THE PREVALENCE OF *COXIELLA BURNETII* IN BEEF AND DAIRY CATTLE COTYLEDONS

By

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ABSTRACT

To assess the potential public health risk of the zoonosis *Coxiella burnetii* in cattle (coxiellosis in cattle and Q fever in humans) we assessed the overall prevalence in beef and dairy cattle.

We extracted DNA and performed real-time PCR on 286 cotyledons from both beef (n= 150) and dairy (n= 136) cattle from individual live operations and a single beef processing plant. These locations included Texas, New Mexico, Kansas, Arizona, and Nebraska. Prevalence of *C. burnetii* detected was 5.6%. Chi-square analysis indicated that prevalence did not differ (p=0.75) between beef and dairy cattle. Likewise concentration of bacterial cells per gram of cotyledonary tissue was similar (p=0.36) for beef and dairy cattle.

Of the 16 positive samples, 13 were obtained from a beef processing plant. This indicates a great necessity to wear personal protective equipment in processing plants to prevent the indirect, direct, or airborne transmission of infectious particles to humans while working with livestock animals. Furthermore, any person working with livestock animals on a day to day basis should wear Personal Protective Equipment (PPE) to prevent the transmission of *C. burnetii.*

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CHAPTER 1: INTRODUCTION

In 1935 there was minimal knowledge about *Coxiella burnetii,* while forthcoming exposure to it in processing plants in Australia had raised concern. The disease came to be known as query fever (Q fever) in humans, and coxiellosis in livestock. In 2008, Q fever was sub-divided into acute and chronic forms due to differing symptoms. The patient's immune status as well as their overall condition predisposes acute or chronic state of Q fever.

C. burnetii has two development cycles, small-cell variant (SCV) and large-cell variant (LCV). The SCV form is resistant to heat, desiccation, ultraviolet light (UV) and chemical agents, making it nearly impossible to kill. The LCV is the growing and replicating phase in the parasitophorous vacuole (PV).

This bacterium has many reservoir hosts, including humans, birds, reptiles, domesticated animals, livestock, ticks, and wild animals. For humans, ruminants are the primary source of infection. Shedding and excretion of *C. burnetii* (which can last for several months) occurs through milk, feces, urine, and most commonly through birth or aborted tissues. Transmission of *C. burnetii* occurs through many routes, but the primary way is by inhaling contaminated particles or aerosols. This is problematic for individuals living in a close proximity to animal facilities or operations even without direct. These individuals with lack of contact can still be at risk of encountering the pathogen. Animal-to-animal, human-to-human, and animal-to-human routes of *C. burnetii* have all been

documented. The intracellular lysosome pH protects *C. burnetii* from most antibiotic treatments because of a decrease of antibiotic activity at an acidic pH [1]. This makes it difficult for broad-spectrum antibiotics to work against *C. burnetii.* Human antibiotic selection depends on the type of Q fever but are typically recommended for both acute and chronic. There is little knowledge of antibiotics for the treatment of coxiellosis and even less evidence to suggest that antibiotics are beneficial. Currently, there is no FDA-approved vaccine for *C. burnetii* available in the United States for humans or cattle. However, there are a multitude of ways to control and prevent an infection from *C. burnetii*.

Numerous studies have been conducted in the U.S. and internationally to examine the prevalence of *C. burnetii*. Overall, studies have shown significant variation in prevalence of this organism from various tissues in cattle, goats, sheep, caprine, and humans. Blood, placental tissue, milk, vaginal mucus, and feces were all used for detection. These studies will be discussed in more detail in the Discussion.

We compared the overall prevalence of *C. burnetii* to previous research and the prevalence in beef vs dairy to assess the potential health risk of zoonosis *C. burnetii* (coxiellosis in cattle and Q fever in humans). We hypothesize that there is no difference in the prevalence of *C. burnetii* in beef and dairy cattle cotyledon samples.

