inflammation and bone and joint destruction that characterize osteoarticular brucellosis may be primarily a result of the inflammatory reaction elicited by brucellae. Inflammatory mediators are also likely the cause of CNS damage in patients with neurobrucellosis. \textit{Brucella abortus} can infect microglia and astrocytes \textit{in vitro} eliciting the production of pro-inflammatory cytokines and matrix metalloproteinases. Metalloproteinases can degrade CNS tissue matrix and directly damage neurons. There is also \textit{in vivo} evidence for their role in \textit{Brucella} pathobiology; matrix metalloproteinases have been detected in the cerebrospinal fluid of patients with neurobrucellosis (Miraglia \textit{et al.}, 2013).

1.6e) Virulence Factors

The virulence of \textit{Brucella} species depends on their ability to persist within host cells. Since natural hosts of \textit{Brucella} almost exclusively shed bacteria in reproductive secretions, brucellae must persist long enough in the host for sexual contact or birth to occur. This allows for transmission to subsequent hosts. Cattle, goats, sheep, and swine, breed only once or twice a year, requiring brucellae to remain hidden from the host immune system for a substantial length of time. Establishment of persistent infection is dependent on the initial interactions between \textit{Brucella} and the host. Brucellae do not to cause overt toxicity, but instead stealthily invade. As a result, brucellae lack classical bacterial virulence factors including exotoxins, endotoxic LPS, cytolysins, a capsule, functional flagella, fimbriae, plasmids, and inducers of apoptosis (Seleem \textit{et al.}, 2008). Instead, \textit{Brucella} virulence factors function in three ways: to hide brucellae from immune detection, to protect from any brucellacidal mechanisms employed by the host, and to disrupt the host immune response.

Before brucellae even cross host mucosal barriers, in many cases they must survive transit through the stomach. The zoonotic \textit{Brucella} species produce urease, a virulence factor that facilitates the degradation of urea.
One product of this reaction is ammonia, which functions to buffer pH and facilitate survival in the acidic environment of the stomach. Interestingly, *B. ovis* does not express urease, potentially explaining why the primary route of infection for this species is venereal rather than oral (Tsolis *et al.*, 2009).

Perhaps the best characterized *Brucella* virulence factor is the T4SS encoded by the *virB* operon. Interestingly, related α-Proteobacteria, which tend to establish chronic infections including *Bartonella, Rickettsia, Wolbachia, Ehrlichia,* and *Anaplasma,* possess orthologous T4SS (Barquero-Calvo *et al.*, 2009). Type IV secretion in the context of brucellosis has been recently reviewed by de Jong and Tsolis (2012). The *virB* genes are conserved among all species of *Brucella* underscoring their importance for the genus. Only a small number of T4SS effectors have been discovered thus far and their functions in pathogenesis only hypothesized. However, mutant strains lacking a functional T4SS are attenuated *in vitro* in macrophages and *in vivo* in mice and goats. Studies have shown the T4SS to play critical roles in intracellular trafficking and replication of brucellae within macrophages in the time period shortly after infection (Celli *et al.*, 2003). The T4SS has also recently been shown to play a vital part in the early stages of placental colonization in cattle (Mol *et al.*, 2014). While intense, acute inflammation resulting in abortion is the ultimate outcome of *Brucella* localization in the pregnant uterus, an anti-inflammatory environment within the placenta is necessary for the initial establishment of infection (Carvalho Neta *et al.*, 2008). *VirB* mutants are unable to subvert the host inflammatory response in the uterus, suggesting that T4SS effectors not only function in intracellular trafficking of brucellae but also in immune evasion. A recent study of *B. microti* has demonstrated that the T4SS is also vital to this non-classical *Brucella* species (Hanna *et al.*, 2011). *Brucella microti* is unique among brucellae in that it causes high mortality in laboratory mice. This lethality is not demonstrated by *virB* mutants.

Other virulence factors of *Brucella* species are BtpA/TcpB and BtpB (Atluri *et al.*, 2011). These proteins contain Toll/interleukin-1 receptor (TIR) domains and actively inhibit TLR stimulation by degrading an adaptor molecule in the signaling cascade. One outcome is the inhibition of dendritic cell activation. Activated dendritic cells play key roles in bridging innate and adaptive immunity by secreting cytokines and presenting MHC-bound microbial antigens to T lymphocytes. Thus, these virulence factors represent yet another means by which *Brucella* actively interferes with the host immune system. At least BtpB may be a T4SS effector as its translocation into
macrophages has been shown to be dependent on virB. BtpB has also been shown to play an essential role in immune system evasion during brucellae colonization of the bovine trophoblast, indicating that its effects extend beyond macrophages and dendritic cells (Mol et al., 2014).

Another essential virulence factor is LPS, and it serves all three necessary functions for brucellae: hiding the organism, protecting from bactericidal mechanisms, and disrupting the immune response (Jimenez de Bagues et al., 2005). Unlike the LPS of Salmonella and Escherichia coli, Brucella LPS is poorly endotoxic allowing evasion of innate immunity. Similar to other related α-Proteobacteria, brucellae possess LPS with a non-canonical lipid A characterized by very long chain fatty acids (Barquero-Calvo et al., 2009). This LPS structure results in only meager stimulation of TLR4 and weak binding of complement. The result is minimal release of pro-inflammatory cytokines, minimal respiratory burst activity, and limited opsonization of bacteria. The O-side-chain of Brucella LPS protects smooth bacteria from both extracellular and intracellular bactericidal components. Brucellae are resistant to complement, cationic peptides, and neutrophil extracts (Barquero-Calvo et al., 2007). LPS may also play a role in inhibition of apoptosis, induction of IL-10 production, and interference with MHC class II antigen presentation (Franco et al., 2007, Skendros et al., 2011, Forestier et al., 2000).

Numerous other putative virulence factors have been described that are beyond the scope of this review. Among these are the BvrS/BvrR two component regulatory system, cyclic β-1,2-glucans, phosphatidylcholine, and unknown inhibitors of apoptosis (Seleem et al., 2008).

1.7) Host Immune Response

Much of our knowledge of Brucella immunology is derived from study of murine infection, but mice are not an ideal model since they are naturally resistant to infection with most species of Brucella. Only B. microti, B. neotomae, and select biovars of B. suis are known to naturally infect rodents. Murine studies can provide valuable information on the components of an immune response necessary for the development of protective immunity in the host. However, in order to characterize the defects in host immune response induced by brucellae, human and ruminant studies are necessary. Few studies in natural hosts have been performed. Data come from study of the immune response in human patients, vaccination studies in ruminant hosts, or in vitro studies of human and rarely
ruminant macrophage cell lines. The overarching premise derived from this work is that a Th1 response is necessary for control of Brucella infection, while up-regulation of IL-10 and development of a Th2 response results in prolonged infection (Baldwin & Parent, 2002). Human infection is characterized by an impairment of Th1 immunity, a defect that likely begins early during infection (Jimenez de Bagues et al., 2005, Dornand et al., 2002).

1.7a) Innate Immunity

The innate immune system is the first line of defense against invading pathogens. It plays a crucial role in both inhibiting the initial replication of bacteria and influencing the development of a protective adaptive immune response. Components of innate immunity include: anatomical barriers, secreted proteins (chemokines, cytokines, and complement), phagocytes (neutrophils, monocytes/macrophages, and dendritic cells), and innate types of lymphocytes (natural killer [NK] cells and gamma-delta [γδ] T cells).

i) Brucella avoids strong activation of the innate immune system via limited stimulation of TLRs

Detection of pathogens by the cellular components of the innate response occurs in a non-specific manner by recognition of microbial structures such as lipoproteins, LPS, flagellin, and DNA by TLR2, TLR4, TLR5, and TLR9, respectively. TLR signaling serves to activate bactericidal mechanisms of phagocytes, stimulate cytokine release, and enhance antigen-presenting properties of dendritic cells so that the adaptive immune system can be primed. Brucella LPS is only a weak stimulator of TLR4 due to modification of the lipid A moiety in the LPS molecule and active interference with TLR signaling via the BtpA/TcpB and BtpB proteins (Atluri et al., 2011). These proteins also interfere with TLR2 signaling but do not inhibit signaling by TLR9. Several studies have used TLR-deficient mice to elucidate the importance of TLR signaling to the outcome of Brucella infection. These experiments have suggested that TLR9 plays a central role in determining host resistance (Macedo et al., 2008, Oliveira et al., 2008). TLR9 is expressed intracellularly within endosomal membranes and serves to detect bacterial DNA rich in CpG motifs. However, Brucella DNA is only detectable by TLR9 if the bacterial envelope is breached. TLR9 signaling in murine macrophages and dendritic cells results in IL-12 production, which is crucial for stimulation of an adequate Th1 adaptive immune response and control of Brucella infection. Signaling through TLR9, as well as other TLRs, is predominately via the adaptor MyD88 following TLR-binding of Brucella products. MyD88 knockout mice infected with Brucella demonstrate impaired dendritic cell maturation, absence of IL-12 and
TNF-α production by macrophages and dendritic cells, reduced levels of inflammatory chemokines, reduced nitric oxide, reduced numbers of IFN-γ producing T lymphocytes, and more severe disease (Macedo et al., 2008, Oliveira et al., 2008). The role of other TLRs including TLR2, TLR4, and TLR5 seems to be minor although this is subject to some debate. The discrepancies in study results are likely due to different cell types utilized, the various Brucella species or strains studied, the mouse strains chosen, and whether gene polymorphisms in human or ruminant hosts are investigated (Grillo et al., 2012, Macedo et al., 2008, Prakash et al., 2014, Rezazadeh et al., 2006).

ii) Brucella induces minimal production of pro-inflammatory cytokines

Cytokine expression is necessary for development of a protective immune response. TNF-α, IL-12, and IFN-γ are the primary cytokines critical for defense against Brucella. Additionally, IL-1, IL-6, and IL-8 are common components of the general cytokine response to gram negative bacteria. Brucella abortus-infected mice produce minimal IL-1β, IL-6, and TNF-α in comparison to Salmonella-infected mice (Barquero-Calvo et al., 2007, Barquero-Calvo et al., 2009). In mice this effect does not appear to be due to active inhibitory mechanisms, but instead is likely related to the poor induction of TLR signaling by Brucella. While levels of pro-inflammatory cytokines induced by Brucella infection in mice are more than 10-fold lower than that induced by Salmonella, IL-1, IL-6, and TNF-α are all still present in murine brucellosis models. In human macrophages, however, TNF-α production is severely impaired (Dornand et al., 2002). Since heat-killed brucellae are able to stimulate TNF-α secretion, it appears that inhibition of TNF-α expression in humans is an active process by live brucellae. Similar evidence of TNF-α inhibition is emerging in cattle. In a calf ligated ileal loop model, expression of TNF-α and IL-12 by bovine Peyer’s patch cells remained unchanged during invasion of B. melitensis (Rossetti et al., 2013). In early stages of placental trophoblast colonization in cattle, B. abortus also actively inhibits expression of pro-inflammatory genes including members of the TNF superfamily (Carvalho Neta et al., 2008). The putative inhibitor of TNF-α expression is a Brucella outer membrane protein, Omp25 (Jubier-Maurin et al., 2001).

Via both autocrine and paracrine signaling TNF-α functions to stimulate macrophage functions. The importance of TNF-α is shown by: exacerbated Brucella infection in mice deficient in TNF-α receptor genes, TNF-α stimulation of IL-12 and IFN-γ production in mice, control of Brucella replication via synergistic activity of TNF-α and CD8+ T lymphocytes in IFN-γ knockout mice, and inhibition of the activity of IFN-γ stimulated murine
macrophages by addition of anti-TNF-α antibodies (Dornand et al., 2002). A recent study also provides evidence for the importance of TNF-α and IL-12 in cattle. In macrophages from Zebu cattle, which are better able to control *B. abortus* replication *in vitro* than macrophages from European cattle, TNF-α and IL-12 expression is stimulated by infection. In macrophages from the more susceptible breed, *B. abortus* appears to inhibit TNF-α and IL-12 expression below basal levels (Macedo et al., 2013).

Although the literature emphasizes the importance of IFN-γ in the control of *Brucella* infection (Baldwin & Parent, 2002, Grillo et al., 2012), this cytokine is produced primarily during the adaptive immune response by CD4⁺ and CD8⁺ T lymphocytes. Natural killer cells and γδ T cells could produce some IFN-γ during the innate response, but TNF-α production would precede even this activity of innate-like lymphocytes. TNF-α production occurs just hours after invasion and shapes the future outcome of bacterial infection. Thus, although IFN-γ plays a crucial role in *Brucella* immunology, TNF-α functions immediately after infection, activating macrophages and inducing future production of IFN-γ.

**iii) Brucella resists and evades the action of neutrophils**

Neutrophils are among the first cells to respond to invasion by gram negative bacteria. Murine, human, and bovine neutrophils efficiently take up *Brucella*, internalizing far more bacteria than macrophages (Ackermann et al., 1988, Celli et al., 2003, Barquero-Calvo et al., 2007). However, *Brucella* infection of neutrophils stimulates only minimal degranulation and respiratory burst activity compared to *Salmonella* infection. As a result, bacterial survival rates for brucellae in human neutrophils are approximately 70%, compared to less than 25% for *Salmonella* (Barquero-Calvo et al., 2007). Murine studies have shown that neutrophils play a negligible role in *Brucella* infection since depletion of neutrophils by treatment with a monoclonal antibody does not influence bacterial load in the spleen (Barquero-Calvo et al., 2007).

Gram negative bacterial infection is typically characterized by marked neutrophilia. Classical gram negative bacteria recruit neutrophils to the site of infection and induce their activation, which serves to increase bactericidal activity and prolong neutrophil lifespan. In contrast, *B. abortus* infection of mice does not result in blood neutrophilia or recruitment of neutrophils to the site of infection (Barquero-Calvo et al., 2007). In humans, as
well, normal or decreased leukocyte counts are most often associated with brucellosis (Troy et al., 2005). The failure of neutrophil recruitment to the site of Brucella invasion may be a function of impaired TLR signaling and insufficient chemokine production, as well as insufficient activation of the complement cascade. The neutropenia observed also is likely due to Brucella-induced neutrophil death. In a recent study, B. abortus was shown to selectively kill human neutrophils (Barquero-Calvo et al., 2015). Death was induced by intracellular release of LPS by both live and dead Brucella, and was specific to neutrophils, as monocytes, macrophages, dendritic cells, and lymphocytes remained unaffected. The lipid A moiety of Brucella LPS appears to induce neutrophil death via a reactive oxygen species mediated mechanism, and cell death occurred silently without the release of inflammatory mediators. By inducing neutrophil death, brucellae may be better able to reach their preferred replicative niche. Neutrophil fragments containing brucellae are removed by macrophages and dendritic cells.

iv) Brucella resists macrophage killing

Macrophages are a double-edged sword in Brucella pathogenesis. While functioning as the primary line of defense against Brucella infection, these cells also provide a favorable intracellular niche for brucellae. Adequate stimulation via TLR binding and other mechanisms help to sway the outcome of infection in favor of the host. Brucellae, however, have several stealth mechanisms to both avoid and actively interfere with the proper functioning of macrophages as alluded to in the previous discussion of virulence factors. The outcome of this battle between bacterium and macrophage is predictive of the outcome of infection for the host. In cattle naturally resistant to Brucella infection, B. abortus shows poor survival within macrophages. However, in cattle susceptible to infection, abortion, and shedding of Brucella, macrophages are unable to kill B. abortus (Qureshi et al., 1996).

Macrophages employ a number of strategies to directly kill pathogens including: phagocytosis and autophagy with subsequent degradation by hydrolytic lysosomal enzymes, oxidative burst with subsequent killing by reactive oxygen and nitrogen species, and antimicrobial peptides with various mechanisms of action. Macrophages also function to stimulate and shape the adaptive immune response via cytokine production and antigen presentation. The efficacy of these direct and indirect mechanisms against Brucella depends on the host species, the Brucella species, and a multitude of factors specific to the individual infected. These factors ultimately determine the outcome of infection. In general phagocytosis and autophagy are ineffective means of killing B.
abortus, B. melitensis, and B. suis. While the majority of internalized bacteria may be killed, the surviving members of these species are able to ultimately exclude lysosomal components from the BCV and establish a replicative niche (de Figueiredo et al., 2015). There is also evidence that brucellae actually utilize the autophagy pathway to their benefit in order to spread from cell to cell (Celli, 2015). Brucellae are also highly resistant to cationic peptides (Barquero-Calvo et al., 2007). The role nitric oxide (NO) plays in controlling Brucella replication is debated. While NO production is one of the primary bactericidal mechanisms employed by macrophages, Brucella stimulates bovine macrophages to produce only low levels of NO compared to Salmonella (Macedo et al., 2013). Macrophages from some breeds of cattle, however, are better able to control Brucella infection. These resistant bovine macrophages exhibit higher levels of inducible NO synthase expression and NO production (Macedo et al., 2013). The bactericidal activity of macrophages is enhanced following activation. LPS, TNF-α, and IFN-γ all function to activate macrophages. Interestingly, a study of B. abortus infection in murine macrophages found that pre-activation of macrophages prior to infection resulted in the death of 80% more brucellae compared to untreated macrophages. However, if macrophages were activated after brucellae had already established a replicative niche, the brucellae were resistant to the enhanced bactericidal mechanisms (Barquero-Calvo et al., 2007). This has important implications since Brucella infection of natural hosts results in limited TLR stimulation via LPS and limited TNF-α production. If macrophage activation is dependent on IFN-γ production, this occurs later during infection likely after brucellae have established a replicative niche. A last ditch effort employed by macrophages unable to control intracellular bacterial replication is induction of apoptosis. Brucella inhibits this bactericidal mechanism as well, facilitating the establishment of chronic infection. In murine macrophages, B. abortus prevents apoptosis by a mechanism independent of TLR signaling (Barquero-Calvo et al., 2007). In human macrophages, B. suis can protect against Fas-ligand, IFN-γ, and TNF-α induced apoptosis. The proposed mechanism is via induction of A1, an anti-apoptotic gene from the Bcl-2 family (Gross et al., 2000). Inhibition of apoptosis has also been demonstrated ex vivo in macrophages from infected cattle and from humans with both acute and chronic brucellosis (Galdiero et al., 2000, Tolomeo et al., 2003). In humans, treatment with antibiotics reverses the inhibition of macrophage apoptosis in patients with acute but not chronic brucellosis.
v) *Brucella* interferes with dendritic cell mediated induction of adaptive immunity

Much of the work in *Brucella* immunology has focused on the macrophage; however, dendritic cells are the primary cell type involved in presentation of antigen to naïve T lymphocytes. As such dendritic cells are a bridge between innate and adaptive immunity and are essential in shaping a protective Th1 response. Dendritic cells seem to be highly permissive to *Brucella* replication. A study of human dendritic cells subjected to *in vitro* infection with *B. abortus*, *B. suis*, and *B. melitensis* found that regardless of the species, *Brucella* are actually better able to invade and persist in dendritic cells than in macrophages (Billard et al., 2005). This is striking as most other intracellular bacteria that target macrophages are unable to replicate within dendritic cells. The ability to persist in dendritic cells may represent a specific virulence strategy of *Brucella*, allowing for dissemination throughout the host within these highly-migratory cells. While persistence of *Brucella* in murine dendritic cells has also been demonstrated (Macedo et al., 2008), evidence for survival in bovine dendritic cells is lacking. The one study performed with bovine monocyte-derived dendritic cells found that *B. abortus* was eliminated by 24 hours post-infection (Heller et al., 2012). This result, however, is likely due to the utilization of cells from cattle vaccinated with RB51 and high levels of B- and T- lymphocyte contamination in the dendritic cell cultures.

Once established within murine or human dendritic cells, *Brucella* interferes with cell maturation, production of TNF-α and IL-12, and presentation of antigen to T lymphocytes (Billard et al., 2007, Macedo et al., 2008, Barquero-Calvo et al., 2009). In an *in vitro* dendritic cell infection study, expression of cell membrane components involved in antigen presentation and co-stimulation (including MHC I and MHC II and CD40, CD80, and CD86) was minimal after *Brucella* infection compared to *E. coli* infection. As a result, *Brucella*-infected dendritic cells were unable to induce proliferation of naïve T lymphocytes in co-culture. The inhibition of dendritic cell maturation appears to be at least partially due to *Brucella*’s ability to prevent TNF-α production in human dendritic cells (Jubier-Maurin et al., 2001, Billard et al., 2007). IL-12 production by *Brucella*-infected dendritic cells is also low, and this cytokine is required for development of a protective Th1 adaptive immune response. In Balb/c mice, which have increased sensitivity to *Brucella* infection, there is a temporary reduction in IL-12 receptor expression during the chronic phase of infection (Grillo et al., 2012). The result is a brief hiatus in Th1 immunity and IFN-γ production. Treatment with recombinant IL-12 resolves this suspension of Th1 immunity with a subsequent 1000-fold reduction in *Brucella* concentration (Sathiyaseelan et al., 2006).
vi) Innate-like lymphocytes play a critical role in controlling infection

Innate-like lymphocytes, including γδ T cells and NK cells, function at the interface of innate and adaptive immunity. These lymphocytes are characterized by the absence of αβ T cell receptors (TCR), which bind antigen in CD4⁺ and CD8⁺ T lymphocytes. Instead innate-like lymphocytes recognize PAMPs, signals of cellular stress, and in the case of γδ T cells potentially antigen presented by dendritic cells (Guzman et al., 2012). The innate-like lymphocytes play critical roles in the control of intracellular bacterial infection since they are able to produce IFN-γ before the adaptive immune response develops. While humans and mice have low levels of circulating γδ T cells (1-5% of total lymphocytes), in ruminants γδ T cells are a major T lymphocyte subtype (Skyberg et al., 2011). Up to 60% of circulating T lymphocytes in calves are γδ T cells (Guzman et al., 2012, Hein & Mackay, 1991).

The importance of γδ T cells in Brucella immunology is only just beginning to be understood. Bovine γδ T cells have been shown to inhibit the in vitro replication of B. abortus within macrophages in co-culture experiments (Skyberg et al., 2011). This protective effect appears to be mediated by IFN-γ produced by γδ T cells, which serves to enhance the bactericidal activity of macrophages. In humans with acute B. melitensis infection, numbers of γδ T cells in the peripheral blood increase dramatically, reaching 30% of the total lymphocyte population in some patients (Bertotto et al., 1993). Human γδ T cells have been shown to inhibit growth of Brucella within macrophages by contact-dependent (granule- and Fas ligand-mediated cytotoxicity) and contact-independent (IFN-γ-mediated macrophage activation) mechanisms (Skendros et al., 2011, Dornand et al., 2002). The cytotoxic action of γδ T cells could circumvent Brucella’s inhibition of macrophage apoptosis. While γδ T cells are also cytotoxic toward Brucella-infected human dendritic cells, the interaction between γδ T cells and dendritic cells serves additional functions essential for Brucella immunity. As discussed above, brucellae inhibit dendritic cell maturation interfering with antigen presentation to T lymphocytes. Human γδ T cells have recently been shown to restore this function (Ni et al., 2012). Through contact-dependent mechanisms, Brucella-infected dendritic cells activate γδ T cells. These activated γδ T cells then function to induce differentiation of the infected dendritic cells into fully mature, IL-12 producing antigen presenting cells with enhanced capacity to promote proliferation of naïve CD4⁺ T lymphocytes.
The contribution of NK cells to control of *Brucella* infection appears to be host specific. In mice experimental depletion of NK cells does not influence the outcome of infection (Baldwin & Parent, 2002). However, in humans NK cells, like γδ T cells, appear to contribute to protection against *Brucella* via cytotoxic activity toward *Brucella*-infected macrophages. Natural killer cell production of TNF-α and IFN-γ may serve additional roles in *Brucella* immunity (Dornand *et al.*, 2004). In patients with acute brucellosis, normal numbers of NK cells are present; however, they show a functional deficit in cytotoxicity (Salmeron *et al.*, 1992). There is evidence that the deficit in cytotoxicity is due to impairment of NK cell maturation. This may be a direct inhibitory effect or may be due to *Brucella*’s inhibition of TNF-α and IL-12 production by macrophages and dendritic cells, as both cytokines serve to activate NK cells. The pathogenic implications for this deficit in NK cell function are unknown but may contribute to intracellular survival of brucellae in human hosts.