CHAPTER 2: LITERATURE REVIEW

Edward Holbrook Derrick, a medical practitioner and director of the Queensland Department of Health Laboratory of Microbiology and Pathology, was invited to investigate the first outbreak of *Coxiella burnetii* (*C. burnetii*) that took place in an abattoir located in Brisbane, Queensland, Australia in 1935 [2]. Two years later, a second case broke out in the same location [3]. At the time of these outbreaks there was no literature describing what Derrick observed. He tried to isolate the agent in guinea pigs but was not successful and perceived it to be a virus because he did not detect bacteria [3]. The organism was thought to be the rickettsial species when Macfarlane Burnet and Mavis Freeman analyzed some of the infectious material sent by Derrick. They were able to reproduce the disease in guinea pigs, mice, and monkeys [3]. *C. burnetii* was similar under microscopic analysis to Gram-negative bacteria in that they possess an outer membrane separated by a periplasmic space but there was no description of the organism at that time [4, 5]. While working on this disease without knowledge of how infectious the bacteria were, Burnet became the first laboratory employee who acquired the disease in January 1937. The three men and their associates were able to investigate the disease and determine epidemiological factors important for further studies [3].

Before the disease name became known as Query (Q fever) named for the puzzle it left researchers during discovery, other names such as abattoir fever and Queensland rickettsial fever were used to describe where it was discovered. In animals it is referred to

as coxiellosis [6]. *C. burnetii* was first recognized in the United States when there was an outbreak in a packing plant in Amarillo, Texas in 1946 [7]. From January 1990 to November 2000, 57% of cases involving *C. burnetii* traced to beef processing plants [8]. *C. burnetii* became a nationally notifiable disease after 19 cases were reported in 2000 and 173 cases in 2007 throughout the U.S. [9]. In 2007-2010 The Netherlands entered an epidemic period where over 4,000 cases were reported. During this time in The Netherlands, Q fever was estimated to be 8-28 times more severe than H1N1 influenza because Q fever caused long term complications and pathological conditions [10]. The Netherlands outbreak was suspected to be due to the increase in goat farming in close proximity to highly populated areas from 2007-2010 [11]. During this same time period in the Netherlands, (2005-2009) large herds were experiencing coxiellosis causing an "abortion storm" [2]. The mortality rate of this epidemic was low, with six deaths reported out of more than 2,300 cases in 2009 with no information on the cause of death [2, 10, 12]. Many infected patients had lingering symptoms that were untreatable [12].

In 2008, the case definition of Q fever was sub-divided into acute and chronic forms and allowed individuals to report the cases separately. Centers for Disease Control and Prevention (CDC) data for Q fever cases currently are available through the calendar year for 2017. From the last recording in 2017, 153 cases of acute Q fever were reported, and 40 chronic cases were reported in the US [9]. The number of annual cases of Q fever reported to the CDC has increased over the last 17 years, with the greatest number recorded in 2017 [9]. The data shows an increasing number of cases as age increases. The group that has been reported most frequently is 60-64 years old [9]. The peak number of reported cases for humans occurs in the months of April, May, and June [9].

Today, Q fever is a worldwide zoonosis that affects every race and ethnicity. It has been discovered in every country except New Zealand [2]. In 2016 the Animal Welfare Regulations in New Zealand ruled that no live animal can be exported for slaughter without approval from the Ministry for Primary Industry (MPI) [13]. The MPI, only available in New Zealand, allows veterinarians to inspect all animals before and after departure to ensure safety when shipping cattle to make sure they are fit to travel [13]. Because Q fever is not on the Nationally Notifiable Disease list in New Zealand, it is rarely reported and the cases that are reported generally come from outbreaks or from laboratories [3]. There were sufficient laboratory-associated *C. burnetii* infections to classify this bacterium as a Risk Group 3 (RG3) pathogen requiring Biosafety Level 3 facilities for research studies. The National Institutes of Health (NIH) defines RG3 pathogens as agents that "are associated with serious or lethal human disease for which preventive or therapeutic interventions may be available" [14].