1.7b) Adaptive Immunity

The innate immune system serves to limit *Brucella* replication and stimulate an adaptive immune response, which is more effective at clearing infection. Adaptive immunity is defined by antigen-specific recognition of pathogens by T- and B- lymphocytes and secreted antibody. The adaptive immune response attempts to control *Brucella* infection by three primary mechanisms. First, IFN-γ produced by CD4⁺ and CD8⁺ T cells serves to activate macrophages, enhancing their bactericidal capacity. Second, cytotoxic CD8⁺ T cells directly kill infected macrophages. Third, B lymphocytes secrete antibody, which has limited effect on the outcome of infection but is useful for diagnosis of disease (Ko & Splitter, 2003, Skendros & Boura, 2013).

i) Th1 immunity defined by IFN-γ production is critical to defense against *Brucella* infection

Th1 cells are a subset of CD4⁺ T lymphocytes that produce the cytokines IFN-γ and IL-2 and are primarily involved in activating macrophages and CD8⁺ T cells. The major competing immune response is the Th2 response which is characterized by CD4⁺ T lymphocytes that produce the cytokines IL-4, IL-5, IL-10, and IL-13 and function to stimulate B cells to produce antibody. The importance of the Th1 immune response in *Brucella* infection was first realized in the mouse model and has since been observed in humans and natural hosts. IFN-γ is the principal cytokine secreted from *in vitro* cultures of splenocytes, T cells, and peripheral blood mononuclear cells (PBMCs) of mice, humans, and cattle stimulated with *Brucella* antigen (Fernandes *et al.*, 1996, Weynants *et al.*, 1998, Rafiei *et
A deficit in IFN-γ production results in decreased clearance of *Brucella* and prolonged infection. This effect is observed naturally in susceptible mouse strains and in human patients with chronic disease (Fernandes et al., 1996, Baldwin & Parent, 2002, Rafiei et al., 2006, Skendros et al., 2011). A deficit in IFN-γ production can also be reproduced in mouse models by using anti-IFN-γ antibodies or knockout strains. Neutralization of IFN-γ in mice infected with *B. abortus* results in increased bacterial colonization of the spleen (Fernandes & Baldwin, 1995). Deficits in IFN-γ production and the Th1 response in whole are also observed in some ruminant species following vaccination with *B. abortus* strain RB51, and this is believed to explain the poor efficacy of the vaccine in elk and water buffalo (Olsen et al., 2002, Diptee et al., 2005).

Deficits in IFN-γ production likely result from defects in other components of the host immune response. Naïve lymphocyte requires three signals in order to differentiate into Th1 effector cells: binding of a foreign peptide-MHC complex to the TCR, binding of co-stimulatory molecules, and binding of cytokines, particularly IL-12. As mentioned in previous sections, a natural defect in IL-12 stimulation in the Balb/c mouse causes a deficit in IFN-γ production until 6 weeks post-infection. This explains the greater susceptibility of this mouse to *Brucella* infection compared to C57BL/6 mice, which produces high levels of IFN-γ throughout the course of infection (Fernandes et al., 1996, Baldwin & Parent, 2002, Sathiyaseelan et al., 2006). Inhibition of both IL-12 production and expression of MHC and co-stimulatory molecules has also been observed in vitro in human dendritic cells infected with *Brucella* (Billard et al., 2007). If this is replicated in vivo, a reduction in all three signals required for the activation of T lymphocytes would result in a substantial reduction in IFN-γ production.

Deficits in IFN-γ also frequently reflect an imbalance between Th1 and Th2 immunity. While both responses are necessary to avoid an overzealous inflammatory response, an abundance of Th2 cytokines is associated with a poor disease outcome. A Th2 response is induced via IL-4 stimulation of naïve T lymphocytes during interaction with an antigen presenting cell. The subsequent Th2 response elicited is characterized by production of IL-4, IL-5, IL-10, and IL-13. IL-10 production in the context of *Brucella* infection has been best studied. This immunoregulatory cytokine is produced by T lymphocytes including Th2 effector cells and regulatory T cells as well as B cells, neutrophils, macrophages, and some dendritic cells. IL-10 functions to inhibit the Th1 response at many steps by interfering with antigen presentation and T cell cytokine production (Sabat et al., 2010).
Both the pro-inflammatory Th1 cytokine IFN-γ and the anti-inflammatory cytokine IL-10 are produced by splenocytes in Brucella-infected mice (Fernandes & Baldwin, 1995). Experimental neutralization of IL-10 results in higher levels of IFN-γ, TNF-α, and IL-6 production and a reduction in Brucella replication; however, increased disease pathology is observed as a result of the high levels of pro-inflammatory cytokines (Fernandes & Baldwin, 1995, Xavier et al., 2013). A recent murine study indicates that B. abortus may induce regulatory T cells to produce IL-10 early in infection (Xavier et al., 2013). In the absence of this early IL-10 expression, Brucella is unable to escape the phagolysosome and establish a replicative niche within the macrophage. Thus, while IL-10 protects the host from substantial disease pathology, excess production of this anti-inflammatory cytokine may promote chronic infection.

Neutrophils may also inhibit the Th1 response. Initially believed to act exclusively during innate immunity, recent evidence suggests neutrophils may also influence the adaptive response. In a mutant neutropenic mouse strain infected with B. abortus, the absence of neutrophils actually favored bacterial elimination at later time points of infection (Barquero-Calvo et al., 2013). Clearance of brucellae in the neutropenic model seemed to be a function of a faster and more efficient adaptive immune response. There were increased numbers of activated lymphocytes present, increased recruitment of monocytes and dendritic cells to the spleen, and increased production of Th1 cytokines.

ii) Deficiencies in CD8+ T lymphocytes may contribute to Brucella persistence

The relative importance of CD4+ versus CD8+ T lymphocytes in the control of Brucella infection is debated; however, both populations likely play a role in immunity (Ko & Splitter, 2003, Skendros & Boura, 2013). CD8+ T cells function to inhibit Brucella replication within macrophages via both IFN-γ production and Fas- or perforin-mediated cytotoxicity. CD8+ T cell numbers seem to increase during certain stages of infection, likely to compensate for lapses in CD4+ T cell responses. This has been observed in Balb/c mice as well as in human patients with chronic brucellosis (Fernandes et al., 1996, Skendros et al., 2007). Although their numbers increase, CD8+ T may not be able to control infection (Vitry et al., 2012). A recent study suggests that B. melitensis actively inhibits the CD8+ T cell response via secretion of the virulence factor TcpB (Durward et al., 2012). This virulence factor was discussed in a previous section in reference to its effects on intracellular trafficking and innate immunity, but
has now been indicated in evasion of adaptive immunity as well. TcpB is released by *B. melitensis* within infected macrophages and functions to exclude phosphatidylinositols from the immune synapse formed during macrophage-CD8+ T cell binding. This prevents CD8+ T cell mediated killing, but also has effects beyond the immediate survival of *Brucella* within the contacted macrophage. The dampening of the resulting immune response appears to prevent the differentiation of CD8+ effector T cells into a long-lived memory pool. In a Balb/c mouse model, few CD8+ T cells retained a memory cell phenotype during chronic *Brucella* infection. The few persisting memory cells displayed an exhausted phenotype characterized by deficiencies in IFN-γ, TNF-α, and IL-2 production.

**iii) Limited role of B lymphocytes in *Brucella* infection**

Although the role of humoral immunity in *Brucella* infection has also been a subject of debate, the emerging consensus is that the primary humoral response is not protective (Vitry *et al.*, 2012, Skendros & Boura, 2013). An effective secondary immune response, such as that which occurs upon exposure post-vaccination or upon repetitive exposure in disease endemic areas, however, seems to require both humoral and cell-mediated responses (Vitry *et al.*, 2014). Antibodies can serve opposing roles in the control of intracellular bacterial infection. They can aid the host by opsonizing the pathogen during intermittent extracellular phases and induce complement activation or phagocytosis. However, antibody-mediated phagocytosis can also benefit the pathogen by providing easy access to intracellular niches (Vitry *et al.*, 2014). A recent study of *Brucella* infection in a B cell-deficient mouse strain suggests that the humoral response actually impedes the control of infection via an antibody-independent mechanism (Goenka *et al.*, 2011). In addition to producing antibody, B cells serve a regulatory role secreting the anti-inflammatory cytokines IL-10 and transforming growth factor (TGF)-β, which attenuate the Th1 response. B cell knockout mice produced higher levels of IFN-γ and exhibited disease resistance.

In the secondary immune response, circulating antibodies do appear to play a critical role (Vitry *et al.*, 2014). Early during infection antibodies limit dissemination of *Brucella* via the bloodstream. Together with a CD4+ Th1 memory response, they also serve to control subsequent splenic replication of *Brucella* in a mouse model. Absence of either a humoral or cell-mediated response leads to *B. melitensis* persistence in mice following Rev. 1 vaccination. The importance of the humoral response in natural hosts, however, remains questionable as antibody titers in cattle post vaccination often do not correlate with protective immunity (Nicoletti, 1990).
iv) Th1/Th2 imbalance and cellular anergy in human brucellosis

A collection of studies on patients with brucellosis are beginning to elucidate peculiarities of the human immune response that may predict disease outcome. Patients with acute brucellosis generally have a strong Th1 immune response characterized by elevated levels of IL-1, IL-2, IL-6, IL-12, TNF-α, and IFN-γ (Rodriguez-Zapata et al., 2010). IFN-γ concentrations in the serum of patients can be more than 100 times greater than that in healthy controls. Levels of anti-inflammatory cytokines including IL-4, IL-10 and IL-13 are typically low (Rodriguez-Zapata et al., 2010, Rafiei et al., 2006, Akbulut et al., 2005). Interestingly, monocytes isolated from blood of patients with acute brucellosis have defective phagocytic activity despite the strong Th1 response (Rodriguez-Zapata et al., 2010). In contrast to patients with acute brucellosis responsive to therapy, patients with either acute disease unresponsive to therapy or chronic disease display a switch to a Th2 response (Rafiei et al., 2006, Skendros & Boura, 2013, Akbulut et al., 2005). These patients have decreased levels of IFN-γ and increased levels of IL-13. Patients with chronic brucellosis also display deficits in the activation state of CD4⁺ T lymphocytes as measured by CD25 expression (Skendros et al., 2007). CD25 is the alpha chain of the IL-2 receptor, which is expressed on activated T cells as well as many other cell types. IL-2 stimulation is necessary for clonal expansion, and the apparent defect in IL-2 signaling in chronic brucellosis patients could contribute to the deficit in Th1 response.

v) Ruminant T cell response to Brucella infection and vaccination

The immune response against Brucella is poorly studied in cattle and small ruminant hosts. Most work has investigated the T cell proliferative response and the humoral response following vaccination. Lymph node cells from vaccinated cattle demonstrate a considerable proliferative response upon stimulation with Brucella antigen (Stevens et al., 1996, Stevens et al., 1995). This work, however, provides little information on the lymphocyte types that provide protective immunity. A recent study investigating the activity of T lymphocyte subsets following vaccination of cattle found proliferation of both CD4⁺ and CD8⁺ T cells (Dorneles et al., 2014). CD4⁺ T cells were the primary source of IFN-γ post vaccination. The cytokine response was characterized by a moderate but significant increase in the percent of T cells producing IFN-γ and no IL-4 production. Analysis of cytokine expression levels following RB51 vaccination of cattle has also demonstrated the presence of IFN-γ and absence of IL-4 expression (Polci et al., 2006). While most work has been done in vaccinated animals, a similar response has been noted in a single cow infected with virulent B. abortus (Weynants et al., 1998). Elevated levels of IFN-γ were
demonstrated in PBMC cultures from this cow post-infection by both enzyme-linked immunoassay (ELISA) and intracellular staining flow cytometry. Again the primary IFN-γ producers were CD4⁺ T cells, with a lesser contribution by CD8⁺ T cells. An in vitro assay of CD4⁺ T cell function indicates that lymphocytes isolated from vaccinated cattle are fully capable of inducing apoptosis in antigen-stimulated and Brucella-infected autologous macrophages (Wyckoff & Potts, 2007). Few studies have looked at the response of small ruminants to B. melitensis infection. Recently, Perez-Sancho et al. reported that the IFN-γ response to both Rev. 1 vaccination and B. melitensis challenge in sheep is similar to that shown in vaccinated cattle (2014).

1.8) Vaccination and Disease Control Methods

Vaccination remains at the cornerstone of disease prevention in livestock and human hosts (Yang et al., 2013). While there is currently no safe and efficacious vaccine for human use, prevalence of disease in humans is directly correlated with levels of disease in livestock. Thus, livestock vaccination is vital for preventing human disease. This is underscored by the difficulties associated with treatment of brucellosis in humans. Prevention of disease in livestock is also justified by economic motives. These include increasing productivity in disease endemic areas such as Africa and parts of Asia where livestock are an important source of subsistence, as well as eliminating the costs associated with trade and market restrictions that are placed on disease positive animals.

While the emphasis of this section is on vaccination, it must be noted that there are two other critical components to disease control: test and slaughter programs and implementation of management practices that reduce exposure to Brucella. Vaccination alone has never been effective at eradicating brucellosis from any livestock population (Olsen & Tatum, 2010).

The brucellosis vaccines currently in use are all live attenuated Brucella strains. While a nonliving brucellosis vaccine is desirable for reasons of safety, killed vaccines are typically ineffective at stimulating the Th1 immune response that is necessary for efficacy (Nicoletti, 1990). The attenuated Brucella strains utilized in live vaccines replicate and persist within the host for a limited period of time, stimulating development of a Th1 response and likely CD4⁺ T memory cells. The optimal attenuated vaccine strain must possess enough residual virulence to stimulate a protective response but not enough to cause disease or abortion (Yang et al., 2013).
The primary vaccines currently used for prevention of bovine brucellosis worldwide are *B. abortus* strain 19 (S19) and *B. abortus* strain RB51. S19 is a spontaneous mutant discovered in 1923 and first introduced for field use in the U.S. in 1941 (Graves, 1943, Olsen & Tatum, 2010). S19 is highly effective at preventing abortion and subsequent transmission of disease; however, it is less successful at preventing infection. Although vaccine efficacy is largely dependent on field conditions and exposure dose, S19 is estimated to protect 65-75% of vaccinated animals from infection and reduce the occurrence of abortion in the remaining animals (Manthei, 1968). The primary limitation of this vaccine is the occasional persistence of circulating antibodies post-vaccination. Since S19 is a smooth strain and expresses the O-side-chain on its LPS, the antibody response of vaccinated animals cannot be differentiated from that of infected animals by most serological tests. Thus S19 vaccination makes the implementation of a test and slaughter program difficult. Another drawback of the S19 vaccine is that it is sufficiently virulent to induce abortion when administered during pregnancy. Solutions to these two problems include restricting vaccination to heifer calves and utilizing a reduced dose of vaccine. These practices reduce abortion rates and the retention of antibody titers. Both the full and reduced dose calfhood vaccines are protective against brucellosis, the full dose offering protection until at least 9 years of age (Manthei, 1968). S19 may occasionally persist in the udder resulting in shedding, although it is reportedly only infectious for humans upon accidental inoculation and not upon ingestion (Nicoletti, 1990).

The development of the RB51 vaccine allowed many of the limitations presented by S19 to be overcome. In the U.S. RB51 has been used almost exclusively since 1996 (Olsen & Tatum, 2010). RB51 is a rough mutant derived from wild type *B. abortus* 2308, and since it lacks the O-side-chain, the antibody response induced by vaccination does not interfere with serological tests. This vaccine is also safer to use in pregnant animals; however, RB51 does retain some virulence and abortion and fetal infection has been noted in a vaccinated pregnant heifer (Olsen & Tatum, 2010, Van Metre *et al.*, 1999). This is a public health concern, as RB51 remains infectious for humans as evidenced by reported cases in a farmer, veterinarians, and veterinary students following cesarean section and necropsy of an infected stillborn calf in Kansas (1998). The retained virulence of RB51 is also of potential concern since this strain is resistant to rifampin (Schurig *et al.*, 1991), a highly effective antibiotic typically utilized in treatment regimens (Vrioni *et al.*, 2014). There is some controversy over the protection provided by RB51 in comparison to S19. One study reported RB51 to prevent 59% of abortions, 59% of cow infections, and 61% of fetal
infections (Poester et al., 2006). Simultaneous comparison of the vaccines in a small number of animals indicates that S19 may be slightly more efficacious (Olsen & Tatum, 2010). While the U.S., Europe, and some South American nations have adopted the use of RB51, S19 is still used in many countries worldwide for control of bovine brucellosis.

The primary vaccine used in small ruminants for protection against \textit{B. melitensis} is the Rev. 1 vaccine. This vaccine was created in 1957 by sequential passage of a wild type \textit{B. melitensis} strain in streptomycin-containing media until a streptomycin-resistant strain was isolated (Elberg & Faunce, 1957). This isolate, Rev. 1, was found to have reduced virulence and protect against infection in small ruminants. Similar to the other live attenuated \textit{Brucella} vaccines, vaccination of pregnant sheep and goats with Rev. 1 can result in abortion and shedding in vaginal secretions and milk in some animals (Alton et al., 1967, Entessar et al., 1967, Blasco, 1997). This presents a potential public health risk since, like S19 and RB51, Rev. 1 retains some virulence to humans (Blasco & Diaz, 1993). Again there are treatment challenges associated with infection of humans with Rev. 1 since the strain is streptomycin resistant, and this antibiotic is commonly used in treatment regiments (Vrioni et al., 2014). Applying a reduced dose of Rev. 1 via the conjunctival route decreases the risk of abortion and shedding, as well as persistence of antibody titers (Jimenez de Bagues et al., 1989). Like the \textit{B. abortus} S19 vaccine, Rev. 1 is a smooth strain with an O-side-chain on its LPS, which interferes with diagnosis on serological tests. Full-dose vaccination produces long lasting protection against abortion and infection. In goats Rev. 1 was found to provide protection for at least 4.5 years, preventing 100\% of abortions and 92\% of infections, as measured by colonization of mammary tissue or uterus. Non-vaccinated goats in this study had a 45\% abortion rate and only 16\% of control animals demonstrated a lack of tissue colonization (Alton, 1968). Use of this Rev. 1 has drastically reduced disease prevalence among small ruminants in many countries, although insufficient vaccine coverage or lapses in vaccination have resulted in re-emergence (Yang et al., 2013, Banai, 2002).

Other \textit{Brucella} vaccines have been developed and utilized in select countries. The \textit{B. suis} strain 2 vaccine is an attenuated \textit{B. suis} strain used in China for vaccination of small ruminants, cattle, and swine (Yang et al., 2013). The vaccine is administered orally via drinking water. The \textit{B. abortus} strain 82 vaccine has been utilized exclusively in the Russian federation (Ivanov et al., 2011). Other vaccines are in the research pipeline. There is
currently no shortage of studies on genetically engineered strains and subunit vaccines; however, most candidates have been evaluated only in mouse models. There has been little progression from laboratory models to natural hosts (Yang et al., 2013, Olsen & Tatum, 2010). There is a significant need for improved livestock vaccines that are of reduced virulence yet high immunogenicity and do not interfere with serological tests. In addition, effective vaccines for use in swine are lacking (World Organization for Animal Health, 2009c).

Much work has also been devoted to development of a vaccine for humans. There is considerable need for vaccination of humans in disease endemic areas and vaccination of U.S. military personnel, as Brucella is a category B select agent and has previously been weaponized (Pappas et al., 2006, Perkins et al., 2010). While there has been historic use of live attenuated vaccine strains in humans in the former Soviet Union and China, these vaccines often caused disease and hypersensitivity reactions and were of limited efficacy (Perkins et al., 2010). As with the livestock vaccines in the research pipeline, most human vaccine candidates have only been applied in mouse models. While subunit vaccines are attractive because of their safety, the immunogenic antigens of Brucella are poorly characterized. Furthermore, there is evidence that there may be differential recognition of Brucella antigens by different hosts (Liang et al., 2010). As a result, efficacy of vaccine candidates in mouse models may be a poor predictor of efficacy in human or livestock hosts.

1.9) Diagnosis of Infection

Similar to the scenario with vaccine development, a perfect test for the diagnosis of brucellosis remains to be developed. Definitive diagnosis in livestock and humans is provided only by detection of Brucella organisms or DNA via culture or polymerase chain reaction (PCR) techniques, respectively. Although culture is the gold standard, this technique often has poor sensitivity depending on the host species and duration of infection. It also carries considerable risk for laboratory staff and is optimally performed in a high security laboratory by skilled personnel. As a result, diagnosis has relied on indirect methods, primarily the detection of anti-Brucella antibodies in serum. The choice of serological test depends on the host species, the diagnostic goal, and the laboratory capabilities. The absence of pathognomonic signs in human and livestock hosts make laboratory tests essential for accurate diagnosis of brucellosis.
1.9a) Serological Testing

There are a number of excellent reviews on the serological tests utilized in diagnosis of brucellosis in livestock (Nielsen, 2002, Nielsen et al., 2004, Nielsen & Yu, 2010, Gall & Nielsen, 2004, Godfroid et al., 2010). The OIE Manuals of Diagnostic Tests and Vaccines for Terrestrial Animals also provide detailed descriptions of the approved tests for each livestock species (World Organization for Animal Health, 2009a, World Organization for Animal Health, 2009b, World Organization for Animal Health, 2009c). The diagnostic tests currently in use for the diagnosis of human brucellosis are often identical to those utilized in livestock and have been reviewed by Araj (Araj, 2010). In livestock diagnostic tests are performed for the purposes of screening or prevalence studies, confirmatory diagnosis, certification for the purpose of trade, and surveillance post-disease eradication. The importance of false positives and false negatives differs for each of these objectives, thus influencing test choice. Serological tests are primarily derived from research on bovine brucellosis, and whole killed \textit{B. abortus} organisms or \textit{B. abortus} LPS are typically utilized as antigen in the tests. The humoral immune response to smooth \textit{Brucella} species is predominately to the O-polysaccharide of the LPS molecule. Due to similarities in their LPS structure, the diagnostic tests currently used generally work equally well in detection of \textit{B. abortus}, \textit{B. melitensis}, and \textit{B. suis} (Godfroid et al., 2010). Diagnosis of infection caused by the rough species \textit{B. canis} and \textit{B. ovis} requires specific tests that utilize outer membrane proteins as antigens (Araj, 1999). Since the conventional serologic tests utilized for human diagnostics do not detect \textit{B. canis}, infection with this species may be underdiagnosed in humans (Lucero \textit{et al.}, 2010).