Bacteriology of *C. burnetii*

Herald R. Cox, a bacteriologist, was the first to isolate *C. burnetii* by growing it in a fertilized chick membrane and discovered that it is an intracellular pathogen that strictly replicates in human and animal eukaryotic cells within a lysosome [2, 4]. *C. burnetii* is an obligate intracellular organism, meaning it cannot replicate outside of the host cell and relies on intracellular resources to reproduce and replicate.

Bacteria are normally classified as Gram-positive or Gram-negative. Both Gram-negative and Gram-positive bacterial cell walls are made up of a polymer, peptidoglycan monomers which is a polysaccharide made of two glucose derivatives, N-acetylglucosamine and N-acetylmuramic acid [15]. In Gram-negative bacteria, there are

peptide interbridges composed of L-alanine, D-glutamine, L-lysine, and D-alanine [15]. These four amino acids combine to form a tetrapeptide, which is linked by the amide linkage to the carboxyl group of N-acetylmuramic acid of the glycan chains [16] Gram-negative bacterial cell wall structure is illustrated on the left side of Figure 1. There is typically a thin peptidoglycan layer that is found between the outer membrane and the plasma membrane [17]. The outer membrane is a membrane that consists of proteins, phospholipids, and lipopolysaccharides [17]. During the Gram staining procedure, Gram-negative bacteria cannot retain the crystal violet/iodine complex and this results in a loss of color due to-their relatively thin peptidoglycan layer. Gram-positive bacteria, represented on the right side of Figure 1, have a thick peptidoglycan layer and cell wall mainly composed of peptidoglycan but lack an outer membrane. Teichoic acids, polyol phosphate polymers, are linked to peptidoglycan layer in Gram-positive bacteria but are generally absent in Gram-negative bacteria [18]. These polymers function as a cation-sequestering mechanism and also aid in anchoring the peptidoglycan to the cytoplasmic membrane [16]. Gram-positive bacteria do not allow crystal violet/iodine to be removed during Gram staining, because the layer is dehydrated by ethanol, thereby trapping the crystal violet/iodine stain [16]. Gram-positive bacteria, therefore, remain purple.

Coxiella burnetii is unique due to it being a Gram-negative bacterium while possessing a thick peptidoglycan layer, similar to a Gram-positive bacterium. This bacterium is also unique in that it cannot be stained by the Gram technique but only with the Gimenez method [2]. *C. burnetii* still possesses an outer membrane and lipopolysaccharide equivalent to typical Gram-negative bacteria. The lipopolysaccharide is made up of the O-antigen which is the outermost part of the LPS, the core

polysaccharide, and lipid A [15]. In Gram-negatice bacteria, the Lipid A later is also known as an endotoxin because of fever and shock effects [19]. Lipid-A structure is composed of acyl chains linked to glucosamine by ester or amide linkages [20]. The O-antigens are composed of heteropolymers with a variety of monosaccharides and amino acids. The O- antigen is highly varied among species and used to identify certain organisms in the laboratory [19]. Many Gram-negative bacteria such as *B. pertussis* and *B. bronchiseptica* do not contain the O-antigen [21]. The Lipid-A section of the LPS could have structural variability, while the O-antigen has a higher degree of structural variability as well as in the number of repeating units. The LPS provides protection to the cell by blocking access of antibacterial agents to parts of the cell wall, aiding in stabilizing the outer membrane by contributing to the negative charge of the cell, and has a role in the host response to pathogenic bacteria [15].