The most common serological tests utilized in both livestock and humans are the serum agglutination test (SAT), the complement fixation test (CFT), buffered \textit{Brucella} antigen tests including the Rose Bengal test (RBT) and the U.S. Card test, ELISA, and the fluorescence polarization assay (FPA) (Araj, 2010, Godfroid \textit{et al.}, 2010). The SAT was developed in 1897, and while this test is no longer recommended for the diagnosis of bovine brucellosis by the OIE, it still is utilized in human medicine (Araj, 2010, World Organization for Animal Health, 2009a). The test is performed in tubes or microtiter plates by reacting a solution of whole \textit{Brucella} cell antigen with serum dilutions. With serum from seropositive individuals, large antigen-antibody complexes form and precipitate at the bottom of the tube, the detection of which is facilitated with a dye. The required overnight sample incubation, low specificity, and relative inability to diagnose infection in chronic cases due to detection of primarily IgM are
major drawbacks of the SAT. The CFT offers the advantage of improved specificity; however, it requires a large number of reagents and is technically challenging. This test has historically been used as a confirmatory test for bovine brucellosis but is beginning to be replaced by ELISA and FPA tests (World Organization for Animal Health, 2009a). The CFT allows for the detection of IgM and IgG anti-*Brucella* antibodies that are able to activate complement. In contrast, the buffered *Brucella* antigen tests are among the simplest to perform, inexpensive, highly sensitive, and among the few serological tests that can be used in a field setting. This is advantageous as many brucellosis endemic areas have minimal laboratory capabilities. To perform these tests, stained whole *Brucella* cell antigen is utilized at an acidic pH to encourage agglutination by IgG over IgM and improve specificity. The antigen mixture is mixed with a serum sample on a plate and the degree of agglutination assessed over a period of several minutes. The buffered *Brucella* antigen tests are commonly used as screening tests for bovine and caprine brucellosis, and there is renewed interest in this test for human diagnostics especially in resource poor disease endemic areas. When compared with other more sophisticated and expensive serological tests including the SAT, competitive ELISA, and others, the RBT was found to perform equally well in the diagnosis of both acute and chronic human brucellosis (Diaz *et al*., 2011). The ELISA assays utilized in the diagnosis of brucellosis include the indirect (iELISA) and competitive (cELISA) varieties. These tests have high sensitivity and specificity and are good choices for diagnosis of focal and chronic brucellosis. ELISA is also commonly utilized for diagnosis in livestock, and the cELISA is highly useful in countries that use the smooth *B. abortus* S19 or *B. melitensis* Rev. 1 vaccines. The cELISA and FPA assays are the only serologic tests available in which antibody resulting from vaccination typically will not react giving a false positive result (Nielsen & Yu, 2010, Nielsen *et al*., 1996). The FPA is a recently developed serological test that allows for diagnosis of infection based on the property that smaller molecules rotate at faster speeds in liquid medium. When serum from a *Brucella* positive animal is added to a solution containing fluorescently-conjugated *Brucella* antigen, the large antigen-antibody complexes that form will rotate slower than unbound antigen. The change in rotational speed of the fluorescent molecule is measured with an instrument using polarized light (Nielsen *et al*., 1996). The FPA assay has high sensitivity and specificity in humans, cattle, and goats (Lucero *et al*., 2003, McGiven *et al*., 2003, Ramirez-Pfeiffer *et al*., 2006), is relatively simple to perform, and testing can be done in a field setting with handheld FPA instruments.
Of final note, the serological tests described here have poor specificity in swine (World Organization for Animal Health, 2009c). The control of swine brucellosis suffers from the lack of an effective diagnostic test as well as the absence of a vaccine. The primary reason for the poor performance of the *Brucella* serological tests in pigs is cross-reaction with other bacterial species. The O-side-chains on the LPS molecules of *Yersinia enterocolitica* O:9, *Vibrio cholerae*, *Francisella tularensis*, *Salmonella* O:30, *E. coli* O:157 and several other species are similar in structure to those of brucellae (Araj, 2010, Diaz *et al.*, 2011). The antibodies produced by a host upon infection with these bacteria will bind to the antigen used in *Brucella* diagnostic tests leading to false positive results. *Y. enterocolitica* O:9 infection is common in swine, and thus positive results on *Brucella* serological tests could be an indication of either *Brucella* or *Y. enterocolitica* infection. The cross-reacting pathogens are less common in other livestock species, but false positives are still a concern in these hosts. In human diagnostics, cross-reactivity is of little concern since these pathogens cause different symptoms than those associated with *Brucella* infection.

1.9b) Culture as a Gold Standard

The detection of anti-*Brucella* antibodies indicates only potential exposure to *Brucella* species. A positive serological test does not necessarily indicate current infection. Conversely, both human and livestock hosts can be antibody-negative but disease positive (Godfroid *et al.*, 2010, Ogredici *et al.*, 2010). Only culture and molecular techniques can definitively establish the presence of infection as well as determine the *Brucella* species responsible. Valid clinical samples for culture include blood, bone marrow, joint fluid, semen, and cerebral spinal fluid in humans and aborted fetuses, fetal membranes, vaginal secretions, sperm, milk, blood, and joint / hygroma fluid in animals (Franco *et al.*, 2007, Troy *et al.*, 2005, Godfroid *et al.*, 2010). Farrell’s medium is most commonly used for culture of potential *Brucella* species from tissue samples; however, various other selective media have been utilized in an attempt to increase yield (Her *et al.*, 2010, Marin *et al.*, 1996a, Marin *et al.*, 1996b). These media contain several antibiotics capable of inhibiting the growth of other bacteria present in clinical samples. Blood samples have traditionally been cultured using biphasic medium such as Castaneda medium or various subculture techniques. However, these methods detect *Brucella* species in only 40-90% of acute human cases and 5-20% of chronic or focal cases. Diagnosis may also be substantially delayed as occasionally incubation times as long as 6 weeks are necessary (Araj, 2010, Franco *et al.*, 2007). For human samples, automated culture systems have largely replaced these traditional methods (Yagupsky, 1999). The result has been higher levels of detection and shorter incubation
times. The duration of bacteremia in patients is poorly characterized; however, once focal infection develops fewer
brucellae are present in the blood resulting in decreased sensitivity of blood culture in patients with chronic
brucellosis. The duration and degree of bacteremia is also likely species specific, with swine and canine hosts
exhibiting bacteremia for months to years after infection (Hollett, 2006, Poester et al., 2013).

1.9c) PCR and Other Molecular Techniques

A number of PCR methods exist for detection and differentiation of Brucella species. While the techniques
were originally developed for detection of Brucella DNA extracted from cultures, PCR has been successfully
applied for direct detection of Brucella in clinical samples (O'Leary et al., 2006, Di Giannatale et al., 2009, Hinic et
1997, Meltzer et al., 2010, Vrioni et al., 2008). Both genus- and species-specific assays are available, with
primarily the genus-specific assay performed directly on clinical samples. The most utilized techniques are based on
detection of 16S rRNA, the IS711 insertion sequence, or a 31 kDa outer membrane protein (BCSP31) (O'Leary et
al., 2006, Queipo-Ortuno et al., 1997, Godfroid et al., 2010). Species-specific assays include: the AMOS PCR
method, which is a multiplex assay named for the species it can detect (B. abortus, B. melitensis, B. ovis, and B.
suis) (Bricker & Halling, 1994, Bricker, 2002); the Bruce-ladder method, which can differentiate all but one of the
currently known Brucella species and the vaccine strains RB51, S19, and Rev. 1 (Garcia-Yoldi et al., 2006, Lopez-
Goni et al., 2008, Mayer-Scholl et al., 2010); and a new method developed by Hinic, which can differentiate the
classical Brucella species by both conventional and real-time systems (Hinic et al., 2008). A multiplex real-time
PCR assay has also been developed for differential diagnosis of extrapulmonary tuberculosis and complicated
brucellosis, as both present with similar clinical signs and are endemic in Africa, Latin America, the Middle East,
and India (Sanjuan-Jimenez et al., 2013).

It is difficult to assign sensitivity and specificity values to PCR techniques since there is no standardized
method applied. While some authors have reported improved levels of detection over culture methods (Queipo-
Ortuno et al., 1997, Hinic et al., 2009), this is not universal (O'Leary et al., 2006). An early application of the
BCSP3 genus-specific PCR assay for detection of Brucella DNA in peripheral blood samples from human patients
found PCR to have 100% sensitivity compared to 70% sensitivity of culture (Queipo-Ortuno et al., 1997). Brucella
DNA has also been detected in the blood of many patients several years after completion of antibiotic regimens and disease eradication based on absence of symptoms, negative serological tests, and negative culture results (Vrioni et al., 2008). This is further evidence for the high sensitivity of molecular techniques.

PCR methods are utilized to diagnose infection, monitor treatment response in human patients, and identify the *Brucella* species responsible for infection. Since PCR methods are not able to distinguish biovars within a species, various DNA typing techniques have also been developed. When used during brucellosis outbreaks, these techniques often allow the source of infection to be determined. For example, multi-loci variable number tandem repeat assays (MLVA) have been used to determine that elk are the primary source of *Brucella* infection in cattle and farmed bison in the GYA of the western U.S. (Rhy an et al., 2013) and that Alpine ibex were the source of a spill-over event of *B. melitensis* into cattle and humans in France (Mick et al., 2014)

1.10) Treatment of Disease

There are a limited number of antibiotics available for the treatment of brucellosis due to the localization of brucellae in a protected intracellular niche. A long-term multi-drug regimen is necessary to prevent treatment failure and later relapse in human patients. While antibiotic treatment of livestock has been shown to successfully eliminate infection based on cessation of shedding in milk and absence of brucellae in tissues cultured at necropsy (Radwan et al., 1993, Radwan et al., 1992), slaughter of positive animals is recommended for public health reasons. Treatment regimens in humans have changed little over the years. A recent review of clinical trials indicates that the WHO recommended combination therapy of doxycycline for 45 days and streptomycin for 14 days remains the most effective treatment for uncomplicated cases (Corbel, 2006, Solis Garcia del Pozo & Solera, 2012). While streptomycin is the most common aminoglycoside utilized in this combination therapy, it can be replaced with gentamicin and administration shortened to 7 days with no impact on treatment outcome. A combination of doxycycline and rifampin for 45 days is actually the most commonly prescribed treatment, although it is associated with higher relapse rates than the former strategy (Solis Garcia del Pozo & Solera, 2012). The replacement of an aminoglycoside with rifampin is more convenient since aminoglycosides require parenteral administration and thus daily visits to a healthcare provider. In a limited number of studies rifampin has also been used in combination with a quinolone such as ciprofloxacin with similar treatment success (Solis Garcia del Pozo & Solera, 2012).
Doxycycline is contraindicated in young children due to the potential for inhibition of bone growth and permanent staining of deciduous teeth, and combinations of co-trimoxazole and either rifampin or an aminoglycoside have been successfully used (Solis Garcia del Pozo & Solera, 2012, Corbel, 2006). Shortening the treatment regiments described above or use of monotherapy is associated with unacceptably high relapse rates (Solis Garcia del Pozo & Solera, 2012). Treatment failure is not believed to be associated with antibiotic resistance (Turkmani et al., 2006).

Treatment of complicated brucellosis including osteoarticular disease and neurobrucellosis often requires months of treatment and triple antibiotic regimens (Troy et al., 2005, Vrioni et al., 2014, Erdem et al., 2012). A retrospective study of neurobrucellosis treatment in 215 patients reported average treatment duration of 4.5-6.5 months depending on the antibiotic regimen administered (Erdem et al., 2012). The use of the extended-spectrum cephalosporin ceftriaxone in combination with rifampin and doxycycline was found to be the preferred treatment choice due to ceftriaxone’s high diffusion rate into the cerebral spinal fluid. In skeletal and cardiac complications associated with brucellosis, surgical intervention is often necessary in combination with antibiotic treatment. In a retrospective study of brucellosis cases in San Diego, California, 5 of 7 patients with radiographic evidence of osteoarticular disease and/or positive joint or bone cultures required surgical debridement of the infected bone (Troy et al., 2005). In a retrospective study of endocarditis in Spain, 8 of 11 patients required cardiac surgery (Reguera et al., 2003). The authors indicated that valve replacement is the treatment of choice in patients presenting with severe aortic valve insufficiency and left ventricular failure.

While treatment strategies have not changed dramatically in recent years, the definition of a successful treatment outcome has been newly questioned. Advances in PCR techniques have allowed for the measurement of microbial load upon disease presentation and then again post-treatment, providing a definitive assessment of treatment success for the first time. Since antibody titers often persist following treatment and in many cases \textit{Brucella} cultures are negative in diseased patients, serological tests and culture results give ambiguous measurements of a disease cure. Detection of \textit{Brucella} DNA in patients years after “successful” treatment suggests \textit{Brucella} may persist in some individuals in a latent state much like in tuberculosis (Ogredici et al., 2010, Castano & Solera, 2009, Vrioni et al., 2008, Young et al., 2002). The goal of treatment may have to be redefined. The choice of antibiotic therapy likely depends on whether the treatment objective is resolution of symptoms or microbial...
eradication. A recent study is the first to compare the efficacy of two antibiotic regimens based on their success at eliminating \textit{Brucella} DNA from the peripheral blood (Vrioni \textit{et al.}, 2014). The results indicate that a triple treatment regimen is superior to the OIE recommended standard in terms of microbial eradication.

\textbf{1.11) Rationale for Current Study}

The overall aim of the research described in the next three chapters of this dissertation was to study \textit{Brucella} infection in the natural host, both in a field and laboratory setting. Much of the current knowledge on brucellosis originates from work in a mouse model; however, mice are resistant to infection with \textit{B. abortus}, \textit{B. melitensis}, and \textit{B. suis} biovars 1-4, the \textit{Brucella} species which are of concern in livestock and human hosts. Only minor species such as \textit{B. suis} biovar 5, \textit{B. neotomae}, and \textit{B. microtii} are naturally found in rodents (Godfroid \textit{et al.}, 2011). As a result of this natural resistance, mice do not develop the characteristic reproductive lesions observed in ruminants, and instead mount an effective immune response to clear infection (Grillo \textit{et al.}, 2012). The study of disease pathogenesis and immune defect thus necessitates a ruminant host.

Findings from field observations and \textit{Brucella} experimental infection studies in ruminants have primarily been published. Rates of abortion and levels of tissue colonization (Alexander \textit{et al.}, 1981, Manthei & Carter, 1950, Thomsen, 1950); the degree of shedding in uterine secretions and milk (Emminger & Schlam, 1943, Alexander \textit{et al.}, 1981); and the histological changes in placental membranes and aborted fetuses resulting from infection have been investigated (Xavier \textit{et al.}, 2009). Most of these studies, however, were published 25-75 years ago and were almost exclusively performed in cattle. When goats were utilized, it was as a model organism for bovine brucellosis. There are more studies conducted on \textit{B. abortus} infection in goats (Anderson \textit{et al.}, 1986a, Anderson \textit{et al.}, 1986b, Meador \textit{et al.}, 1989, Meador & Deyoe, 1986, Meador \textit{et al.}, 1988) than on the naturally occurring \textit{B. melitensis} infection. The select studies performed with \textit{B. melitensis} have primarily been carried out in sheep (Shimi & Tabatabayi, 1981, Tittarelli \textit{et al.}, 2005), which for the most part do not develop as severe an infection as goats (Alton, 1990a). While some vaccination and challenge studies have provided basic information on \textit{B. melitensis} infection in small ruminants (Alton, 1968, Elberg & Faunce, 1957, Entessar \textit{et al.}, 1967, Edmonds \textit{et al.}, 2002, Elzer \textit{et al.}, 1998, Elzer \textit{et al.}, 2002, Perry \textit{et al.}, 2010, Phillips \textit{et al.}, 1997, Roop \textit{et al.}, 2001), much of the text on caprine disease is simply extrapolated from work on bovine brucellosis. Thus, there remains a need to study basic
pathogenesis of *B. melitensis* infection in goats including rates of abortion, degree of shedding in uterine secretions and milk, and tissue distribution and burden. In the present study these basic characteristics of infection were compared in goats infected with virulent *B. melitensis* and the attenuated *B. melitensis* vaccine strain Rev. 1. The results are presented in chapter 3.

The subsequent objective was to determine the components of the immune response that are differentially active in groups of goats infected with virulent or attenuated *B. melitensis* strains. To the author’s knowledge, this is the first study to directly compare the “successful” immune response elicited by vaccination with the “unsuccessful” response elicited after infection with a fully virulent strain. It is also the first study to look at specific components of the cell-mediated immune response *in vivo* in *B. melitensis*-infected goats, including the activity of CD4+ T lymphocytes and γδ T cells. The findings of this work are described in chapter 4.

The laboratory components of this dissertation were informed by field research in Mongolia. A gap in knowledge exists in Mongolia and much of central Asia on disease prevalence, the species of *Brucella* circulating in livestock and humans, and optimal control strategies. I sought to fill some of these gaps by studying disease prevalence and risk factors for infection, as well as by evaluating a new diagnostic strategy. The conclusions drawn from my laboratory studies on disease pathogenesis also serve to benefit the work in Mongolia, providing knowledge on the specific public health risks associated with various herding practices.
2.1) Introduction

Brucellosis is one of the most common zoonotic diseases worldwide (Pappas et al., 2006). Caused by gram-negative bacteria of the genus *Brucella*, brucellosis is predominately a disease of domestic animals; however, it is highly transmissible to humans. Cattle, small ruminants, and pigs are among the primary hosts. Endemic in the Middle East, Mediterranean Basin, Central Asia, and parts of Africa and South and Central America, brucellosis affects the world’s poorest populations. With significant impacts directly on human health as well as on the productivity of livestock that serve as these people’s main source of subsistence, brucellosis has major health and socioeconomic effects worldwide. In a study of over 75 diseases affecting livestock, brucellosis was determined to be one of the 10 most important in terms of impact on impoverished people (Perry, 2002).

Mongolia is one country in which brucellosis has an ongoing and significant impact. Mongolia was considered to have the second highest incidence of human brucellosis in the world a decade ago (Pappas et al., 2006), but knowledge of current disease incidence and epidemiology in this country and all of Central Asia remains poorly described (Dean et al., 2012). Brucellosis is a re-emerging disease in countries of the former Soviet Union and former Eastern Bloc satellite states, including Mongolia. Economic instability following the collapse of the Soviet Union in 1990 led to a lapse in veterinary care and disease control measures (Pappas et al., 2006, Foggin et al., 2000).

Vaccination of cattle and small ruminants with S19 and Rev. 1 vaccines in Mongolia began in the 1960s with assistance from the World Health Organization (Denes, 1997, Kolar, 1995). The Mongolian vaccination program in small ruminants from 1975 to 1985 is regarded as one of the largest and most successful brucellosis control programs conducted worldwide, with approximately 33 million animals vaccinated. Human incidence of brucellosis was reduced from 48 per 100,000 in 1974 to 0.23 per 100,000 in 1981 (Elberg, 1996). Although control programs reduced both animal and human brucellosis during the period of Soviet influence, vaccine coverage was insufficient for eradication. Persistence of disease in livestock allowed for re-emergence of brucellosis when
vaccination ceased after the collapse of the Soviet Union, and cases of brucellosis in both humans and animals spiked in the early 1990’s. According to Mongolian ministry of health data, human brucellosis incidence rates in 1992 were 115 per 100,000 (Racloz et al., 2013), however, it is estimated that only 2-3% of cases are actually diagnosed and recorded in official ministry of health data (Roth et al., 2012).

In response to the re-emergence of brucellosis a formal national control program was put in place from 2000 to 2010. The goal of this program, eradication of brucellosis by 2010, was not achieved, in part due to insufficient vaccine coverage (Roth et al., 2012). The current national livestock brucellosis elimination program assures significant funding for brucellosis control from 2010 to 2021 with the goal of eradicating brucellosis in all animals by 2015 and achieving official brucellosis-free status by 2021. With the most recent national brucellosis serosurvey in livestock conducted by the government in 2011, and the only cross-sectional surveillance study in Mongolia published to date performed in 2010 (Zolzaya et al., 2014), the current picture of brucellosis in Mongolian livestock requires further investigation. It is essential that prevalence be monitored so that the control program can be evaluated and adjusted. Without constant assessment of disease status and vaccination coverage the goals of the current control program will again not be realized.

Results of the 2011 national brucellosis serosurvey and the 2010 cross-sectional surveillance study conducted in two of Mongolia’s 21 provinces were inconsistent. According to government data, the countrywide brucellosis seroprevalence was 1.8% in cattle, 0.7% in sheep, 0.5% in goats, and 0.7% in camels (Tsend et al., 2014). The cross-sectional surveillance study, however, found seroprevalences in Sukhbaatar and Zavkhan provinces to be 16.0% in cattle, 6.2% in sheep, 5.2% in goats, 2.5% in camels, 8.3% in horses, and 36.4% in dogs (Zolzaya et al., 2014). Since only two provinces were sampled, and Sukhbaatar is considered to be a region of high brucellosis prevalence within Mongolia (Zolzaya et al., 2014), the question remains as to whether brucellosis is maintained in livestock to these levels countrywide. The aim of the present study was to expand the current understanding of brucellosis epidemiology in Mongolia by studying prevalence of disease in two additional Mongolian provinces, Khuvsgul and Bulgan. Several districts in the province of Khuvsgul are highly mountainous and semi-nomadic herders in the area utilize yak and cow-yak hybrids (heinag) for milk, meat, fiber, and transportation of possessions. Although yak play an important role in brucellosis epidemiology and public health in
regions of India (Bandyopadhyay et al., 2009) and Nepal (Jackson et al., 2014), little is known about brucellosis in yak and heinag within Mongolia.

In 2001, a field based test for detection of *Brucella* antibodies in cow milk by fluorescence polarization assay (FPA) was validated (Nielsen et al., 2001). This assay is highly underutilized, and to our knowledge, this test has not been applied in Mongolia. Advantages of milk FPA include: applicability to field testing, high sensitivity (100%) and specificity (99%), no cross-reaction with antibody elicited by vaccination, and the ability to utilize a sample that can easily be acquired by non-invasive means and without veterinary assistance (Nielsen & Gall, 2001). Thus, the objective of this study was to use FPA to identify antibodies in milk samples and estimate the proportion of *Brucella* positive cattle, yak, and heinag in three regions of Mongolia. Additionally, prevalence of brucellosis in herd owners was assessed via questionnaire. The final aim was to acquire information from herd owners regarding animal husbandry practices and herd health and to utilize this information to identify individual- and herd-level characteristics that are predictive for brucellosis.

2.2) Materials and Methods

2.2a) Study Design

This cross-sectional study was conducted during June and July 2014 in two provinces (*aimags*) of northern Mongolia, Bulgan and Khuvsgul (Figure 2.1). Within Bulgan province, sampling was performed exclusively in the district (*soum*) of Orkhon. Khuvsgul province is divided into more governmental districts, from which two sampling locations were selected: Bayanzurkh and Renchinlkhumbe.

i) Inclusion Criteria

Households were selected for convenience sampling by a local veterinarian or governmental official. Four sampling sites (villages (*bags*) 1, 2, 3, and 4) were selected in Orkhon and three sampling sites were selected in both Bayanzurkh and Renchinlkhumbe (*bags* 2, 3, and 5 and *bags* 1, 4, and 6 respectively). These sites were chosen using a combination of factors including established relationships with herders in these areas, local interest in our study, and accessibility. Participation in the study was voluntary, but no households declined inclusion. A total of 77 households participated in this study including 34 from Orkhon, 24 from Bayanzurkh, and 19 from
Renchinkhumble. A total of 549 milk samples were obtained, 279 from Orkhon, 169 from Bayanzurkh, and 101 from Renchinlkhumbe. Animals included in the study were cattle, yak, and *heinag*.

**ii) Milk Sampling**

Milk collection vials (1 vial per animal) were distributed to the selected households, and herd owners allowed to choose the animals sampled. Up to ten animals were sampled per household, depending on the number of milking animals owned. From each, 10 ml of milk was collected.

![Sampling Areas](image)

**Figure 2.1.** Location of provinces (light grey) and districts (dark grey) in which sampling was conducted.

**2.2b) Testing for Anti-*Brucella* Antibodies by Milk FPA**

Milk was tested individually from each animal selected. In most cases the milk was stored overnight to allow lipids to separate to the surface, and the sample was tested the subsequent day. A *Brucella abortus* Antibody Test Kit (*Brucella FPA*, Ellie LLC, Wisconsin, USA) was utilized on all samples. The FPA assay was performed according to manufacturer's instructions. Briefly, 10 μl of milk serum (whey) was added to sample diluent. Blank
intensity readings were obtained on a Sentry 100™ FPA Instrument (Diachemix, Wisconsin, USA). A second intensity reading was subsequently taken after addition of 10 μl of antigen-fluorescein conjugate. The instrument automatically subtracts the background reading and presents a result in millipolarization units (mP). Since polarization readings are affected by temperature, all test results were reported as ΔmP, which was calculated by subtracting the average mP reading of a negative control from each sample mP. A single positive control and three replicates of a negative control were run every hour or if a temperature change was observed. Any samples with positive ΔmP readings were re-tested in duplicate using 20 μl of sample.

The assay was modified slightly in Khuvsgul province to accommodate yak milk, which has a higher fat content. In an attempt to improve sample transparency, 500 μl of milk was added to 30 μl of ClearMilk™ Buffer (Ellie LLC, Wisconsin, USA), which causes milk fat to congeal. After an incubation period, 10-20 μl of milk serum was removed and tested using the FPA protocol described above.

2.2c) Characteristics Associated with Brucella Infection

For each animal included in the study, species and age were recorded, as well as the GPS location where sampling occurred. Basic herd level data were also collected by administration of a survey to the head of household or a family member responsible for milking. Information collected included herd size for each livestock species owned and the number of abortions observed for each species over the past year.

Human disease was also studied via administration of a questionnaire. Heads of households were asked of the presence of symptoms of joint pain or fever in the family and the number of family members ever previously diagnosed with brucellosis by serological test at a local hospital. Additionally, a subset of sampled households in Renchinlkhumbe were questioned about husbandry practices and other risk factors including consumption of raw milk, disposal of aborted fetuses, assistance with calving and use of gloves, and herd movement following abortions.

2.2d) Data Analysis

Prevalence of anti-Brucella antibodies in milk were calculated both at the individual animal and herd levels. Numbers of test positive and suspect animals were combined to calculate individual animal prevalence.
Herd prevalence was determined by considering any herd with at least one positive or suspect animal a *Brucella* positive herd.

On initial FPA test, all samples with mP readings below the negative control were determined to be negative for anti-*Brucella* antibodies and were not subjected to re-test. For re-tested milk, samples were classified as negative, suspect, or positive using the criteria in Table 2.1.

### Table 2.1. Criteria used for classifying milk samples as negative, suspect, and positive by FPA.

<table>
<thead>
<tr>
<th></th>
<th>Negative</th>
<th>Suspect</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial test:</td>
<td>&lt;0 mP; no re-test</td>
<td>Re-test: Both samples 10-20 mP</td>
<td>Re-test or Initial Test: Two samples &gt;20 mP</td>
</tr>
<tr>
<td>Re-test:</td>
<td>Both samples &lt;10 mP</td>
<td>Re-test: One sample 10-20 mP, one sample &lt;10 mP</td>
<td>Re-test: One sample &gt;20 mP, one sample 10-20 mP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Re-test: One sample &gt;20 mP, one sample &lt;10mP</td>
<td></td>
</tr>
</tbody>
</table>

*a Readings given are ΔmP, calculated by subtracting negative control mP from corresponding sample mP readings.*

Animal age and species were evaluated as potential characteristics associated with brucellosis at the individual animal level, while numbers of abortions and herd size were evaluated as potential herd level factors. Characteristics of test positives were compared to those of test negatives. Descriptive analysis was utilized since the number of positive and suspect samples was small.

The study was approved by the Institutional Animal Care and Use Committee of Colorado State University. The questionnaire component of the study was part of a larger assessment of health of Mongolian herders conducted by Montana State University, and approval for human sampling was by the Institutional Review Board of Montana State University. Information regarding test positive animals was shared with local veterinarians.

### 2.3) Results

#### 2.3a) Households and Animals Studied

Milk from a total of 549 animals was collected from 77 households. Approximately equal numbers of animals were sampled in Bulgan and Khuvsgul provinces; however, more households were sampled in Khuvsgul.
due to smaller average herd size in this province. Overall, cattle comprised the majority of animals in the study. Of the milk samples collected, 56% were from cattle, 24% from yak, and 13% from heinag (Table 2.2). The remaining 7% (n=39) of samples were brought to the testing site by herd owners, and the animal source of the sample was not specified. These samples were included in the analysis since they originated from one of the three species represented in our study; all 39 of these samples were found to be test negative by FPA.

Animal types were not equally represented across all sampling locations (Table 2.2). In Orkhon, cattle were exclusively sampled due to the scarcity of yak and heinag in that region of Mongolia. In the more mountainous areas of Bayanzurkh and Renchinlkhumbe, only 11% of sampled animals were cattle, while 48% were yak and 26% were heinag. This sampling distribution represents the approximate prevalence of these livestock types in the region.

Table 2.2. Sample size by species and study region.

<table>
<thead>
<tr>
<th></th>
<th>Cattle</th>
<th>Yak</th>
<th>Heinag</th>
<th>Spp. Unknown</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bulgan Province</strong></td>
<td>279 (100%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>279</td>
</tr>
<tr>
<td>Orkhon</td>
<td>279 (100%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>279</td>
</tr>
<tr>
<td><strong>Khuvsgul Province</strong></td>
<td>30 (11%)</td>
<td>130 (48%)</td>
<td>71 (26%)</td>
<td>39 (15%)</td>
<td>270</td>
</tr>
<tr>
<td>Bayanzurkh</td>
<td>5 (3%)</td>
<td>83 (49%)</td>
<td>44 (26%)</td>
<td>37 (22%)</td>
<td>169</td>
</tr>
<tr>
<td>Renchinlkhumbe</td>
<td>25 (25%)</td>
<td>47 (46%)</td>
<td>27 (27%)</td>
<td>2 (2%)</td>
<td>101</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>309 (56%)</td>
<td>130 (24%)</td>
<td>71 (13%)</td>
<td>39 (7%)</td>
<td>549</td>
</tr>
</tbody>
</table>

*Percentage of the total number of animals sampled within that region is given in parentheses.

2.3b) Prevalence of Antibodies in Milk

The apparent overall prevalence of anti-Brucella antibodies in milk was 2.0% (95% CI 1.0-3.6). Renchinlkhumbe had more than twice the prevalence of the other districts; however, confidence intervals are large and overlapping (Table 2.3). Herd level prevalence was higher with 10.4% (95% CI 4.6-19.4) of the households sampled owning at least one positive or suspect animal. Widespread presence of antibodies in milk within Brucella positive herds was not observed; only two herds were found to have multiple test positive or suspect animals.
Test positive and suspect herds were also isolated cases. In general, we did not find groups of neighboring households to own *Brucella* positive animals (*Figures 2.2 – 2.4*). In Orkhon, however, animals sampled to the west and south of the district center were all negative, while the five animals found to be *Brucella* positive or suspect were north of the district center near the neighboring district of Bugat and the city of Erdenet (*Figure 2.1*).

**Table 2.3.** Prevalence of anti-*Brucella* antibodies in milk at the individual animal and herd levels.

<table>
<thead>
<tr>
<th></th>
<th>Number Sampled</th>
<th>Number Positive</th>
<th>Number Suspect</th>
<th>Prevalence*</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overall Individual</strong></td>
<td>549</td>
<td>3</td>
<td>8</td>
<td>2.0</td>
<td>1.0 - 3.6</td>
</tr>
<tr>
<td><strong>Bulgan Province</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orkhon</td>
<td>279</td>
<td>1</td>
<td>4</td>
<td>1.8</td>
<td>0.6 - 4.1</td>
</tr>
<tr>
<td><strong>Khuvsgul Province</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bayanzurkh</td>
<td>169</td>
<td>0</td>
<td>2</td>
<td>1.2</td>
<td>0.1 - 4.2</td>
</tr>
<tr>
<td>Renchinlkhumbe</td>
<td>101</td>
<td>2</td>
<td>2</td>
<td>4.0</td>
<td>1.1 - 9.8</td>
</tr>
<tr>
<td><strong>Overall Herd</strong></td>
<td>77</td>
<td>2</td>
<td>6</td>
<td>10.4</td>
<td>4.6 - 19.4</td>
</tr>
<tr>
<td><strong>Bulgan Province</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orkhon</td>
<td>34</td>
<td>1</td>
<td>3</td>
<td>11.8</td>
<td>3.3 - 27.4</td>
</tr>
<tr>
<td><strong>Khuvsgul Province</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bayanzurkh</td>
<td>24</td>
<td>0</td>
<td>2</td>
<td>8.3</td>
<td>1.0 - 27.0</td>
</tr>
<tr>
<td>Renchinlkhumbe</td>
<td>19</td>
<td>1</td>
<td>1</td>
<td>10.5</td>
<td>1.3 - 33.1</td>
</tr>
</tbody>
</table>

*Prevalence was calculated by combining test positive and suspect results. Herds with at least one positive or suspect animal were considered positive.

### 2.3c) Characteristics Associated with Brucellosis in Livestock

Animal age and species were evaluated as potential characteristics associated with brucellosis. Animals found to have anti-*Brucella* antibodies in their milk were older than *Brucella* negative animals. Mean age of the 11 test positive and suspect animals was 8.3 ± 3.2 years. All of these animals were over 6 years of age, except one test suspect cow in the district of Orkhon that was 3 years of age and a test positive *heinag* in Renchinlkhumbe for which age information was not recorded (*Table 2.4*). Mean age of test negative animals was 6.4 ± 2.3 years.

Yak and *heinag* were observed to have higher prevalences of antibodies in milk compared to cattle (*Table 2.5*). *Heinag*, although comprising only 13% of the animals tested, represented 27% of the test positive and suspect animals. In Khuvsgul, where all 3 livestock types were present, no cattle were found to be positive or suspect.
Potential herd characteristics associated with brucellosis including herd size and numbers of abortions were also evaluated. Herd sizes were highly variable in the regions studied; however, this was not found to influence brucellosis prevalence. Herd sizes of cattle, yak, and *heinag*, which are kept together in mixed herds, varied from 7 to 200 animals in the households sampled. Median herd size was 30 animals in test positive/suspect herds and 29 animals in negative herds. Size of goat and sheep herds ranged from zero to 550 and zero to 1200 animals, respectively. Households with test positive cattle owned marginally more goats (median = 110) than households with test negative cattle (median number of goats = 100). Conversely, sheep ownership was slightly lower in households with test positive cattle (median number of sheep = 115) than households with test negative cattle (median number of sheep = 123).

**Figure 2.2.** Locations and test results of the 34 households sampled in the Orkhon district of Bulgan province. The color of the point represents the herd level test result; 1 positive and 3 suspect herds were observed. Locations of 3 herds south of the district center were not recorded; these herds were negative.
Figure 2.3. Locations and test results of the 24 households sampled in the Bayanzurkh district of Khuvsgul province. The color of the point represents the herd level test result; 2 herds were found to be suspect. Herds were in summer grazing area, which often fell outside of the district boundary.

Figure 2.4. Locations and test results of the 19 households sampled in the Renchinlkhumbe district of Khuvsgul province. The color of the point represents the herd level test result; 1 positive and 1 suspect herd were observed.
Abortions in cattle, yak, and *heinag* were also not found to be a predictor of brucellosis in the herd. Of the households sampled, 6.3% reported abortions in these three animal types over the past year. All herds in which abortions were reported tested negative for brucellosis by milk FPA. Levels of abortion in sheep were nearly twice that in cattle, yak, and *heinag* with 11.5% of households reporting at least one sheep abortion in the past year.

Again, all of these households were negative for brucellosis in the cattle, yak, and *heinag* sampled. High prevalence of abortion was observed in goats with 36.5% of households reporting at least one abortion. Of households with cattle, yak, or *heinag* that were test positive or suspect by milk FPA, 50% reported abortions in their goat herd.

While the maximum within herd abortion rate in goats was reported at 35%, the three households that owned both test positive cattle and aborting goats reported herd abortion rates of 1.5 – 3% (*Table 2.4*).

**Table 2.4.** Animal and herd level characteristics of test positive and suspect animals.

<table>
<thead>
<tr>
<th>Animal ID&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Herd ID</th>
<th>Location&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Species</th>
<th>Age</th>
<th>Cattle Herd Size&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Sm. Rum. Herd Size&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Cattle Abortions&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Goat Abortions&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Human Brucellosis Diagnosis&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>A</td>
<td>Orkhon Bag 4</td>
<td>Cow</td>
<td>8</td>
<td>21</td>
<td>113</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>Orkhon Bag 4</td>
<td>Cow</td>
<td>10</td>
<td>31</td>
<td>500</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>Orkhon Bag 4</td>
<td>Cow</td>
<td>13</td>
<td>31</td>
<td>500</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>Orkhon Bag 2</td>
<td>Cow</td>
<td>6</td>
<td>30</td>
<td>200</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>D</td>
<td>Orkhon Bag 2</td>
<td>Cow</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>E</td>
<td>Bayanzur. Bag 5</td>
<td>Yak</td>
<td>10</td>
<td>30</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>Bayanzur. Bag 3</td>
<td><em>Heinag</em></td>
<td>7</td>
<td>35</td>
<td>600</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>G</td>
<td>Renchin. Bag 1</td>
<td>Yak</td>
<td>6</td>
<td>15</td>
<td>250</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9*</td>
<td>G</td>
<td>Renchin. Bag 1</td>
<td><em>Heinag</em></td>
<td>13</td>
<td>15</td>
<td>250</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10*</td>
<td>G</td>
<td>Renchin. Bag 1</td>
<td><em>Heinag</em></td>
<td>15</td>
<td>250</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>H</td>
<td>RENCHIN. Bag 6</td>
<td>Yak</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Test positive animals are indicated by an asterisk. All others are test suspect.

<sup>b</sup> Bayanzur. = Bayanzurkh; Renchin. = Renchinkhumb.  

<sup>c</sup> Cattle herd size includes number of cattle, yak, and *heinag* owned.

<sup>d</sup> Sm. rum. herd size includes number of sheep and cattle owned.

<sup>e</sup> Numbers of abortions are given for the past reproductive cycle. Cattle abortions include abortions in cattle, yak and *heinag*.

<sup>f</sup> Human brucellosis diagnosis indicates the number of family members that reported previous diagnosis of brucellosis by serological test at a local hospital. Responses to the questionnaire were not received from all households.
Table 2.5. Prevalence of anti-Brucella antibodies in milk by livestock type across the three districts sampled.

<table>
<thead>
<tr>
<th>Type</th>
<th>Number Sampled</th>
<th>Number Positive</th>
<th>Number Suspect</th>
<th>Prevalencea</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>309</td>
<td>1</td>
<td>4</td>
<td>1.6</td>
<td>0.5-3.7</td>
</tr>
<tr>
<td>Yak</td>
<td>130</td>
<td>0</td>
<td>3</td>
<td>2.3</td>
<td>0.5-6.6</td>
</tr>
<tr>
<td>Heinag</td>
<td>71</td>
<td>2</td>
<td>1</td>
<td>4.2</td>
<td>0.9-11.9</td>
</tr>
</tbody>
</table>

a Prevalence was calculated by combining test positive and suspect results.

2.3d) Association between Livestock and Human Brucellosis

Prevalence of brucellosis in herders was assessed via questionnaire. Of all the households sampled, 17% reported that at least one person within the household had previously been diagnosed with brucellosis. No association was found, however, between the presence of anti-Brucella antibodies in cattle, yak, or heinag milk and reported disease in humans. Of the eight households with test positive or suspect herds, none reported previous diagnosis of brucellosis in family members (Table 2.4). While three of these households reported the presence of joint pain in at least one person, and two households reported presence of fevers, these symptoms were ubiquitous among all the households surveyed.

2.3e) Husbandry Practices as Potential Risk Factors for Disease in Humans and Animals

Nine households in the district of Renchinkhunbe were surveyed with an expanded questionnaire to investigate practices in the local nomadic culture potentially associated with disease transmission in animals and humans. All families reported that they boil milk before consumption. Members of the majority of households (89%) assisted with calving, and all handled newborns and placental material with bare hands. Direct contact with fetal/placental material was also documented following abortions. All herders surveyed made an attempt to remove aborted fetuses from the pasture. The majority of households (89%) buried or burned aborted fetuses, while one herder explained that fetuses were put in a trash pile for birds to eat. Occasionally dogs would consume fetal tissues on pastureland before they were found by herders. When disposing of aborted fetuses, only two households reported that they sometimes used gloves or a stick to handle the material. Only one household reported that they made an attempt to move their herds away from pasturelands on which abortions occurred to prevent other animals in the herd from aborting.
2.4) **Discussion**

2.4a) **Significance**

This study expands upon limited previous research on brucellosis in Mongolian livestock (Zolzaya *et al.*, 2014), and provides disease prevalence information for two additional provinces, Bulgan and Khuvsgul. The findings are applicable to the current national brucellosis control program in Mongolia. The study indicates that brucellosis remains present at low levels in Mongolian cattle, yak, and *heinag* despite a nationwide vaccination program initiated in 2000. There are also broader implications. The study is the first to compare brucellosis rates in yak, hybrids, and cattle, and suggests that yak and hybrids may have higher disease prevalences. The study also confirms the applicability of milk FPA for field testing in remote locations.

2.4b) **Prevalence in Livestock and Herders**

Apparent prevalence of anti-*Brucella* antibodies in milk is reported since to our knowledge FPA has never been performed with samples from yak or hybrids, and the sensitivity and specificity of the test in these species is thus unknown. The FPA has been found to perform well across a variety of species, however, supporting the applicability to the current study (Nielsen & Gall, 2001). Brucellosis prevalence ranged from 1.2-4.0% within cattle, yak, and *heinag* in the three study areas. This level of disease was lower than that reported in a previous cross-sectional seroprevalence study; Zolzaya *et al.* found 7.8 and 15.3% of cattle to be seropositive in Sukhbaatar and Zavkhan provinces, respectively (2014). Regional differences in brucellosis prevalence likely explain both the differences in prevalence reported by the present study and Zolzaya *et al.* as well as observed differences in prevalences in Orkhon, Bayanzurkh, and Renchinlkhumbe districts. Officially reported levels of disease in cattle are 0.7% in Bulgan province and 2.3% in Khuvsgul province, according to a 2011 national brucellosis surveillance study in which 2981 and 4096 cattle in Bulgan and Khuvsgul, respectively, were tested by cELISA (Sugir, 2013). Orkhon is one of 16 districts within Bulgan province, and Bayanzurkh and Renchinlkhumbe are two of 24 districts within Khuvsgul. The higher level of disease in cattle observed in Renchinlkhumbe compared to Orkhon in the present study may be explained by differences in vaccination coverage. Orkhon is closer to the capital city of Ulaanbaatar, and more consistently receives a supply of brucellosis vaccine. According to a veterinarian in Renchinlkhumbe, vaccine coverage in the district was only 50%. Vaccination status of the herds sampled was not recorded since conflicting reports were obtained from veterinarians and herders.
Herd level prevalence within cattle, yak, and hybrids in the three areas sampled was 10.4%. Herd prevalence is most important from a public health perspective, a point illustrated by high levels of human disease in the area. According to questionnaire results, 17% of households reported prior diagnosis of brucellosis in a family member. In a previous questionnaire study, 15-18% of individuals in Zavkhan and Sukhbaatar provinces reported prior diagnosis of brucellosis, while serological testing of the same individuals revealed prevalence rates of 26-29% (Zolzaya et al., 2014). A human seroprevalence study in Khuvsgul found 11.5 and 42.7% of individuals and households, respectively, to be positive for brucellosis (Tsend et al., 2014). In a pilot project, which we conducted in 2009 in the Renchinlkhumbe district of Khuvsgul province, 11% (n=163) of herders tested positive by card test (unpublished data).

While human prevalence was only assessed by questionnaire in the present study, the results suggest levels of disease are considerably higher than those officially reported. Levels of reported disease increased as officials from national to regional to local levels were consulted. According to National Official Statistical Data, in 2012 provincial labs in Bulgan and Khuvsgul detected only 1 and 18 cases, respectively. However, the health department in the provincial capital of Bulgan reported 4 cases in 2012, and the local hospital in Orkhon district alone reported that 20-25 people are diagnosed with brucellosis annually. Brucellosis is a reportable disease within Mongolia, but the differences in case numbers indicate that cases diagnosed in district hospitals are often not included in national statistical data. Discrepancies in case numbers are also due to new and relapse cases being combined in some instances. This likely explains the high levels of disease reported by the Orkhon district hospital. Rates of relapse are high in Mongolia with delay in diagnosis and insufficient treatment regimens playing a role. Treatment of patients with acute brucellosis is reported to be for 7-10 days at the Orkhon district hospital and for a minimum of 20 days at the Bulgan provincial hospital, durations that fall well short of the World Health Organization recommended 6 weeks of treatment (Solis Garcia del Pozo & Solera, 2012, Corbel, 2006). These condensed treatment regimens have been reported by others within Mongolia (Zolzaya et al., 2014, Ebright et al., 2003). Effective antibiotics are also not applied in some cases. The Orkhon district and Bulgan provincial hospitals report the use of doxycycline in combination with either a cephalosporin or gentamicin. The use of cephalosporins have been associated with high relapse rates (Corbel, 2006).
2.4c) Test Choice and Performance

The FPA applied in the present study was the best diagnostic test available for the existing conditions, although a perfect antibody-based test for diagnosis of brucellosis is not available. FPA has the advantage of being both highly sensitive (100%) and specific (99%) and thus is a good choice when only a single test can be performed, for example due to financial and time constraints. Additionally, the FPA is the only brucellosis test currently available that can be both performed in a field setting and distinguish between infected and vaccinated animals (Nielsen & Gall, 2001, Nielsen et al., 1996). In many areas of Mongolia, a cold chain cannot be easily maintained, making field testing of samples advantageous. A mixture of vaccinated and unvaccinated herds is also present. These conditions are likely common worldwide in areas where brucellosis is endemic. Milk is an attractive sample for antibody testing and is highly underutilized. Milk is easily obtainable by non-invasive means and is especially desirable in remote areas where there are shortages of animal restraint facilities or veterinarians to perform venipuncture. Milk is also more suitable for subsequent downstream tests, for example, if culture or PCR are performed on antibody-positive samples. Finally, there are indications that the presence of anti-\textit{Brucella} antibody in milk can be used as a predictor of shedding of bacteria, and thus public health risk (Boraker \textit{et al.}, 1981).

We found that the milk FPA requires further optimization. According to the manufacturer’s protocol (Ellie LLC, Wisconsin, USA), the FPA works on both clear and cloudy particulate-free samples. Developers of the assay report that milk can be tested directly, after storage, after freezing, or in a deteriorated state (Nielsen & Gall, 2001). In the present study, however, samples that remained turbid after dilution in buffer were found to have higher background values and lower sample mP readings. To decrease turbidity, a dilution factor of 1:100 was used. This is the dilution typically used for cattle serum, but differs from the 1:25 dilution used in the original milk FPA (Nielsen & Gall, 2001, Nielsen \textit{et al.}, 2001). Yak milk which is of higher fat content, required additional treatment with ClearMilk™ Buffer to decrease sample turbidity and improve FPA results. Several of the animals sampled had recently calved, and the colostrum was not found to be suitable for testing either because of the turbidity or color of the sample. An additional limitation of the test procedure was its poor performance at high ambient temperatures. This may be unique to the FPA instrument used in the present study.
2.4d) Characteristics Associated with Brucellosis in Livestock

Animals in which anti-Brucella antibodies were detected were found to be older than test negative animals. This difference may be an early indicator of success of the vaccination program despite continued low level prevalence of brucellosis in livestock. Assuming vaccine is available and effective (a cold chain is often unavailable), all female calves are vaccinated according to the current national control program. As the control program progresses, this pool of immunized animals should increase. Theoretically, older animals are more likely to be infected as they did not benefit from calfhood vaccination. Mass vaccination is performed every few years, but older animals may already be Brucella positive prior to vaccination. An alternative explanation for the finding that mean age of test positive animals is increased, is that older animals are simply more likely to be test positive since they have had greater opportunity to be exposed to Brucella. In a region in Nepal where vaccination is not practiced, brucellosis prevalence was also observed to be higher among older animals (Jackson et al., 2014).

In the present study, yak and heinag were more likely to have anti-Brucella antibodies in milk than cattle. Yak are known to have high levels of brucellosis infection; seroprevalence levels of 24% and 22% and been reported among yak in regions of India and Nepal, respectively (Bandyopadhyay et al., 2009, Jackson et al., 2014). To our knowledge, however, this is the first study to compare brucellosis prevalence among cattle, yak, and hybrids. Sample sizes were too low and the distributions of species too uneven to fully assess susceptibility to Brucella infection, but the data suggest that heinag and yak may be more susceptible to infection than cattle. Further research is needed on brucellosis in these species. Differences in susceptibility to Brucella infection among ruminants have previously been noted; bison appear to be more susceptible than cattle to infection (Olsen & Johnson, 2011).

Herd size and presence of abortions were evaluated as potential herd-level characteristics associated with brucellosis. Although other studies have found risk of infection to increase as herd size increases (Muma et al., 2007, Jackson et al., 2014), we did not find this to be the case in Mongolia. Stocking density may have been a more appropriate parameter to evaluate; however, this could not be determined since herds graze on open pastureland.

Abortions were also not found to be associated with the presence of anti-Brucella antibodies in milk. No abortions were reported among cattle, yak, or heinag over the past year in the 8 herds found to be test positive or
suspect. While others have found abortions to be associated with higher odds of *Brucella* infection (Jackson *et al.*, 2014) and abortion is a characteristic clinical sign of brucellosis in livestock, cattle typically abort only during the parturition immediately following infection. Subsequent parturitions are often normal (Carvalho Neta *et al.*, 2010). With the exception of one 3 year-old cow, all test positive animals in the study were at least 6 years of age. These animals may have been infected many years previously with abortion occurring at that time. Abortions in sheep and goats were also recorded. Fifty percent of households with *Brucella* positive or suspect cattle, yak, or heinag, reported abortions in their goat herd. In comparison only 35% of households with *Brucella* negative cattle, yak, or heinag reported abortions in their goats. Herders in Mongolia maintain mixed-species herds, and the presence of brucellosis, and abortions, in small ruminants has been associated with increased risk of disease in cattle (Holt *et al.*, 2011). Whether cattle and yak are infected with *B. melitensis* in Mongolia must be further investigated. The etiology of the high levels of abortion observed in goats also warrants additional research. While local veterinarians believe the primary causes are nutritional deficiencies and cold winters, these animals should be tested for brucellosis and other infectious diseases that cause abortions, as many of these diseases are also of importance to public health.

### 2.4e Potential Risk Factors for Brucellosis in Herders

The presence of anti-*Brucella* antibodies in milk of cattle, yak, or heinag was not found to be associated with disease in humans. This must be interpreted with caution, however, since human brucellosis was assessed via questionnaire. Although brucellosis is highly infectious to humans, relationships between animal and human disease are complex. Zolzaya *et al.* (2014) also failed to find a significant correlation between animal and human brucellosis seroprevalence in Zavkhan and Sukhbaatar provinces. In Kyrgyzstan, small ruminant seroprevalence but not cattle seroprevalence was associated with brucellosis in humans (Bonfoh *et al.*, 2012).

Transmission of brucellosis to humans likely occurs primarily through direct contact with infected material. The majority of herders surveyed reported that they assisted with calving as well as removed aborted fetuses from pastureland. Herders do not have access to gloves and often do not wash their hands after handling potentially infected material. In a serosurvey conducted among rural Mongolians, contact with aborted fetuses and placentas as well as being a veterinarian were found to be associated with increased odds of infection (Tsend *et al.*, 2014). While
none of the herders in the present study reported consumption of raw milk, it is possible that herders are infected during the process of milking. Zolzaya et al. (2014) found women, who are responsible for milking, to be at higher risk of Brucella infection. While cultural practices of food preparation prevent most foodborne sources of brucellosis infection in Mongolia, herders should be educated about simple hygienic measures such as hand-washing as a means of decreasing risk of brucellosis transmission.

2.5) Conclusions

This study indicates that brucellosis remains endemic in cattle, yak, and hybrids within Bulgan and Khuvsgul provinces of Mongolia despite a national control program. High levels of human disease were also reported. The milk FPA is advantageous for testing in remote field sites, especially when concerns of false positive results from vaccination exist. Diagnostics based on antibody response indicate only exposure to Brucella, however, and not necessarily current infection. Future studies should employ milk FPA as a screening test with downstream PCR analysis. It is also essential that the species of Brucella responsible for infection in cattle, yak, and hybrids as well as in humans be determined. Identifying reservoirs of infection for both human and animal disease will lead to more effective disease control strategies. The Mongolian brucellosis control program must be critically evaluated and necessary adjustments quickly implemented if the goal of obtaining brucellosis-free status by 2021 is to be realized.
CHAPTER 3: EVALUATION OF SHEDDING, TISSUE BURDENS, AND HUMORAL IMMUNE RESPONSE IN GOATS AFTER EXPERIMENTAL CHALLENGE WITH BRUCELLA MELITENSI

3.1) Introduction

*Brucella melitensis*, a gram-negative bacterial pathogen, is the causative agent of brucellosis in small ruminants. This is an economically important disease endemic in many sheep and goat-raising countries worldwide. Infection spreads rapidly among flocks causing abortion storms and chronic disease. While goats are considered the true natural hosts of *B. melitensis*, many breeds of sheep are also highly susceptible (Alton, 1990a). In much of the Mediterranean basin and Middle East, *B. melitensis* is also the *Brucella* species most commonly isolated from bovine, camelid, and equine hosts (Refai, 2002).

Many of the *Brucella* species are highly pathogenic in humans, with *B. melitensis* typically cited as the agent responsible for the majority of human cases (Pappas et al., 2005). Brucellosis is one of the most common zoonotic diseases worldwide with over half a million new cases reported annually (Pappas et al., 2006). Infection most commonly results from ingestion of unpasteurized milk or contact with infected animals, and a chronic debilitating disease often develops. Acute febrile illness is followed by focal disease affecting the joints, reproductive organs, nervous system, and rarely the heart (Franco et al., 2007, Reguera et al., 2003, Troy et al., 2005).

*Brucella melitensis* was the first of the brucellae discovered, isolated from soldiers with Mediterranean fever on the island of Malta in 1887 and later from goats on the same island. Extensive research on the development of diagnostic tests and a suitable vaccine was pursued in Malta in the first half of the 20\(^{th}\) century. France and Russia also experienced outbreaks of *B. melitensis* in the early 1900s and research centers were established in these nations as well (Alton, 1990b). While early accounts of the pathogenesis of *B. melitensis* infection in goats may have been published in French or Russian, there is a paucity of information currently available on basic clinical aspects of disease in goats.
Considerable advances have recently been made in the field of brucellosis research, but most studies have been conducted in mouse models. Mice are naturally resistant to Brucella infection (Grillo et al., 2012). Study of reproductive lesions, tissue colonization, and shedding of organism necessitates a ruminant host, as these aspects of disease pathogenesis cannot be extrapolated from mouse models. Studies performed in ruminants, however, have focused primarily on B. abortus infection in cattle (Ackermann et al., 1988, Alexander et al., 1981, Capparelli et al., 2009, Emminger & Schlam, 1943, Manthei, 1968, Xavier et al., 2009). When goats have been utilized in experimental infection studies, this has primarily been as a model for B. abortus infection (Anderson et al., 1986a, Anderson et al., 1986b, Meador et al., 1989, Meador & Deyoe, 1986, Meador et al., 1988).

While the study of B. abortus infection in the goat may be an appropriate model for bovine brucellosis (Meador & Deyoe, 1986), there is little evidence available to show that it is a good representation of B. melitensis infection in goats. Brucella abortus only rarely causes disease in small ruminants in a natural setting. There are also clear differences between B. abortus and B. melitensis from a public health and disease control perspective. Brucella melitensis is arguably a more frequent cause of human disease than B. abortus. The control of disease in humans depends on the control of disease in livestock populations; however, B. melitensis eradication efforts have proven more difficult than control of B. abortus. No major country with endemic B. melitensis infection has been able to successfully eradicate this pathogen (Olsen & Palmer, 2014).

Control of caprine and ovine brucellosis is currently dependent on vaccination with B. melitensis strain Rev. 1 as well as test and slaughter programs. Rev. 1 is a live, attenuated strain with unknown mutations. The vaccine was created in 1957 by sequential passage of a wild type B. melitensis strain in streptomycin-containing media until a streptomycin-resistant strain was isolated (Elberg & Faunce, 1957). The resulting isolate, Rev. 1, was found to have reduced virulence and to protect against infection in small ruminants. Much of what is known of the pathogenesis of B. melitensis infection in small ruminants comes from research on the efficacy of the Rev. 1 vaccine. These studies were conducted in Malta and Iran in the 1950s and ‘60s (Alton et al., 1967, Entessar et al., 1967, Elberg & Faunce, 1957, Alton, 1968). While the primary goal of the work was to determine the rate of abortion in goats vaccinated with Rev. 1 and subsequently challenged with virulent B. melitensis, information on the pathogenesis of the attenuated Rev. 1 strain was also published. Study designs that utilized un-vaccinated goats as
controls in *B. melitensis* challenge studies also allowed some information on the pathogenesis of fully virulent *B. melitensis* to be inferred. These early studies, however, used variable infection doses and routes of inoculation or simply challenged animals by exposing them to “donors,” which were infected with *B. melitensis*. Over the past 20 years, advances in genetic techniques have sparked a new wave of *B. melitensis* vaccination studies in goats (Edmonds *et al.*, 2002, Elzer *et al.*, 1998, Elzer *et al.*, 2002, Phillips *et al.*, 1997, Roop *et al.*, 2001, Perry *et al.*, 2010). As with the early work, unvaccinated controls are often utilized in these vaccination and challenge studies. Use of standard infection doses and routes of exposure have allowed important information to be inferred on the pathogenesis of *B. melitensis* in the goat. Rates of abortion and colonization have been published, but since the objective of these studies is to access efficacy of new vaccine candidates, key elements of *B. melitensis* pathogenesis remain undefined. The few strict pathogenesis studies published on virulent *B. melitensis* have been conducted in sheep (Shimi & Tabatabayi, 1981, Tittarelli *et al.*, 2005), while goats are more susceptible to infection (Alton, 1990a).

The goal of the present research was to study the pathogenesis of *B. melitensis* infection in goats by comparing disease caused by the fully virulent *B. melitensis* strain 16M and the reduced virulence strain Rev. 1. Objectives were to (1) determine the clinical effects of infection by each strain including rate of abortion, changes in body temperature, and changes in hematological parameters, (2) evaluate tissue distribution and level of colonization resulting from infection, (3) quantify shedding of *B. melitensis* 16M and Rev. 1 in milk and uterine secretions, and (4) evaluate the humoral immune response triggered in response to infection, especially the stage of infection during which antibodies are first detected.

### 3.2) Materials and Methods

#### 3.2a) Experimental Design

**i) Animals**

Fifteen mixed-breed female goats (aged 2-9 years) were utilized in this study. The animals had not been vaccinated previously with any *Brucella* vaccines and were seronegative prior to challenge. The estrous cycles of the does were synchronized using two injections, 10 days apart of prostaglandin F2α (Lutalyse, Zoetis, Inc.), followed by mating to a fertile male. All animals were found to be pregnant by ultrasound examination at either 44
or 88 days of gestation. Pregnant goats were transferred to a biosafety level 3 containment facility at least one week prior to experimental challenge, where they were housed for the remainder of the study. All animals were fed a complete pelleted diet and hay daily with nutritional supplements added in late gestation. Animals were group-housed in rooms of adequate size (2-4 goats per room), and all stages of the study were conducted with consideration for animal welfare. The experimental procedures were approved by the Institutional Animal Care and Use Committee of Colorado State University.

ii) Experimental Challenge

This study was carried out over the course of 2 years with 5 goats challenged in March 2014 (group 1) and the remaining 10 animals challenged in January 2015 (groups 2-4). Challenge was conducted at either 11 (group 1) or 14 weeks (groups 2 and 3) of gestation. Since animals within each group were housed together, factors such as body size, presence of horns, and attitude were considered when assigning animals to groups 2-4 in the 2015 study. Animals were infected by instillation of $8 \times 10^6 - 8 \times 10^7$ CFU of *Brucella* onto their conjunctiva (50 μl of inoculum per eye). The infection dose was determined by serial dilution of the inoculum in saline and standard plate count on brain heart infusion (BHI) agar plates. Nine animals (5 goats in 2014 and 4 goats in 2015) were challenged with virulent *B. melitensis* strain 16M (groups 1 and 2), and 4 animals were challenged with the attenuated vaccine strain *B. melitensis* Rev. 1 (group 3). The remaining two goats were kept as uninfected controls (group 4) in order to determine a normal baseline for clinical and serological results. Animals were monitored closely for clinical signs of infection, such as changes in mentation and elevation of body temperature. Temperature was measured either per rectum or via an iButton (Maxim Integrated, San Jose, CA), which were administered *per os* prior to the 2015 study and then subsequently recovered at necropsy from the reticulum. The iButtons were programmed to record core temperature every 4 hours. In goats from which rectal temperature was recorded, measurements were taken prior to infection to establish a baseline, once daily for the first 2 weeks post-infection, and then weekly until time of parturition.
3.2b) Sampling Procedures

i) Samples from Does

Blood samples were collected by jugular venipuncture from all animals prior to challenge and following challenge on days 3, 7, 14, 21, 28, 35, and at time of parturition. Blood samples were utilized for bacteriological culture, assessment of humoral immune response, and complete blood count analysis (CBC). Aseptic technique was utilized during venipuncture to prevent contamination of samples for culture.

Approximately 10 ml of milk was collected on the day of parturition and over each of the subsequent 3 days. Vaginal swabs were collected on the day of parturition and at necropsy. All does were humanely euthanized 3 days following abortion or normal parturition by intravenous administration of pentobarbital. Maternal samples collected aseptically at necropsy for bacteriological determination included: spleen, liver, lung, mammary tissue, uterine caruncle, muscle, and lymphatic tissue (bronchial, mediastinal, hepatic, internal iliac, mesenteric, mandibular, parotid, retropharyngeal, prescapular, and supramammary lymph nodes).

ii) Samples from Kids

Fetuses were necropsied within 24 hours of abortion. Live kids were kept together with the group of does and allowed to nurse until euthanasia and necropsy at 3 days of age. Samples collected at necropsy included spleen, liver, lung, abomasum, abomasal contents, and rectal swab. Placental tissue was also collected for culture.

3.2c) Bacteriological Tests

Culture of blood, milk, vaginal swabs, fetal rectal swabs, and all tissue samples was performed on selective medium agar formulated from *Brucella* medium base (Oxoid CM0169), *Brucella* selective supplement (Oxoid SR0083A), and 10% fetal bovine serum. All cultures were incubated at 37 °C and 5% CO₂. In most cases, growth was observed in 3-4 days. *Brucella* cultures were identified based on colony morphology and growth characteristics. Isolates from Rev. 1 infected goats were subcultured onto BHI agar containing streptomycin (2.5 μg/ml) and incubated for 2 weeks at 37 °C in air. This allowed for the differentiation of Rev. 1 and 16M strains since only Rev. 1 is capable of growing on streptomycin (World Organization for Animal Health, 2009b).
i) **Blood Culture**

At each time point, 10 ml of whole blood was collected in EDTA for culture. This samples were diluted 1:1 in BHI broth containing amphotericin B (1 μg/ml), vancomycin (20 μg/ml), and sodium citrate (1% v/v) (World Organization for Animal Health, 2009a). Of this dilution, 100 μl was plated directly on the *Brucella* selective media described above. The remaining enrichment culture was maintained at 37 °C with weekly subcultures performed for 4 weeks. Plates were examined regularly for growth and declared negative if no colonies were observed after one month.

ii) **Milk**

Milk samples were centrifuged (13,000 × g, 5 min) and cream and precipitate layers combined for culture. Serial dilutions of the cream/precipitate mixture were plated on *Brucella* selective media for semi-quantitative determination of shedding.

iii) **Vaginal and Rectal Swabs**

Vaginal and fetal rectal swabs were directly streaked on *Brucella* selective media. In these samples shedding was assessed by the presence/absence of growth rather than quantification of colony counts.

iv) **Tissues**

Tissue samples weighing approximately 0.1 g were added to 2 ml snap-top tubes containing 900 μl of homogenization media (BHI broth with 10% glycerol) and stainless steel beads. Samples were homogenized using a mixer mill (Retsch Mixer Mill MM 400, Düsseldorf, Germany) for 5 min at a frequency of 25 Hz. Colonization (CFU/g) of tissues was determined by plating serial dilutions of tissue homogenate on *Brucella* selective media. The homogenate consisted of a 1:10 dilution of tissue and additional dilutions were subsequently made extending to 10⁻⁸.

3.2d) **Serological Tests**

Sera were tested for antibodies to *Brucella* antibodies using the Card Test (National Veterinary Services Laboratories [NVSL], Ames, IA, USA) performed with *B. abortus* antigen of 3% cell concentration according to the
protocol provided by NVSL. Briefly 30 μl of serum was mixed with an equal volume of antigen suspension and the degree of agglutination determined after a 4 min incubation period.

3.2c) Complete Blood Count Analysis

Blood from a subset of *B. melitensis* 16M-infected goats was subjected to CBC analysis (HemaTrue Hematology Analyzer, Heska, Loveland, CO, USA) throughout the course of infection. Four measurements were taken prior to experimental challenge to establish normal baseline values, and parameters were subsequently measured on days 3, 7, 14, and 35 post-infection as well as at the time of parturition.

3.3) Results

3.3a) Clinical Results

i) Abortion

Pregnant does were infected with either the fully virulent *B. melitensis* strain 16M (groups 1 and 2) or the attenuated strain Rev. 1 (group 3). Differences between the groups are described in Table 3.1. Six of the 7 (86%) pregnant does infected with *B. melitensis* 16M aborted. The remaining animal (No. 9) delivered one dead and one live full-term kid. While the live kid (No. 9-2) was weak, it survived until necropsy at 3 days post-infection. Of the 4 animals infected with the attenuated *B. melitensis* strain Rev. 1, all delivered full-term kids. A gestation length of 21 weeks was used to define full-term birth. Two (29%) of the full-term kids from the Rev. 1 group, both from the same doe (No. 12), were dead at birth. Another kid (No. 10-1) was found dead 3 days after birth, however, whether death was due to *Brucella* infection is unknown. Three does (No. 5, 7, and 11) showed no evidence of abortion and were not pregnant at necropsy. These animals were initially determined to be pregnant by ultrasound observation prior to challenge. Uninfected controls remained healthy and delivered live, full-term kids. Median time between challenge and parturition was 42.5 days (range 34-64), 36 days (range 35-53), and 55 days (range 53-58) for goats in groups 1, 2, and 3, respectively. Goats in group 2 that were infected with 16M were further along in gestation than the goats in group 1 at time of parturition; however, group 2 was also infected at a later stage of gestation.
Table 3.1. Effect of *B. melitensis* 16M and Rev. 1 on parturition in goats.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Doe No.</th>
<th>Challenge Strain</th>
<th>Challenge Dose (CFU)</th>
<th>Week of Gestation at Challenge</th>
<th>Week of Gestation at Parturition</th>
<th>Kid No.</th>
<th>Fetus Condition</th>
<th>Isolation of <em>B. melitensis</em> at Parturition</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>16M</td>
<td>8 x 10^6</td>
<td>11</td>
<td>17</td>
<td>1-1</td>
<td>Dead</td>
<td>+</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-2</td>
<td>Dead</td>
<td>+</td>
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<tr>
<td></td>
<td>2</td>
<td>16M</td>
<td>8 x 10^6</td>
<td>11</td>
<td>16</td>
<td>2-1</td>
<td>Dead</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2-2</td>
<td>Dead</td>
<td>+</td>
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<tr>
<td></td>
<td>3</td>
<td>16M</td>
<td>8 x 10^6</td>
<td>11</td>
<td>20</td>
<td>3-1</td>
<td>Dead</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3-2</td>
<td>Dead</td>
<td>+</td>
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<td>4</td>
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<td>8 x 10^6</td>
<td>11</td>
<td>17</td>
<td>4-1</td>
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<td>+</td>
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<tr>
<td></td>
<td>5</td>
<td>16M</td>
<td>8 x 10^6</td>
<td>11</td>
<td>NP^a</td>
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<td>8 x 10^6</td>
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<td>19</td>
<td>6-1</td>
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<td>+</td>
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<tr>
<td></td>
<td>7</td>
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<td>14</td>
<td>NP^b</td>
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<tr>
<td>2</td>
<td>8</td>
<td>16M</td>
<td>8 x 10^6</td>
<td>14</td>
<td>19</td>
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<td>Dead</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td>8-2</td>
<td>Dead</td>
<td>NC</td>
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<td></td>
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<td>+</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>9-2</td>
<td>Live,Weak</td>
<td>+</td>
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<tr>
<td>3</td>
<td>10</td>
<td>Rev. 1</td>
<td>8 x 10^7</td>
<td>14</td>
<td>21</td>
<td>10-1</td>
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<td>8 x 10^7</td>
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<td>NP^b</td>
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<tr>
<td></td>
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<td>Rev. 1</td>
<td>8 x 10^7</td>
<td>14</td>
<td>22</td>
<td>12-1</td>
<td>Dead</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
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<td>+</td>
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<tr>
<td></td>
<td>13</td>
<td>Rev. 1</td>
<td>8 x 10^7</td>
<td>14</td>
<td>21</td>
<td>13-1</td>
<td>Live</td>
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<td></td>
<td></td>
<td>13-3</td>
<td>Live</td>
<td>-</td>
</tr>
</tbody>
</table>

^a NP = not pregnant at time of necropsy despite being declared pregnant on ultrasound at 6 weeks of gestation  
^b NP = not pregnant at time of necropsy despite being declared pregnant on ultrasound at 12 weeks of gestation  
^c Kid 10-1 was found dead 3 days after parturition  
^d + = *B. melitensis* isolated, − = *B. melitensis* not isolated, NC = sample not collected

ii) Body Temperature

After a normal baseline was established, rectal temperature was recorded for goats in group 1 once daily for the first 2 weeks post-infection and then weekly until time of parturition. Normal temperature was defined as 100 – 104 °F. Rectal temperatures remained within the normal range in these five 16M-infected goats throughout the course of the study (Figure 3.1a).

Use of iButtons for measurement of core temperature every 4 hours allowed a more thorough examination of changes in body temperature. However, despite applying lead sinkers to the iButtons in an attempt to maintain them within the rumen-reticulum, the devices were passed by many of the goats and excreted in feces. The iButtons were successfully utilized in one group 2 doe (No. 9) and two group 3 does (No. 10 and 11). In these goats core
body temperatures were highly variable and often fell outside the normal range (Figure 3.1b). This was not exclusively an effect of infection, as temperatures above 104 °F were also observed prior to challenge in does 10 and 11. While iButtons were utilized in only a small number of animals, an interesting trend was noted. Core body temperatures of Rev. 1 vaccinated goats (No. 10 and 11) seemed to fluctuate around a set point unique to each of the does for the duration of the study. In a 16M-infected goat (No. 9), however, a general increase in body temperature was observed from the time of challenge to the time of parturition. The fever was remittent in nature. The sharp spike in temperature observed in Figure 3.1b corresponds to a reading of 108.7 °F, which occurred 2 days after parturition in doe 9.

### iii) Complete Blood Count Analysis

Hematology was utilized in group 1 animals to evaluate whether any changes in the leukogram consistently occurred that could be indicative of infection. Analysis revealed that 3 of 5 animals had marked leukocytosis and neutrophilia prior to challenge. Median leukocyte count over the 4 pre-infection time points in these animals (No. 2, 4, and 5) was $18.6 \times 10^3$ cells/µl (range $11.7 – 25.1 \times 10^3$ cells/µl). Median granulocyte count in the same animals was $10.0 \times 10^3$ cells/µl (range $7.1 – 17.6 \times 10^3$ cells/µl). These cell concentrations were nearly double those measured in the other 2 animals (No. 1 and 3), which maintained total leukocyte and granulocyte counts of $9.6 \times 10^3$ cells/µl and $5.6 \times 10^3$ cells/µl, respectively. Pre-infection lymphocyte and monocyte parameters fell within the normal range for all goats. Leukocytosis and neutrophilia in does 2, 4, and 5 is suggestive of pre-existing infection.

Analysis of the leukogram at days 3, 7, and 14 post-infection showed a decrease in both total leukocyte count and granulocyte count among the two does (No. 1 and 3) with normal pre-infection parameters. For example, the leukocyte count of doe 1 decreased from $9.7 \text{ to } 7.8 \times 10^3$ cells/µl over the first 2 weeks of infection and the animal’s granulocyte count fell from $6.3 \text{ to } 4.1 \times 10^3$ cells/µl. The lymphocyte count was also decreased from pre-infection measurements at days 3 and 7 post-infection, but had increased above baseline levels by 14 days post-infection. Monocyte numbers changed little over the first two weeks of infection. A postpartum measurement was also taken for doe 3, which revealed a neutrophilic leukocytosis relative to pre-infection values (leukocyte concentration of $12.2 \times 10^3$ cells/µl and granulocyte concentration of $8.7 \times 10^3$ cells/µl).
Figure 3.1. Effect of *B. melitensis* 16M and Rev. 1 on body temperature in pregnant does. Temperature was measured by rectal thermometer (A) or gastric iButton (B). Does 1-5 (A) and 9 (B) were infected with $8 \times 10^6$ CFU of 16M. Does 10 and 11 (B) were infected with $8 \times 10^7$ CFU of Rev. 1.
3.3b) Bacteriological Results

i) Does

*Brucella melitensis* was isolated from tissue samples or milk of all 13 infected does, indicating that experimental challenge was successful in all animals. The three does (No. 5, 7, and 11) that showed no evidence of abortion and were not pregnant at necropsy had minimal tissue colonization. In the non-pregnant 16M-infected does, *Brucella* was isolated from the spleen and mandibular, retropharyngeal, and internal iliac lymph nodes of one animal (No. 5) and exclusively the hepatic lymph node of the other animal (No. 9). Colonization in these tissues was minimal (100 – 400 CFU/g of tissue). The non-pregnant Rev. 1-challenged doe (No. 11) showed no evidence of systemic infection. A small volume of serous fluid was collected from this animal’s teat at necropsy, and the “milk” was culture positive (30 CFU/ml).

A similar colonization pattern was observed in a pregnant Rev. 1-infected goat. *Brucella melitensis* was not cultured from any tissue from doe 10 including the mammary gland and associated lymph nodes, uterus, and placenta, yet this doe exhibited low level excretion (10 CFU/ml) of Rev. 1 in milk on days 2 and 3 postpartum, shed organisms in vaginal secretions, and one kid showed low levels of colonization (Table 3.1). Likewise, another Rev.1-infected goat (No. 13) showed minimal colonization with just 100 CFU/g and 10 CFU/ml of *B. melitensis* isolated from uterine tissue and milk, respectively. Rev. 1 does have the capacity to cause significant generalized infection as shown by the final Rev. 1-infected animal (No. 12). *Brucella* was isolated from 9 different maternal tissues, placenta, and milk of this animal with high levels of colonization noted. A higher concentration of *B. melitensis* was present in the placenta of this animal than in any of the 16M-infected goats. All isolates from Rev. 1-challenged goats grew on streptomycin-containing media, confirming that no 16M contamination occurred in group 3 goats. Rate of *B. melitensis* isolation and tissue burden in pregnant animals is shown in Table 3.2 and Figure 3.2.

Generalized infection was present in all pregnant 16M-challenged goats, with rates of isolation varying from 4/16 to 15/15 tissues in the 7 goats sampled. A trend of higher rates of colonization, in terms of both number of *Brucella*-positive tissues and degree of tissue burden, was observed in animals challenged at week 11 of gestation (group 1) compared to does challenged at 14 weeks (group 2) (Figure 3.2).
In 16M-infected goats, *B. melitensis* displayed a definite preference for various tissues. Interestingly, major organs such as liver, spleen and lung showed low levels of colonization. *Brucella* was more frequently isolated from lymph nodes, with all 7 pregnant goats showing colonization of the internal iliac lymph node. Lymph nodes of the head, which drained the site of inoculation, as well as the hepatic lymph node, the mesenteric lymph node, and the supramammary lymph node also showed high rates of colonization. Uterine and placental infection were noted in all pregnant 16M-infected goats, and these two tissues were the most heavily colonized, with *B. melitensis* at concentrations of $10^5 – 10^{10}$ CFU/g of tissue. Concentration of organism in lymph node tissue was typically in the range of $10^3 – 10^5$ CFU/g of tissue. Unexpectedly, muscle tissue was positive in 2 of 3 pregnant 16M-challenged goats from which cultures were taken with tissue load similar to that in lymph nodes. While only performed in group 1 goats, all blood cultures were negative throughout the course of infection (cultures taken on days 3, 7, 14, and 28 post-infection and day 1 postpartum).

**Table 3.2.** Rate of isolation of *B. melitensis* 16M and Rev. 1 from tissues of pregnant does at necropsy.

<table>
<thead>
<tr>
<th>Tissue</th>
<th><em>B. melitensis</em> 16M Infected Animals&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>B. melitensis</em> Rev. 1 Infected Animals&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>2/3</td>
<td>1/3</td>
</tr>
<tr>
<td>Liver</td>
<td>2/7</td>
<td>1/3</td>
</tr>
<tr>
<td>Spleen</td>
<td>1/7</td>
<td>1/3</td>
</tr>
<tr>
<td>Lung</td>
<td>2/7</td>
<td>0/3</td>
</tr>
<tr>
<td>Mammary Tissue</td>
<td>4/7</td>
<td>0/3</td>
</tr>
<tr>
<td>Uterus</td>
<td>7/7</td>
<td>2/3</td>
</tr>
<tr>
<td>Supramammary LN</td>
<td>5/7</td>
<td>1/3</td>
</tr>
<tr>
<td>Parotid LN</td>
<td>3/7</td>
<td>1/3</td>
</tr>
<tr>
<td>Mandibular LN</td>
<td>7/7</td>
<td>1/3</td>
</tr>
<tr>
<td>Retropharyngeal LN</td>
<td>4/7</td>
<td>1/3</td>
</tr>
<tr>
<td>Prescapular LN</td>
<td>3/7</td>
<td>0/3</td>
</tr>
<tr>
<td>Mediastinal LN</td>
<td>3/7</td>
<td>0/3</td>
</tr>
<tr>
<td>Bronchial LN</td>
<td>3/4</td>
<td>0/2</td>
</tr>
<tr>
<td>Hepatic LN</td>
<td>5/7</td>
<td>0/3</td>
</tr>
<tr>
<td>Internal Iliac LN</td>
<td>7/7</td>
<td>1/3</td>
</tr>
<tr>
<td>Mesenteric LN</td>
<td>4/7</td>
<td>0/3</td>
</tr>
<tr>
<td>Placenta</td>
<td>5/5</td>
<td>1/3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Only data from pregnant animals are included in the table.
Figure 3.2. Level of tissue colonization by *B. melitensis* 16M and Rev. 1 in pregnant does. Colonization is measured in CFU of *B. melitensis* 16M (groups 1 and 2) or Rev. 1 (group 3) per gram of tissue. Level of detection is 100 CFU. Data from does 5, 7, and 11 are not included since these animals were not pregnant.

*Brucella melitensis* 16M was shed at high levels in milk of all pregnant animals challenged with this virulent strain (Figure 3.3). Three does were consistent, high level shedders with *B. melitensis* present in milk at a concentration of $10^3 – 10^6$ CFU/ml throughout the 4 days of sampling. The remaining 4 does showed a declining trend in the degree of shedding postpartum. In these animals *Brucella* was shed at a concentration of $10^3 – 10^4$ CFU/ml in colostrum, but by day 4 numbers of organisms were near the limit of detection. Shedding in milk was minimal in all but one of the Rev. 1-infected goats. This doe (No. 12) excreted organism at levels similar to goats infected with *B. melitensis* 16M. Shedding in vaginal secretions occurred in all 16M-infected goats on both days 1 and 3 postpartum. In Rev. 1-infected does shedding was observed in 2/3 animals, but at lower levels.
ii) Kids

A 92% fetal infection rate was observed among kids born to *B. melitensis* 16M-infected does (*Table 3.3*). Infection was systemic; organism was isolated from liver, spleen, lung, and abomasum in all animals. Tissue burden ranged from $10^3$ – $10^9$ CFU/g of tissue with severely autolyzed fetuses typically having lower concentrations of *Brucella*.

Infection rate among kids born to *B. melitensis* Rev. 1-infected does was 43%. Doe 12, which showed the high levels of maternal, uterine, and mammary infection gave birth to two dead, heavily infected kids. Low level infection was also observed in one kid born to doe 10. *Brucella melitensis* Rev. 1 was isolated from the spleen and abomasal fluid of this kid at concentrations of 100 and 900 CFU/g, respectively. The animal was also potentially shedding organism in feces since *Brucella* was cultured from a rectal swab.
Table 3.3. Isolation of *B. melitensis* 16M and Rev. 1 from fetal tissues at time of parturition.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Kid No.</th>
<th>Fetus Condition</th>
<th>No. of samples containing <em>B. melitensis</em></th>
<th>Tissue Burden (CFU/g)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Liver</td>
<td>Spleen</td>
</tr>
<tr>
<td>1-1</td>
<td>1-1</td>
<td>Dead</td>
<td>6/6</td>
<td>+ + + + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>Dead</td>
<td>6/6</td>
<td>++ + + + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>2-1</td>
<td>2-2</td>
<td>Dead</td>
<td>6/6</td>
<td>+ + + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td></td>
<td>3-1</td>
<td>Dead</td>
<td>4/4</td>
<td>++ + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>3-2</td>
<td>3-2</td>
<td>Dead</td>
<td>4/4</td>
<td>+++ + +++ + +++ + +++ + +++ + +++ + +++ + +++ + + + + + + +</td>
</tr>
<tr>
<td>4-1</td>
<td>4-1</td>
<td>Dead</td>
<td>6/6</td>
<td>+ + + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>5-1</td>
<td>5-1</td>
<td>Live, Weak</td>
<td>6/6</td>
<td>+ + + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>6-1</td>
<td>6-1</td>
<td>Dead</td>
<td>6/6</td>
<td>+ + + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>8-1</td>
<td>8-1</td>
<td>Dead</td>
<td>6/6</td>
<td>+++ + +++ + +++ + +++ + +++ + +++ + +++ + +++ + + + + + + +</td>
</tr>
<tr>
<td>9-1</td>
<td>9-1</td>
<td>Dead</td>
<td>6/6</td>
<td>+++ + +++ + +++ + +++ + +++ + +++ + +++ + +++ + + + + + + +</td>
</tr>
<tr>
<td>9-2</td>
<td>9-2</td>
<td>Live, Weak</td>
<td>0/6</td>
<td>-- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- --</td>
</tr>
<tr>
<td>10-1</td>
<td>10-1</td>
<td>Live&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3/6</td>
<td>-- + -- -- -- -- -- -- -- -- -- -- -- -- --</td>
</tr>
<tr>
<td>10-2</td>
<td>10-2</td>
<td>Live</td>
<td>0/6</td>
<td>-- -- -- -- -- -- -- -- -- -- -- -- -- -- -- --</td>
</tr>
<tr>
<td>12-1</td>
<td>12-1</td>
<td>Dead</td>
<td>6/6</td>
<td>+ + + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>12-2</td>
<td>12-2</td>
<td>Dead</td>
<td>5/5</td>
<td>+++ + +++ + +++ + +++ + +++ + +++ + +++ + +++ + + + + + + +</td>
</tr>
<tr>
<td>13-1</td>
<td>13-1</td>
<td>Live</td>
<td>0/6</td>
<td>-- -- -- -- -- -- -- -- -- -- -- -- -- -- -- --</td>
</tr>
<tr>
<td>13-2</td>
<td>13-2</td>
<td>Live</td>
<td>0/6</td>
<td>-- -- -- -- -- -- -- -- -- -- -- -- -- -- -- --</td>
</tr>
<tr>
<td>13-3</td>
<td>13-3</td>
<td>Live</td>
<td>0/6</td>
<td>-- -- -- -- -- -- -- -- -- -- -- -- -- -- -- --</td>
</tr>
</tbody>
</table>

<sup>a</sup> Kid 10-1 was found dead three days after parturition

<sup>b</sup> += 1 x 10<sup>2</sup> – 1 x 10<sup>4</sup> CFU/g
++ = 1 x 10<sup>3</sup> – 1 x 10<sup>5</sup> CFU/g
+++ = 1 x 10<sup>5</sup> – 1 x 10<sup>7</sup> CFU/g
++++ = 1 x 10<sup>7</sup> – 1 x 10<sup>9</sup> CFU/g
− = *B. melitensis* not isolated, limit of detection = 1 x 10<sup>2</sup> CFU/g
L = lawn
G = some growth
NC = sample not collected

3.3c) Humoral Immune Response

Anti-*Brucella* antibodies were present in all 13 challenged does (Table 3.4). Seroconversion was delayed in the non-pregnant 16M-challenged animals, occurring at 61 and 70 days post-infection. In pregnant animals challenged with virulent *B. melitensis*, median time to seroconversion was 21 days (range 14-64 days); however, 2 animals did not seroconvert until after abortion. All *B. melitensis* Rev. 1-challenged does were seropositive by 4 weeks post-infection; but titers were transient with only the heavily infected doe (No. 12) still seropositive at time of parturition. The two uninfected controls remained seronegative throughout the duration of the study.
Table 3.4. Rate of seroconversion in *B. melitensis* 16M- and Rev. 1-infected does determined by Card Test.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Weeks after Challenge</th>
<th>Post-Parturition / At Time of Necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>B. melitensis</em> 16M Infected</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td><em>B. melitensis</em> Rev. 1 Infected</td>
<td>0/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

\(^a\) Only group 1 animals tested.
\(^b\) Only group 2 animals tested.
\(^c\) NT = not tested

3.4) Discussion

The present study provides a thorough assessment of the clinical effects resulting from *B. melitensis* infection in goats, a natural host. Rates of abortion and fetal death, distribution of organism in tissues, and levels of shedding were investigated. Measurement of body temperature, leukogram parameters, and serological reaction were analyzed for the ability to indicate potential infection. By characterizing these aspects of disease pathogenesis in goats infected with both *B. melitensis* strain 16M and strain Rev. 1, the study provides a starting point from which differences in virulence factors or host immune response can be compared between the two strains.

3.4a) Clinical Disease and Diagnosis of Infection

One objective of the present study was to identify early clinical signs that would stimulate suspicion of infection and subsequent testing for brucellosis. An obvious component of brucellosis control is the identification of infected animals. This is a critical first step in test and slaughter programs; however, even in resource poor countries identification of infected animals allows herders to take proper protective measures to prevent spread of disease. Optimally, diagnosis of *Brucella* infection would be made prior to parturition before massive shedding of organism occurs in vaginal fluids, fetal tissues, and milk.

Routes of infection and doses of exposure are variable in the field and likely influence clinical outcome. Due to difficulties in replicating these factors in a laboratory setting as well as a need to make the findings of this study comparable to previous work, a standard experimental infection dose and route was utilized (Olsen & Johnson, 2011, Elzer *et al.*, 2002). The intended challenge dose was $1 \times 10^7$ CFU for both 16M and Rev. 1 strains.
Serial dilution and standard plate counts of the inocula indicated that infection was actually with $8 \times 10^6$ CFU and $8 \times 10^7$ CFU of 16M and Rev. 1, respectively. The objective was to use Rev. 1 at a dose that was both similar to 16M and comparable to that utilized in vaccination. The standard dose of the Rev. 1 vaccine is $1 \times 10^9$ CFU; however, many countries utilize a reduced dose vaccine, often at a $1 \times 10^6$ concentration (Blasco, 1997).

In pregnant goats challenged by these methods, infection was inapparent for a minimum of 2-3 weeks. In two animals, we were even uncertain if the inocula were successfully administered until time of abortion and tissue culture. In these animals there was no clear indication of infection based on clinical signs, hematology, bacteriology, or serology until after abortion occurred. This underscores the challenges in diagnosing infection.

Although brucellosis in humans is characterized by remittent fevers, which provide an early indication of infection (Franco et al., 2007), body temperature is not typically monitored in ruminant experimental infection studies. In the present study, an obvious elevation in body temperature was absent throughout the course of infection, at least by rectal thermometer recordings. The use of iButtons allowed for nearly continuous monitoring of core temperature without the induction of possible temperature spikes during capture. By these methods, a 16M-infected goat was found to have remittent fever starting at approximately 3 weeks post-infection. In *B. melitensis*-infected sheep, slight elevations in body temperature have been reported around time of abortion (Shimi & Tabatabayi, 1981). Increase in body temperature in the first 10 days after Rev. 1 vaccination have also previously been reported in goats (Elberg & Faunce, 1957).

In addition to body temperature, hematology is often used in ruminants to assess general health and could be an early indicator of possible infection. While changes in leukogram parameters, especially total leukocyte and granulocyte numbers, were observed during acute stages of *B. melitensis* 16M infection, interpretation of these changes was made difficult by underlying infections that were present in many of the animals. Two of three animals with marked neutrophilic leukocytosis prior to challenge had evidence of caseous lymphadenitis at necropsy. This common disease of small ruminants is known to cause neutrophilia (Morris, 2009). While use of specific-pathogen-free animals would make interpretation of any alternations in the leukogram post *B. melitensis* infection more clear, co-infection is commonplace within animals in a field setting.
Analysis of leukograms from two 16M-infected animals with normal pre-infection parameters showed decreases in total leukocyte, granulocyte, and lymphocyte counts over the first week of infection. While lymphocyte numbers rebounded by week 2, granulocyte counts remained low. Changes in leukogram parameters post-infection were small, however, and remained within published normal ranges. Thus, continuous monitoring would be necessary to detect deviations from normal values and create suspicion for infection. This makes the leukogram less useful in a diagnostic setting. From a research perspective, however, the prolonged decrease in total leukocyte and granulocyte counts observed in some animals from days 3-14 post-infection compared to pre-infection values is noteworthy. Although ruminants have small functional reserves of neutrophils in bone marrow, the neutropenic response that often follows acute bacterial infection typically resolves within 4 days of infection (Morris, 2009). This was not observed in the present study and suggests that Brucella infection induces an altered immune response. Humans with acute brucellosis also have normal to decreased neutrophil counts, which may be explained by the ability of *B. abortus* to cause apoptosis of human neutrophils *in vitro* (Barquero-Calvo *et al.*, 2015, Troy *et al.*, 2005).

*Brucella*-specific diagnostic tests also did not provide evidence of infection prior to abortion in all animals. Blood cultures failed to detect *Brucella* in any of the animals sampled. The inability to detect bacteremia may have been due to insufficient sampling volume or frequency, as tissue culture results at necropsy indicated that bacteremia had obviously occurred. Previous work has shown about 25% and 50% of *B. melitensis*-infected goats to be bacteremic at 2 and 3 weeks post-infection, respectively (Elberg & Faunce, 1957). While circulating antibodies were more easily detected than brucellae in the present study, diagnosis of infection by serology in all animals prior to abortion was still not possible. Only 50% of pregnant 16M-infected does had circulating anti-*Brucella* antibodies detectable by the Card Test on week 3 post-infection, and 2 animals did not seroconvert until after abortion. Delay in seroconversion until after abortion has previously been noted in cattle (Thomsen, 1950, O’Hara & Christiansen, 1978). Extensive investigation of abortions in New Zealand during the 1970’s indicated that in 13% of cows, *Brucella* infection could only be diagnosed by culture. In many underdeveloped areas where brucellosis is endemic, however, bacteriological culture is not possible. In these regions, serological tests are the only means of diagnosis and thus detection of infection may be delayed or missed in some animals.
The primary clinical sign of brucellosis is abortion, which was observed in 86% of *B. melitensis* 16M-infected does in the present study, with 92% of offspring infected and dead at birth. This rate of abortion is within the range cited in recent literature (Elzer *et al.*, 2002, Perry *et al.*, 2010, Olsen & Johnson, 2011). Typically, 70-100% of pregnant goats experimentally infected with *B. melitensis* suffer from abortion, while an abortion rate of only 30-50% is observed in goats and cattle infected with *B. abortus*. The variability in abortion rates observed in *B. melitensis*-infected goats is likely due to a number of factors including animal age and underlying health status as well as the stage of gestation at time of infection. In cattle, the incubation period between infection and abortion is inversely proportional to age of the fetus at time of inoculation (Thomsen, 1950). However, if infection occurs late in gestation, there may be insufficient time for *Brucella* to disseminate to the placenta, replicate extensively, and stimulate the inflammatory response that precedes abortion. This may explain the slight difference in abortion rates between group 1 and 2 goats in the present study. One group 2 animal, which was challenged at 14 weeks of pregnancy, had a full-term parturition with one live, un-infected kid and one dead, heavily-infected kid born. This doe also had the lowest level of placental colonization among animals challenged with 16M. Since placental colonization and the lesions that result are the direct cause of abortion, this likely accounts for the absence of abortion in this doe. While all Rev. 1-infected animals had full-term parturitions, infection was not without clinical effects. Two kids, both from the same doe, were dead at birth and another kid was found dead three days later. It is well known that Rev. 1 retains some virulence in goats and vaccination during pregnancy is not recommended. While the inoculum used in the present study was less than the standard vaccine dose, even reduced dose vaccines are known to cause abortion (Blasco, 1997).

### 3.4b) Pathogenesis of *B. melitensis* 16M and Rev. 1 Infection

*Brucella melitensis* 16M was found to produce generalized infection in all pregnant does, with brucellae recoverable from 15 different tissues in one animal. Predictably, the uterus and placenta showed both the highest rates of colonization (100%) and the highest tissue burdens (up to $5 \times 10^{10}$ and $5 \times 10^{8}$ CFU/g in the uterus and placenta, respectively). Although the goat placenta is thus highly infectious for animals or humans that come into contact with this tissue, it seems that the cow placenta presents a slightly greater risk. Tissue load in the placenta of cows naturally infected with *B. abortus* has previously been reported at $5 \times 10^{11} - 1 \times 10^{13}$ CFU/g and the placenta of a cow is obviously larger in size (Alexander *et al.*, 1981).
Bacteriological culture of tissues from pregnant 16M-infected does also yielded some unexpected results with important public health implications. Muscle tissue was cultured from three does with the initial intention of simply obtaining bacteriological confirmation for the safety of muscle tissue. Generally handling and consumption of raw or undercooked organ meat is considered a potential health risk, but muscle meat has been assumed safe (Corbel, 2006). In 2 of the 3 animals from which muscle cultures were performed, however, *Brucella* was isolated at concentrations comparable to that in organs and lymph nodes \(10^2 - 10^4\) CFU/g. This was repeatable and unlikely to be due to contamination. Many other tissues in these two animals were culture negative, indicating that contamination of culture media and equipment was unlikely. Due to small sample size in the present study, infection of muscle tissue should be further investigated. To the author’s knowledge this is the first report of the isolation of *Brucella* from muscle tissue in ruminants. In humans *Brucella*-associated myositis has been reported and may be under diagnosed (Kojan *et al.*, 2012, Turan *et al.*, 2009). Osteoarticular involvement is common in human patients and brucellae may often colonize the associated musculature.

Data on bacterial colonization in pregnant 16M-infected does also indicated that *B. melitensis* has a preference for lymph nodes over the spleen and liver, which are the two organs most heavily colonized in mice (Grillo *et al.*, 2012). This provides further evidence for differential pathogenesis of *Brucella* in mouse models and natural hosts. The internal iliac lymph node was colonized in all of the pregnant 16M-infected does; this lymph node drains the uterus, likely explaining its high colonization rate. Although mammary tissue and the supramammary lymph nodes were also frequently colonized (rates of 57% and 71%, respectively), tissue burden was not always predictive of shedding in milk.

This discrepancy in tissue burden and shedding was also observed among Rev. 1-infected animals. Three does infected with Rev. 1 had no evidence of mammary infection besides shedding in milk; one animal had no evidence of uterine or placental infection yet shed brucellae in vaginal secretions and gave birth to a kid with low levels of infection; and one doe showed the opposite pattern with uterine infection but no evidence of infection in placental tissue, vaginal secretions, or in three healthy offspring. Shedding in milk was also noted in the absence of abortion or birth of full-term dead offspring. Similar findings have been previously reported in Rev. 1 vaccinated goats and sheep (Alton, 1968, Alton *et al.*, 1967, Entessar *et al.*, 1967). This variability in pathogenesis observed in
Rev. 1-infected animals is of potential public health significance. While Rev. 1 is not highly virulent in humans, Rev. 1 contaminated milk is not of negligible public health risk (Banai, 2002). Shedding observed in the absence of abortion is of potential concern since there would be no clinical suspicion for infection.

While maternal infection, fetal infection, and shedding were absent or minimal in most of the Rev. 1-infected animals, one doe was heavily colonized. In this animal Brucella was isolated from 9 maternal tissues at concentrations similar to that in 16M-infected does. Growth characteristics confirmed that the isolates were indeed Rev. 1. The efficacy of this vaccine strain is in part due to its ability to colonize and temporarily persist in tissues; however, previous studies indicate that by 8 weeks post-inoculation Rev. 1 is minimally present (Elberg & Faunce, 1957). Individual variation and underlying health status may explain the heavy tissue burden observed in the one Rev. 1-infected doe at necropsy. This animal was of poor body condition.

3.4c) Shedding and Public Health

Although milk was cultured for only 4 days postpartum in the present study, several conclusions can be drawn from the data. Few reports exist in the literature on levels of B. melitensis present in milk of infected goats. Here we report shedding in 100% of post-parturient 16M-infected does. Those animals can be divided into two groups, however, with approximately half of the animals being consistent, high level shedders and half showing a declining trend in shedding. Without continued monitoring of Brucella concentrations in milk, it is difficult to infer whether these groups would hold throughout the lactation period. A recent study on water buffalo naturally infected with B. abortus indicates that while most animals intermittently shed brucellae at low levels in milk, 16% of infected animals can be classified as “superspreaders” since they consistently shed high levels of organism (Capparelli et al., 2009). These animals are of considerable public health risk, as well as the primary source of disease in the herd. Culling of superspreaders was found to halt disease transmission. The present study suggests that similar groups of high and low level shedders may exist among B. melitensis-infected goats. Studies in cattle and sheep, however, have shown that early shedding of Brucella is not always predictive of bacterial concentration in milk later in lactation (Morgan, 1960, Shimi & Tabatabayi, 1981). In one B. melitensis-infected ewe, for example, milk was culture negative until day 47 postpartum at which time shedding commenced (Shimi & Tabatabayi, 1981). This was unique among the ewes in this small study, as frequency of shedding decreased over the course of lactation in the
other animals. The opposite trend was noted in an early study of infected cattle, with over 50% of animals showing increased levels of shedding during the latter half of lactation (Morgan, 1960).

The present study clearly demonstrates the public health risk associated with dairy products made from *B. melitensis*-infected goats. Shedding was at the level of $10^5 - 10^6$ CFU/ml of milk in approximately half of the goats sampled. While samples were concentrated to a degree by enriching for cream and pellet layers, an 8 oz (237 ml) serving of unpasteurized milk would contain over a million infectious doses. It is essential to determine the period of time over which milk continues to be a public health risk. The risk is likely to extend beyond the length of lactation. While previously published work on excretion of *Brucella* in the milk of goats over a prolonged period is scarce, among *B. abortus*-infected cattle, 90% of animals continue to shed organism at the second lactation following infection (Morgan, 1960). In sheep experimentally infected with *B. melitensis*, shedding in milk persisted for at least three reproductive cycles post-infection (Tittarelli et al., 2005). In that study, the percent of sheep shedding *Brucella* in milk was 79%, 64%, and 38% over the first, second, and third reproductive cycles post-infection, respectively. A higher rate of shedding was observed among goats in the first reproductive cycle in the present study, supporting the notion that goats are more susceptible to infection than sheep (Alton, 1990a). Taken together, it is also likely that goats too excrete high levels of brucellae in milk over subsequent lactation period presenting considerable public health risk. Identification of “superspreaders” and selective culling of these animals, as previously described for management of *Brucella* infection in water buffalo (Capparelli et al., 2009), may be a useful disease control strategy in resource poor areas where test and slaughter programs are not feasible.

3.5) Conclusions

The clinical effects, pathogenesis, and excretion of two strains of *B. melitensis* in goats are reported in the present study. Infection of goats with *B. melitensis* 16M resulted in an 86% abortion rate with all but one kid exhibiting generalized infection leading to fetal death (Table 3.5). *Brucella melitensis* 16M disseminated widely in pregnant does post-infection with none of the 15 sampled tissues spared from colonization. Importantly, we report the first isolation of *B. melitensis* from muscle tissue in ruminants with colonization at levels of $10^2 - 10^4$ CFU/g. Thus, muscle meat must be added to the list of potential foodborne sources of *Brucella* infection in humans. For comparison, levels of shedding in milk reached levels of $10^5 - 10^6$ CFU/ml in several goats, while placental and fetal
tissue would be a source of heavy pasture contamination containing up to \(10^9\) CFU/g. Pathogenesis of Rev. 1 infection was variable with two pregnant does showing minimal colonization and birth of full-term healthy kids and one doe exhibiting tissue colonization and clinical signs similar to those in animals infected with fully virulent 16M. Regardless of maternal tissue burden and outcome of parturition, excretion of Rev. 1 in milk, placental tissue, and vaginal secretions is possible at low levels, and is of potential public health concern. Massive excretion of \textit{B. melitensis}, whether 16M or Rev. 1, occurred only in animals that aborted or gave birth to full-term dead kids. Diagnosis of infection prior to parturition would greatly decrease disease transmission events. Unfortunately body temperature and leukogram parameters were found to be minimally useful in providing suspicion of \textit{Brucella} infection. Even \textit{Brucella}-specific diagnostic tests including blood culture and serological assay were unable to diagnose infection in all animals prior to parturition. Postpartum identification of animals shedding high levels of organism in milk may benefit disease control efforts. Selective culling of high-level shedders may be an effective and feasible alternative to a comprehensive test and slaughter program.

\textbf{Table 3.5. Summary of results.}

<table>
<thead>
<tr>
<th></th>
<th>\textit{B. melitensis} 16M Infected Animals\textsuperscript{a}</th>
<th>\textit{B. melitensis} Rev. 1 Infected Animals\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Frequency of Abortion</td>
<td>86%</td>
<td>0%</td>
</tr>
<tr>
<td>2. Percent of Kids Born Dead</td>
<td>92%</td>
<td>29%</td>
</tr>
<tr>
<td>3. Fetal Infection Rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Any degree of infection</td>
<td>92%</td>
<td>43%</td>
</tr>
<tr>
<td>b) Generalized infection\textsuperscript{b}</td>
<td>92%</td>
<td>29%</td>
</tr>
<tr>
<td>4. Maternal Infection Rate\textsuperscript{c}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Any degree of infection</td>
<td>100%</td>
<td>66%</td>
</tr>
<tr>
<td>b) Generalized infection\textsuperscript{b}</td>
<td>100%</td>
<td>33%</td>
</tr>
<tr>
<td>5. Shedding in Vaginal Secretions</td>
<td>100%</td>
<td>66%</td>
</tr>
<tr>
<td>6. Shedding in Milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Any degree of shedding</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>b) Consistent high level of shedding</td>
<td>43%</td>
<td>33%</td>
</tr>
<tr>
<td>7. Seroconversion Prior to Parturition</td>
<td>71%</td>
<td>100%</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Only data from pregnant animals are included in the table.
\textsuperscript{b} Generalized infection is defined by recovery of \textit{B. melitensis} from three or more tissues.
\textsuperscript{c} Maternal infection is defined by recovery of \textit{B. melitensis} from any maternal sample excluding fluid secretions such as vaginal fluid and milk.
CHAPTER 4: CELL MEDIATED IMMUNE RESPONSE IN GOATS AFTER EXPERIMENTAL CHALLENGE WITH THE VIRULENT BRUCELLA MELITENSI S STRAIN 16M AND THE REDUCED VIRULENCE STRAIN REV. 1

4.1) Introduction

*Brucella melitensis* is the etiologic agent of brucellosis in small ruminants and a common cause of disease in humans. While the protective immune response against this pathogen has been well studied in the mouse model (Grillo *et al.*, 2012), little is known of the immune response triggered by *B. melitensis* infection in natural hosts. The development of an appropriate immune response can protect the host from chronic infection and pathologic effects, such as abortion. An inappropriate response can lead to bacterial persistence or immunopathology. While mice, which are not natural hosts for *Brucella*, develop an effective Th1-mediated response and clear infection, the immune response of goats to *B. melitensis* must be deficient in some way as goats suffer from chronic infection and pathologic effects. When goats are immunized with the live, reduced virulence *B. melitensis* vaccine strain Rev. 1, however, the animals clear infection within several months of inoculation and develop a memory response that is protective against subsequent infection with fully virulent *B. melitensis*.

The Rev. 1 vaccine was developed over 50 years ago (Elberg & Faunce, 1957), yet little is known of the immune response responsible for the protection induced by this *Brucella* strain. Even less information is available on the immune response of goats to fully virulent field strains of *B. melitensis*. Interferon gamma (IFN-\(\gamma\)), the primary Th1 cytokine, is essential for control of *Brucella* infection in mice (Baldwin & Parent, 2002, Fernandes *et al.*, 1996, Grillo *et al.*, 2012). This cytokine is also believed to be an important determinant of the outcome of *Brucella* infection in humans (Akbulut *et al.*, 2005, Rafiei *et al.*, 2006, Skendros *et al.*, 2011). A recent study in sheep showed that exposure to both virulent *B. melitensis* and the reduced virulence Rev. 1 strain resulted in an IFN-\(\gamma\) response, although response was delayed in goats infected with the virulent strain (Perez-Sancho *et al.*, 2014). This is the only study of cell-mediated immune response to *B. melitensis* infection in a natural host. Major gaps in the understanding of immune response to virulent *B. melitensis* and the vaccine strain remain. Perez-Sancho *et al.* (2014) did not investigate the cell types responding to infection or whether anti-inflammatory cytokines were present to counteract the protective IFN-\(\gamma\) response. In cattle infected with virulent *B. abortus* or *B. suis*, CD4\(^+\) T
cells have been shown to be the primary producers of IFN-γ, with a lesser amount produced by CD8+ T cells and almost no contribution from WC1+ γδ T cells (Weynants et al., 1998). Similarly, a study of the B. abortus vaccine strains S19 and RB51 in cattle showed that the IFN-γ response triggered was primarily mediated by CD4+ rather than CD8+ T cells (Dorneles et al., 2014).

The aim of the present research was to build upon the limited knowledge obtained from these experimental infection studies performed in sheep and cattle. Since initial work suggested that bovine CD8+ T cells play a minor role in control of Brucella infection, the present study focused instead on the CD4+ T cell and γδ T cell response to B. melitensis infection in goats. The importance of γδ T cells in Brucella immunology is only just beginning to be understood. In humans, γδ T cells make up only 1 – 5% of circulating lymphocytes in healthy individuals, yet there is evidence that these cells play important roles in controlling Brucella infection (Bertotto et al., 1993, Dornand et al., 2002, Ni et al., 2012). γδ T cells comprise up to 60% of circulating lymphocytes in young ruminants (Guzman et al., 2012, Hein & Mackay, 1991); however, the role of this larger population of γδ T cells is relatively unknown. While the in vivo study in Brucella infected cattle described above indicated γδ T cells play a negligible role in the IFN-γ response (Weynants et al., 1998), bovine γδ T cells have been shown to inhibit replication of Brucella in macrophages following in vitro infection. This effect was IFN-γ mediated (Skyberg et al., 2011). Further studies in ruminant hosts are necessary to explain this apparent contradiction.

Thus, the objectives of the present study were to evaluate the following components of the caprine immune response over the course of infection with the virulent B. melitensis strain 16M or the reduced virulence B. melitensis strain Rev. 1: (1) basic changes in granulocyte, monocyte, and mononuclear cell numbers, (2) changes in numbers of CD4+ T cells and WC1+ γδ T cells, (3) the activation state of T lymphocytes, and (4) the production of the pro-inflammatory cytokine IFN-γ and the anti-inflammatory cytokine IL-10 by CD4+ and WC1+ T cells.

4.2) Materials and Methods

4.2a) Animals and Experimental Design

The cell mediated immune response to B. melitensis infection was investigated in the same goats utilized for a pathogenesis study described in chapter 3. Briefly, 15 mixed-breed female goats were divided into 4 groups:
B. melitensis strain 16M-challenged (groups 1 and 2, does 1-5 and 6-9, respectively), B. melitensis strain Rev. 1-challenged (group 3, does 10-13), and uninfected controls (group 4, does 14-15). This study was carried out over the course of two years with 5 goats challenged in March 2014 (group 1) and the remaining 10 animals challenged in January 2015 (groups 2-4). Animals had not been vaccinated previously with any Brucella vaccines and were seronegative prior to challenge. After estrus synchronization, animals were naturally inseminated. All animals were initially found to be pregnant by ultrasound examination; however, does 5, 7, and 11 subsequently suffered early fetal loss, as these goats showed no evidence of abortion and were not pregnant at necropsy. Animals were transferred to a biosafety level 3 containment facility at least one week prior to experimental challenge. Animals were group-housed in rooms of adequate size (2-4 goats per room), and all stages of the study were conducted with consideration for animal welfare. Goats were fed a complete pelleted diet and hay daily with nutritional supplements added in late gestation.

Animals were infected intraconjunctivally at 80 (group 1) to 100 (groups 2-4) days of gestation (50 μl of inoculum per eye). The infection dose was determined by serial dilution of the inoculum in saline and standard plate count, which showed strain 16M and Rev. 1 inocula doses to be $8 \times 10^6$ and $8 \times 10^7$ CFU, respectively. The experimental procedures were approved by the Institutional Animal Care and Use Committee of Colorado State University.

4.2b) Sample Collection and Processing

Blood samples were collected by jugular venipuncture prior to challenge and following challenge at days 3, 7, 14, 28 (groups 2-4) or 35 (group 1), and at time of parturition. At every time point approximately 8 ml of blood was collected from each goat in BD Vacutainer® CPT™ Cell Preparation Tubes with sodium heparin. These tubes contain a Ficoll cell separation medium and polyester gel barrier, which allow for isolation of peripheral blood mononuclear cells (PBMCs) within the tube via a single centrifugation step. Purified PBMCs were either utilized directly ex vivo for quantification of monocyte and T lymphocyte populations or were cultured in the presence of Brucella antigen for quantification of cytokine production.
4.2c) Direct Ex Vivo Cell Surface Marker Staining

Numbers of CD4⁺ T cells, WC1⁺ γδ T cells, and CD14⁺ monocytes were measured immediately after PBMC isolation. These cell types were identified with monoclonal antibodies against ovine CD4 (clone 44.3, AbD Serotec, Raleigh, NC, USA), bovine WC1 (clone CC15 AbD Serotec), and human CD14 (clone TUK4, AbD Serotec). Antibodies were conjugated with Alexa Fluor 647, fluorescein isothiocyanate (FITC), and R-phycoerythrin (RPE), respectively. In addition, PBMCs obtained from group 1 goats were screened for expression of the CD25 marker on T lymphocytes using anti-bovine CD25-RPE (clone IL-A111, AbD Serotec). A mouse IgG1 antibody was used as an isotype-matched control for CD25. Tri-color staining was performed on PBMCs of goats from all groups; however, the staining protocol differed between group 1 and groups 2-4. PBMCs from group 1 goats were subjected to a 20 min incubation with CD4 and WC1 antibodies. These cells were subsequently split among 3 wells and stained with CD25, CD25 isotype control, or CD14. Since the CD25 marker was not investigated in PBMCs from groups 2-4, a single-step multi-color staining protocol could be followed with CD4, WC1, and CD14 antibodies. Stained cells were sterilized and preserved with 4% paraformaldehyde (PFA) until flow cytometry was performed.

4.2d) Cell Culture and Antigenic Stimulation

PBMCs were cultured in complete RPMI 1640 media for 72 hours at 37 °C, 5% CO₂. Cells from each animal were aliquoted among 3 wells of a 96-well plate (approximately 5 x 10⁵ cells/well) and cultured in the presence of Brucella antigen, concanavalin A (ConA) (positive control, 5 μg/ml), or media alone (negative control). ConA treatment was not included in the analysis of PBMCs from group 1 animals. All three culture treatments were utilized in group 2-4 animals. Brucella antigen was created by γ-irradiation (1.4 x 10⁶ rads) of a B. melitensis 16M suspension.

Following 72 hours of incubation, cells were stained with monoclonal antibodies in a multi-step process. Initially, T lymphocytes were stained with CD4 and WC1 antibodies. These cells were subsequently split between various wells for either further cell surface staining or staining for intracellular cytokines. Activation of T lymphocytes was assessed by CD25 staining after antigenic stimulation in addition to directly ex vivo as described above. Staining for intracellular cytokines was with anti-bovine IFN-γ-RPE (clone CC302, AbD Serotec) and anti-
mouse IL-10-RPE (clone JES5-2A5, AbD Serotec) and appropriate isotype controls (mouse IgG1 negative control for IFN-\(\gamma\) and rat IgG1 negative control for IL-10). A BD Cytofix/Cytoperm\textsuperscript{TM} Fixation/Permeabilization Kit was utilized to prepare cells for intracellular staining. While CD25, IFN-\(\gamma\), and IL-10 staining was performed on cells from group 1 animals, analysis of PBMCs cultured from animals in groups 2-4 was focused on differential measurement of IFN-\(\gamma\) production by CD4\(^+\) and WC1\(^+\) T lymphocytes.

4.2e) Flow Cytometry Studies

Prior to flow cytometry analysis, stained cells preserved and stored at 4 °C in 4% paraformaldehyde were re-suspended, transferred from 96 well plates to polystyrene tubes, and diluted in phosphate buffered saline (PBS) if necessary. Absolute cell counts were measured in samples from groups 2-4 by addition of CountBright\textsuperscript{TM} Absolute Counting Beads (Molecular Probes, Eugene, OR, USA) prior to analysis. Samples were run on a FACSCanto\textsuperscript{TM} II flow cytometer (BD Biosciences, San Jose, CA, USA) with data acquisition and analysis performed using FACSDiva\textsuperscript{TM} (BD Biosciences) and FlowJo software (FlowJo, LLC, Ashland, OR, USA), respectively. Compensation was performed using single-stained sample control tubes. Dead cells were excluded from analysis by adjustment of voltage and by setting appropriate gates on the side scatter and forward scatter plots. Gating strategies are shown in Figures 4.1 and 4.2. Approximately 10,000 events within the mononuclear cell gate were analyzed for each sample. Fluorescence expression was calculated by subtracting readings from samples of unstained cells from antibody-stained samples. For IFN-\(\gamma\), IL-10, and CD25 expression, isotype control measurements were also subtracted. Results are presented as either the percent of cells expressing fluorescence or the total number of cells expressing fluorescence, as calculated from comparison to CountBright\textsuperscript{TM} Bead quantities. Numbers of monocytes, granulocytes, and lymphocyte subtypes were compared between \textit{B. melitensis} 16M-infected, \textit{B. melitensis} Rev. 1-infected, and uninfected goats over the course of infection by the non-parametric Kruskal-Wallis and Mann-Whitney tests (GraphPad Prism 6.0, GraphPad Software, USA). The percent of IFN-\(\gamma\) producing lymphocytes were similarly compared between experimental groups. Comparison between time points was by the Wilcoxon matched-pairs signed rank test. Significance was defined at p<0.05.
Figure 4.1. Gating strategy utilized in analysis of specific leukocyte populations studied directly *ex vivo*. (A) Granulocyte, total mononuclear cell, and monocyte populations were identified based on size and granularity characteristics. For quantification of lymphocyte subpopulations, cells within the mononuclear cell gate were analyzed for expression of CD4 and WC1 (B) and CD25 (C). Cells within the mononuclear cell gate were also analyzed for CD14 staining (D) as an additional strategy for quantification of monocytes. Numbers displayed on the plots represent frequencies of cell populations from a representative animal. Frequencies are either a percent of the total population of cells (A) or the percent of the mononuclear cell population (B-D).
Figure 4.2. Gating strategy utilized in analysis of cytokine production by CD4\(^+\) T cells and WC1\(^+\) \(\gamma\delta\) T cells. (A) A representative plot of cell populations after 72 hours of culture. A broad gate was set around the lymphocyte population for subsequent analysis. (B) The lymphocyte subpopulation was analyzed based on staining for APC (Alexa Fluor 647)-labeled anti-CD4 and Alexa Fluor 488 (FITC)-labeled anti-WC1. (C and D) CD4\(^+\) cells and WC1\(^+\) cells were each analyzed for PE staining to quantify IFN-\(\gamma\) and IL-10 production.

4.3) Results

4.3a) Changes in Monocyte, Granulocyte, and Total Mononuclear Cell Numbers over the Course of Brucella Infection

Granulocytes and mononuclear cells, which include lymphocytes and a smaller population of monocytes, were gated based on flow cytometric characteristics of size and granularity. While a monocyte gate was created within the mononuclear cell population, monocytes were also quantified by CD14 staining in order to differentiate these cells from large, activated lymphocytes. CD14\(^+\) cells were found to make up 0 – 10% of the total mononuclear cell population prior to infection (Figure 4.3). Many goats infected with virulent \textit{B. melitensis} 16M showed an increase in both the percentage of CD14\(^+\) cells and the absolute number of these cells; however, response to infection was highly variable. Changes in monocyte numbers from pre-infection to day 28 post-infection were determined, and while goats infected with 16M showed a trend towards an increase in CD14\(^+\) cells when compared to Rev. 1-infected and uninfected animals, the difference was not significant (p=0.0914).
**Figure 4.3.** Changes in CD14⁺ mononuclear cell numbers over the course of *B. melitensis* infection. (A-C) Mononuclear cells were gated based on size and granularity, and the percent of cells within this gate expressing CD14 determined. Note does 5, 7, and 11 were not pregnant. (D) The change in numbers (cells/μl) of CD14⁺ mononuclear cells between day 0 and day 28 post-infection was determined and compared between *B. melitensis* 16M- and Rev. 1-infected goats and uninfected controls. Only does 6-9 are included in the 16M group of (D) since absolute numbers of cells were not quantified in the other does within this group. CD14⁺ mononuclear cell numbers at day 28 (corrected for quantities at time zero) were not significantly different between infection groups (p=0.0914, Kruskal-Wallis Test).
Granulocytes and total mononuclear cells showed a similar increase from day 0 to 28 post-infection in *B. melitensis* 16M-infected goats (Figure 4.4). The increase in mononuclear cells is most likely due to an increase in lymphocyte numbers since monocytes were found to make up only about 10% of the mononuclear cell population. While trends over the course of infection were again variable between goats, at day 28 post-infection granulocyte and mononuclear cell numbers were near baseline in uninfected and Rev. 1-infected goats and markedly increased in 16M-infected goats. The difference between 16M- and Rev. 1-infected animals was statistically significant (p<0.05).

![Figure 4.4](image)

**Figure 4.4.** Changes in granulocyte (A) and total mononuclear cell (B) numbers over the course of *B. melitensis* infection. Cell populations were gated based on size and granularity characteristics. The change in cell numbers (cells/μl) between day 0 and day 28 post-infection was determined and compared between *B. melitensis* 16M- and Rev. 1-infected goats and uninfected controls. Only does 6-9 are included in the 16M group since absolute numbers of cells were not quantified in does 1-5 within this group. Granulocyte and total mononuclear cell numbers at day 28 (corrected for quantities at time zero) were significantly different between infection groups (p<0.05, Kruskal-Wallis Test) with 16M-infected goats showing greater increases in granulocytes and mononuclear cells over the course of infection than Rev. 1-infected goats (p<0.05, Mann-Whitney Test).

### 4.3b) Characterization of Select Lymphocyte Populations in *B. melitensis*-Infected Goats

Further work was performed to characterize the lymphocyte subpopulations responsible for the observed increase in mononuclear cells over the course of infection. Specifically CD4+ lymphocytes and WC1+γδ T cells were investigated for both proliferation and functional response. Analysis of CD4+ lymphocyte numbers directly *ex vivo* showed a significant increase in these cells at day 28 post-infection in 16M-infected goats compared to animals infected with Rev. 1 (p<0.05, Figure 4.5). WC1+ cells showed a more variable response to infection. While this cell type was minimally increased in some 16M-infected goats at day 28 over baseline levels, there was no significant difference between the experimental groups (p=0.3175).
Figure 4.5. Changes in CD4⁺ and WC1⁺ T cell numbers over the course of *B. melitensis* infection. The percent of mononuclear cells expressing CD4 and WC1 over the course of infection are shown in A-C and E-G, respectively.
The change in CD4\(^+\) (D) and WC1\(^+\) (H) cell numbers (cells/μl) between day 0 and day 28 post-infection was determined and compared between B. melitensis 16M- and Rev. 1-infected goats and uninfected controls. CD4\(^+\) T cell numbers (D) at day 28 (corrected for quantities at time zero) were significantly different between infection groups (p<0.05, Kruskal-Wallis Test) with 16M-infected goats showing greater increases in CD4\(^+\) T cells over the course of infection than Rev. 1-infected goats (p<0.05, Mann-Whitney Test). There was no significant difference in numbers of WC1\(^+\) T cells between experimental groups at day 28 (p=0.3175, Kruskal-Wallis Test).

In group 1 goats CD4\(^+\) and WC1\(^+\) T cells were further studied via flow cytometric methods for determination of CD25 expression and production of IFN-\(\gamma\) and IL-10. Minimal CD25 staining of mononuclear cells from these B. melitensis 16M-infected goats was observed regardless of whether analysis was done directly ex vivo or after stimulation with Brucella antigen. The percent of CD25 staining cells increased from approximately 0.1% to 0.6% from day 0 to time of abortion, however, the change was not significant (p=0.125, Figure 4.6). No change in CD25 expression was observed in one non-pregnant 16M-infected goat.

![Figure 4.6](image_url)

**Figure 4.6.** Percent of mononuclear cells staining positive for CD25 over the course of B. melitensis 16M infection. Analysis was performed directly ex vivo. The change in CD25 expression from day zero to time of abortion was not significant (p=0.125, Wilcoxon matched-pairs signed rank test).

IL-10 response was highly variable among goats and was investigated in only the 16M-infected animals of group 1 (Figure 4.7). In most animals the percent of both CD4\(^+\) and WC1\(^+\) cells producing IL-10 fell over the first 7 days of infection. By day 14, IL-10 production had increased in many goats, but levels typically remained below pre-infection values. The rise in IL-10 production at day 14 mirrored a peak in IFN-\(\gamma\) at this time point (Figure 4.8).
Figure 4.7. Percent of mononuclear cells producing IL-10 over the course of *B. melitensis* 16M infection. Mononuclear cells were either cultured with *Brucella* antigen or remained unstimulated. The percent of IL-10 positive cells in the unstimulated cultures were subtracted out from levels in the stimulated cultures. Trends over the course of infection are shown separately (A-E) for each goat sampled (No. 1-5) due to the variable response. Doe 5 (E) was not pregnant.
An interesting trend was observed in IFN-γ production among group 1 does. Using IFN-γ production by cells in unstimulated cultures as a baseline, *Brucella* antigen stimulation resulted in limited IFN-γ production until day 14, when a response was observed exclusively among WC1⁺ mononuclear cells (Figure 4.8). From day 0 to 14, the percent of WC1⁺ cells producing IFN-γ increased from near 0% to 2-8% in 4 of 5 goats. In all but the non-pregnant doe, IFN-γ production fell back to baseline levels after the peak at day 14. Response by CD4⁺ T cells appeared negligible.

**Figure 4.8.** Percent of mononuclear cells producing IFN-γ over the course of *B. melitensis* 16M infection. Mononuclear cells were either cultured with *Brucella* antigen or remained unstimulated. The percent of IFN-γ⁺ cells in the unstimulated cultures were subtracted out from levels in the stimulated cultures. Trends over the course of infection are shown separately (A-E) for each goat sampled (No. 1-5). Doe 5 (E) was not pregnant.
IFN-γ production by CD4+ and WC1+ T cells was further investigated in *B. melitensis* 16M- and Rev. 1-infected goats as well as in uninfected controls. Cell culture treatments were also expanded. In addition to *Brucella* antigen stimulation and an unstimulated negative control treatment, cells were stimulated with ConA mitogen. Use of absolute counting beads allowed for quantification of the number of CD4+ and WC1+ IFN-γ producing cells per μl of cell culture suspension. In initial work, response by CD4+ cells in terms of the percent of cells producing IFN-γ appeared limited (Figure 4.8); however, since CD4+ cells are present in much higher numbers than WC1+ cells (Figure 4.5), the absolute number of IFN-γ producing CD4+ cells may be functionally significant. Analysis of absolute cell numbers did not reveal this to be the case (Figure 4.9a). CD4+ T cells remained poor producers of IFN-γ after *Brucella* antigen stimulation across infection groups and time points. While ConA mitogen was found to stimulate high levels of IFN-γ production by CD4+ T cells at pre-infection time points (Figure 4.9b), infection with the *B. melitensis* Rev. 1 strain suppressed this response. After normalization for numbers at day 0, the number of CD4+ IFN-γ+ cells present after ConA stimulation was significantly less in Rev. 1-infected goats than in 16M-infected goats at both day 14 and 28 post-infection (p<0.05).

**Figure 4.9.** IFN-γ production by CD4+ T cells in *B. melitensis*-infected goats. (A) The change in numbers (cells/μl) of IFN-γ producing CD4+ cells between day 0 and day 14 post-infection was determined and compared between *B. melitensis* 16M- and Rev. 1-infected goats and uninfected controls. IFN-γ production by CD4+ T cells at day 14 post-infection (corrected for quantities at time zero) was significantly different between infection groups and culture treatments (p<0.05, Kruskal-Wallis Test) with Rev. 1-infected goats showing significant suppression of IFN-γ production by CD4+ T cells after ConA stimulation compared with 16M-infected goats (p<0.05, Mann-Whitney
Test). (B) The number of IFN-γ positive CD4⁺ and WC1⁺ T cells following *Brucella* antigen, ConA, or media only stimulation of PBMCs at pre-infection time points.

*Brucella melitensis* Rev. 1 mediated suppression of IFN-γ production was not observed in WC1⁺ T cells as was the case in CD4⁺ T cells. IFN-γ production by WC1⁺ T cells was highly variable among individual goats; however, similar to the trend observed in group 1 goats (Figure 4.8), *Brucella* antigen stimulated WC1⁺ cells from 16M-infected goats of group 2 showed a spike in IFN-γ production at day 14 post-infection (Figure 4.10). This response, while unique to 16M-infected does, was not statistically significant (p=0.1797). At day 28 post-infection, however, a significant difference was observed in the number of IFN-γ producing WC1⁺ cells between infection groups and culture treatments (p<0.05). The trend observed at day 14 was reversed by day 28 with *Brucella* antigen stimulated WC1⁺ cells isolated from Rev. 1-infected goats showing significantly higher levels of IFN-γ production than cells from 16M-infected goats (p<0.05). WC1⁺ cells from uninfected control goats, however, also showed high levels of IFN-γ production at day 28 following *Brucella* antigen stimulation.
Figure 4.10. IFN-γ production by WC1+ T cells in *B. melitensis*-infected goats. The change in numbers (cells/μl) of IFN-γ producing WC1+ cells between day 0 and either day 14 (A) or 28 (B) post-infection was determined and compared between *B. melitensis* 16M- and Rev. 1-infected goats and uninfected controls. IFN-γ production by WC1+ T cells at day 14 post-infection (corrected for quantities at time zero) was not significantly different between infection groups and culture treatments (p=0.2674, Kruskal-Wallis Test). At day 28 a significant difference between groups was observed (p<0.05, Kruskal-Wallis Test) with Rev. 1-infected goats showing a significant increase in IFN-γ production by WC1+ T cells after *Brucella* antigen stimulation compared with 16M-infected goats (p<0.05, Mann-Whitney Test).

4.4) Discussion

4.4a) Changes in Leukocyte Parameters

The aim of the present study was to characterize the protective immune response elicited by *B. melitensis* Rev. 1 inoculation and compare this response to that following infection with virulent *B. melitensis* 16M so that specific deficits in the immune response of ruminants could be defined. To the author’s knowledge the present study represents the first application of flow cytometric analysis for study of immune response in *Brucella*-infected goats. The results of the series of flow cytometry studies conducted indicate that a stronger pro-inflammatory response is induced in animals infected with 16M than those infected with the Rev. 1 vaccine strain. This response was characterized by significantly higher numbers of granulocytes and mononuclear cells in 16M-infected goats at day 28 post-infection in comparison with Rev. 1-infected goats. An increase in monocyte numbers from day 0 to day 28 post-infection was also observed in 16M-infected goats, while monocyte numbers remained near baseline levels in Rev. 1-infected goats. While the difference in monocyte numbers between infection groups was not significant (p=0.0914), small sample size increases the risk of a type II error. Basic leukocyte parameters have not previously been investigated in ruminants following *Brucella* infection. In human hosts, infection is typically characterized by leukopenia rather than leukocytosis (Troy et al., 2005), but humans are incidental hosts of *Brucella* and likely exhibit an immune response unique from that of ruminants.

The stronger pro-inflammatory response observed in 16M-infected goats may be induced by higher rates of replication of 16M compared to Rev. 1 at day 28 post-infection. Tissue burden was evaluated in the two infection groups but not until parturition occurred at approximately day 55 post-infection. While tissue burden was higher in 16M-infected goats at this time (results described in chapter 3), it is unknown how replication of these strains compare earlier in infection. Rev. 1 has been shown to persist for 3.5 months in goats, but tissue load is decreased by one month post-inoculation (Elberg & Faunce, 1957). Differential expression of virulence factors by 16M and
Rev. 1 strains may also explain the stronger inflammatory response elicited by 16M infection. The genetic basis for the attenuation of the Rev. 1 strain is unknown.

Previous in vitro research on *B. abortus* and *B. suis* has shown that these *Brucella* species can inhibit apoptosis of murine and human macrophages (Barquero-Calvo *et al.*, 2007, Gross *et al.*, 2000). Inhibition of apoptosis has also been demonstrated *ex vivo* in macrophages from infected cattle and from humans with both acute and chronic brucellosis (Galdiero *et al.*, 2000, Tolomeo *et al.*, 2003). While markers of apoptosis were not specifically investigated in the present study, inhibition of apoptosis by *B. melitensis* 16M would explain the increase in monocyte numbers seen in 16M-infected goats at day 28 post-infection. Alternatively, the relative monocytosis may be simply a result of chronic bacterial disease and the presence of granulomatous lesions, which are known causes of elevated monocyte counts in ruminants (Morris, 2009). Further work should investigate the monocyte subtypes present in 16M- versus Rev. 1-infected goats. Measurement of CD16 expression gives an indication of the functional status of monocytes (Hussen *et al.*, 2013). CD14⁺CD16⁺ intermediate monocytes have the highest inflammatory potential and would be most beneficial in defense against *Brucella*.

Granulocytes were serendipitously studied in the present work, as the PBMC purification method utilized was supposed to eliminate granulocytes from the recovered cell fraction. Caprine neutrophils apparently have different density profiles than those of other species maintaining them within the PBMC fraction. Changes in granulocyte numbers were highly variable early in infection among individual goats, so comparisons between infection groups was made only at later time points. The neutropenic response resulting from *Brucella*-induced neutrophil killing was not observed in the present study, as has been recently noted in humans (Barquero-Calvo *et al.*, 2015).

The increase in mononuclear numbers at day 28 post-infection, while significantly greater in 16M-infected goats than Rev. 1-infected animals, is only suggestive of an increase in lymphocyte numbers. This change says nothing of functional response. Since none of the Rev. 1-infected animals aborted in the present study in comparison to an 86% abortion rate among 16M-infected goats (results presented in chapter 3), Rev. 1-infected
goats must be mounting an effective cell-mediated immune response. The focus of the remainder of the study was to investigate this cell-mediated response and define functional differences between lymphocytes in 16M- and Rev. 1-infected goats. While initial leukocyte parameters indicated that 16M-infected animals display a stronger pro-inflammatory response at least in terms of expansion of cell populations, perhaps the activity of these cells is repressed. Alternatively, the stronger initial inflammatory response could contribute to the pathology observed in 16M-infected goats, and be followed by increased immunosuppression later in infection allowing for persistence of *Brucella*.

4.4b) **CD4⁺ and WC1⁺ T Cells: Numbers and Function**

The response of lymphocytes to *B. melitensis* infection was observed to be highly variable between individual goats. Several animals utilized in the present study, including an uninfected control, had evidence of concurrent infections. Elevated white blood cell counts prior to infection were observed in some animals as well as abscesses consistent with caseous lymphadenitis. While use of specific-pathogen-free animals would likely have decreased variability in the lymphocyte responses observed, concurrent infections are a realistic finding in animals naturally infected with *Brucella*. Other experimental infection studies performed in ruminants have also shown individual animals to display markedly different proliferative and IFN-γ responses to infection with *Brucella* (Dorneles et al., 2014, Weynants et al., 1998).

Analysis of lymphocyte numbers showed a clear increase in CD4⁺ T cell numbers at day 14 and day 28 post-infection compared to levels at day 0. This effect was only observed in 16M-infected goats, however, with numbers of CD4⁺ T cells in Rev. 1-inoculated animals remaining relatively stable over the course of infection. It should be noted that cell numbers were analyzed directly *ex vivo* in order to identify the cell type responsible for the observed increase in mononuclear cells. A recent study reported proliferation of CD4⁺ T cells in response to *Brucella* antigen stimulation in *B. abortus* strain S19 vaccinated cattle (Dorneles et al., 2014), however lymphocyte cell types responding to *B. melitensis* infection in small ruminants has not previously been reported. In contrast to the CD4⁺ T cell response, WC1⁺ γδ T cells displayed a limited and variable proliferative response to *B. melitensis* infection. At days 14 and 28 post-infection neither Rev. 1- nor 16M-infected goats showed a significant increase in WC1⁺ γδ T cell numbers over pre-infection values. This is in sharp contrast to the response observed in human
patients. In humans with acute brucellosis circulating γδ T cells have been reported to comprise nearly 30% of the total lymphocyte population, while γδ T cells made up only 4% of the lymphocyte population in healthy controls (Bertotto et al., 1993). The limited response observed among WC1⁺ γδ T cells in the present study was surprising. While a small study of three Brucella-infected cattle also reported a limited WC1⁺ γδ T cell response (Weynants et al., 1998), other studies in cattle have shown increases in WC1⁺ γδ T cell numbers and activation status following *Mycobacterium bovis* infection or BCG vaccination (Guzman et al., 2012).

Activation status of T lymphocytes was accessed in the present study via staining for CD25, the alpha chain of the IL-2 receptor. Expression of CD25 provides an indication of proliferative capacity without use of radioactive thymidine (Caruso et al., 1997). Mononuclear cells isolated from four *B. melitensis* 16M-infected animals showed limited CD25 staining. While activation status increased over the course of infection, less than 1% of mononuclear cells were CD25⁺. Stimulation of cells with *Brucella* antigen for 72 hrs did not further increase activation status. Several studies in human patients have found significant changes in numbers of CD25⁺ T cells via direct *ex vivo* measurement (Bertotto et al., 1993, Skendros et al., 2007). In humans with acute *B. melitensis* infection, over 25% of lymphocytes have been reported to express CD25 compared to 3% of lymphocytes in healthy controls (Bertotto et al., 1993). A study of patients with chronic brucellosis, however, reported a decrease in the percent of lymphocytes expressing CD25, and the unresponsiveness of these cells to mitogen stimulation suggests a state of T cell anergy in chronic brucellosis (Skendros et al., 2007). Cellular anergy should be further investigated in *Brucella*-infected ruminants. Although not yet specifically studied in cells isolated from ruminants, *Brucella* is known to inhibit expression of MHC molecules, co-stimulatory molecules, and secretion of IL-12 by human dendritic cells, which can lead to T cell anergy (Billard et al., 2005).

The presence of anergic T lymphocytes in *B. melitensis*-infected goats would explain the limited IFN-γ response observed in the present study. CD4⁺ T cells isolated from both 16M- and Rev. 1-infected goats failed to produce IFN-γ after stimulation with *Brucella* antigen. While cells stimulated with ConA were found to produce IFN-γ prior to infection, inoculation of goats with *B. melitensis* Rev. 1 in particular, completely inhibited this response at 14 and 28 days post-infection. The unresponsiveness of CD4⁺ T cells to Rev. 1 infection is puzzling given that these cells are believed to be critical to the protective response elicited by vaccination. While a recent
study of IFN-γ production in *B. melitensis* Rev. 1 vaccinated sheep reports a marked response at day 7 post-inoculation, response was transient with negligible IFN-γ detected after 14 days post-inoculation (Perez-Sancho *et al.*, 2014). Interestingly, although IFN-γ production was delayed by one week in sheep infected with virulent *B. melitensis*, the IFN-γ response was stronger and more prolonged in these animals compared to Rev. 1-inoculated sheep. Thus, a more effective IFN-γ response does not seem to be the basis for the protective response elicited by *B. melitensis* Rev. 1 versus virulent strains in small ruminants. To further investigate the IFN-γ response, however, the role of WC1⁺γδ T cells was investigated in the present study.

Large percentages of WC1⁺γδ T cells were found to produce IFN-γ, although response was inconsistent between goats and culture stimulation treatments. A peak in IFN-γ response typically appeared at day 14 post-infection in WC1⁺ cells of *B. melitensis* 16M-infected goats, while many of the Rev. 1-inoculated animals had higher numbers of IFN-γ producing WC1⁺ cells at day 28 post-infection. γδ T cells have been associated with the rapid production of IFN-γ prior to development of an effective adaptive immune response (Guzman *et al.*, 2012), but this does not seem to be the case in *Brucella*-infected goats. The delay in response may be associated with *Brucella*-mediated suppression of dendritic cell function. Direct contact with dendritic cells, or stimulation by dendritic cell produced cytokines, has been shown to be an important signal for γδ T cell activity (Guzman *et al.*, 2012, Ni *et al.*, 2012). Alternatively, cell culture conditions may have been insufficient to mediate this interaction. In the present study, IFN-γ production by WC1⁺γδ T cells was also found to be highly variable in positive (ConA) and negative (media only) control culture treatments. The variability in response may be partially explained by the fact that several subtypes of WC1⁺ cells exist, which are differentially activated by pathogen antigens, mitogens, and cellular stress signals (Guzman *et al.*, 2012). Despite the variability in response, large percentages of WC1⁺γδ T cells were found to produce IFN-γ at times during the course of *Brucella* infection. The functional significance of this activity remains to be defined, since absolute numbers of WC1⁺ cells remained low in infected animals. When considering the pro-inflammatory immune response in its entirety, however, the IFN-γ response by WC1⁺γδ T cells was the single component identified by the present study to be more active in Rev. 1-inoculated goats over 16M-infected animals, at least at later time points of infection.
4.5) Conclusions

The cellular immune response to 2 strains of *B. melitensis* is reported in the present study. Infection of goats with fully virulent *B. melitensis* 16M resulted in the development of a pro-inflammatory response characterized by increased numbers of granulocytes, monocytes, and lymphocytes by day 28 post-infection compared with pre-infection values. The relative lymphocytosis noted was comprised of increases in CD4⁺ but not WC1⁺ T cell types. This was in contrast to the dramatic expansion of γδ T cells reported in human patients. In comparison to 16M-infected goats, granulocyte, monocyte, and lymphocyte numbers remained at baseline levels throughout the course of infection with the *B. melitensis* vaccine strain Rev. 1. Analysis of lymphocyte function suggested a degree of potential T cell anergy, with low levels of CD25 expression noted and unresponsiveness of CD4⁺ T cells to mitogen stimulation post-infection. This absence of a marked CD4⁺ T cell IFN-γ response in *Brucella*-infected goats especially those infected with Rev. 1 is puzzling given that Rev. 1 produces a protective immune response in vaccinated animals. The components of this protective response remain undefined. The present study points to a potential WC1⁺ γδ T cell mediated response, with high percentages of γδ T cells found to produce IFN-γ at various time points over the course of *Brucella* infection. Since WC1⁺ γδ T cell made up less than 10% of the lymphocyte population in 16M-infected goats and less than 5% of the lymphocyte population in Rev. 1-infected goats, however, absolute numbers of IFN-γ producing WC1⁺ cells were low. Thus, the functional significance of WC1⁺ γδ T cells in *Brucella*-infected and vaccinated goats must be further investigated. The present study investigated the systemic immune response to *Brucella* infection. Future work should examine recruitment of WC1⁺ T cells to sites of infection via application of immunohistochemistry. Although systemic percentages of WC1⁺ T cells remained low over the course of *Brucella* infection, large numbers of these cells may be recruited to lymph nodes and reproductive tissues of infected goats. Thus, minor systemic changes in lymphocyte numbers and functional activity may be indicative of strong local immune activity.
CHAPTER 5: CONCLUDING REMARKS

The overarching aim of this dissertation research was the study of the pathogenesis of brucellosis in a natural ruminant host. A complete understanding of disease pathogenesis requires investigation of infection from both pathogen and host perspectives, as it is the interaction between bacterial virulence factors and host immune components that determines the outcome of infection. The present work was primarily focused on host response. Two different strains of *B. melitensis* were utilized and the outcome of infection studied in goats, including clinical disease, tissue burdens, and shedding. Immune response was then investigated in order to determine how differences in host response to these two *Brucella* strains lead to the different disease outcomes observed.

The majority of *Brucella* experimental infection studies are performed in mouse models with limited work conducted in natural ruminant hosts. The expense associated with purchasing and housing ruminants is often prohibitive for this research, especially considering the larger sample sizes required when using outbred animals. Small sample sizes are typical when working with large animals, and as a result, findings have a high chance of type II error. While statistically significant differences may not always be detected when working with limited numbers of research subjects, biologically meaningful data can often still be collected. Compounding the challenges associated with sample size, few biosafety level 3 facilities are available to accommodate large animals. Despite these obstacles, it is essential that *Brucella* studies are conducted in ruminants given the clear differences in disease outcome in ruminants and mice. Goats, the natural hosts of *B. melitensis*, are excellent models for study of *Brucella* infection given their smaller size and shorter gestation period in comparison to cattle. Several studies have previously utilized goats as models for *B. abortus* (Anderson et al., 1986a, Anderson et al., 1986b, Elzer et al., 2002, Meador & Deyoe, 1986, Meador et al., 1989) and *B. melitensis* (Edmonds et al., 2002, Elzer et al., 2002, Perry et al., 2010, Phillips et al., 1997, Roop et al., 2001, Cheville et al., 1996, Olsen et al., 1997) infection. The *B. melitensis* studies previously conducted in goats have focused on development of novel vaccine candidates. In the present study we demonstrate the relative ease of using goats for study of the pathogenesis of *B. melitensis* infection. Once this model was developed in our laboratory, we then utilized goats to investigate the immune response elicited by *B. melitensis*. 

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Cellular immune response to *Brucella* infection is rarely studied in natural hosts. Increased availability of laboratory reagents, such as monoclonal antibodies against ovine and bovine cell markers, now allow advanced immunology studies to be conducted in ruminants. To the author’s knowledge the present study represents the first application of flow cytometric analysis for study of immune response in *Brucella*-infected goats. Using this approach, a fully virulent *B. melitensis* strain was found to trigger a pro-inflammatory response in goats characterized by increases in granulocytes, monocytes, and CD4\(^+\) T lymphocytes over the course of infection. Functionally, however, CD4\(^+\) T cells were found to be in a potential anergic state within goats infected with either virulent or vaccine strains. This is the first description of potential T cell anergy in ruminant hosts, although humans with chronic brucellosis are known to show similar deficits in T cell function. The present work is also among the first to investigate the function of γδ T cells in *Brucella*-infected ruminants *in vivo*. Potential differences in the IFN-γ response of these innate-like lymphocytes may explain differences in disease outcome between goats infected with virulent *B. melitensis* and the reduced virulence *B. melitensis* vaccine strain. While further research is necessary to identify specific immune components responsible for the protective response to the *B. melitensis* Rev. 1 vaccine and immune deficits allowing persistence of field strains of *B. melitensis* in goats, the present study provides proof of concept for a flow cytometric approach to study of immune response to *Brucella* infection in natural hosts.

Use of ruminant hosts for the study of *Brucella* pathogenesis allows for direct translation of findings made in the laboratory to application in a field setting. Millions of cattle and small ruminants are infected with *Brucella* worldwide (de Figueiredo *et al.*, 2015), and the immense disease burden among livestock poses a significant threat to human health. In this dissertation I took a holistic approach to the study of *Brucella* pathogenesis. By studying brucellosis in ruminants and humans in Mongolia, an endemic disease area, the range of disease presentations occurring in a field setting were appreciated, public health dilemmas understood, and challenges to disease diagnosis and control realized. Gaps in knowledge of brucellosis pathogenesis exist that prevent educated assessment of the public health risk posed by certain practices. A poor understanding of the protective components of an immune response to *Brucella* infection hinder development of improved vaccines. In this dissertation research, laboratory studies were designed to address these gaps in knowledge. Thus, investigation of tissue burden and distribution of *B. melitensis* following experimental infection in goats was not purely a question of academic curiosity but findings served to inform herders in an endemic disease area. The present study is the first to report isolation of *Brucella*
from muscle tissue of a ruminant. While reproductive tissues, milk, and vaginal secretions present the greatest risk to human health, *B. melitensis* was isolated from muscle at concentrations similar to that in tissues of the reticuloendothelial system. This study, thus, points to the potential risk associated with butchering and consumption of undercooked muscle meat. Investigation of levels of *B. melitensis* in milk of experimentally infected goats, suggests the potential presence of “super shedding” that pose heightened risk to human consumers.

The research presented in this dissertation builds upon current knowledge of *B. melitensis* pathogenesis and immunology in a natural ruminant host. The work provides a strong framework from which further comparative investigations of immune response to virulent *B. melitensis* and the reduced virulence *B. melitensis* vaccine strain, Rev. 1, can be conducted with the ultimate goal of defining components of a protective versus deficient response to *Brucella* in a natural host.
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