C. burnetii has two development cycles, the small-cell variant (SCV) and large-cell variant (LCV) [2]. The SCV form is 0.2 to 0.5 μM long, resistant to desiccation, heat 71.7 °C, UV light, and chemical agents. The hardiness of this bacteria led to the idea of Pasteurization [2, 22]. Breathing contaminated dust, manure, or air particles typically consists of the SCV form being inhaled rather than LCV. *C. burnetii* replicates intracellularly in a large phagolysosome-like vacuole (PV) which is similar to a lysosome, specifically due to the acidic pH [23-25]. The typical lysosome includes enzymes that break down proteins, nucleic acids, carbohydrates, and lipids in eukaryotic cells. The lysosome degrades material as well as digests phagocytized material that is infectious by endocytosis [26]. The PV is different than a regular lysosome, a membrane-enclosed organelle in eukaryotes, in that it possesses properties of a mature

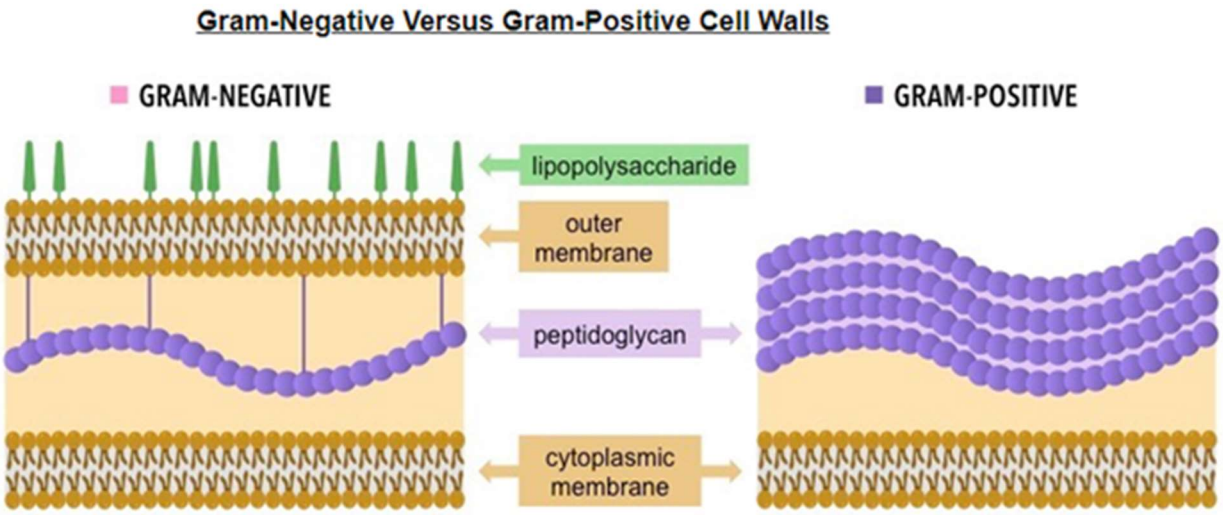

Figure 1. Gram-negative and Gram-positive bacterial cell wall structure [1]

phagolysosome. The lysosome also contains lysosomal proteases such as cathepsin D (CatD). This plays a role in the folding, activation, and delivery of the protein to the lysosome like vacuole [27, 28].

The PV and auto-phagosomes provide nutrients to the bacterium, allowing it to survive longer and have characteristics that resemble a secondary lysosome because of the lower pH (pH 5), allowing the bacteria to replicate [23, 29]. This is different than other lysosomes because it typically fuses with other vacuoles and has the ability to survive in various environments in and outside of the host [27, 30]. Other parasites and pathogens use a PV for developing, which protects them from the host cell. Some intracellular pathogens including *Salmonella*, *Chlamydia*, and *Mycobacterium tuberculosis* replicate in the PV and exit that space and the host cell in a few steps. Other pathogens like *Listeria monocytogenes* and *Shigella flexneri* escape the vacuole and replicate in the host cell cytosol where the replicates are released from the host cell [31]. *C. burnetii,* in contrast, remains inside the lysosome-like PV for its replication [27].

Due to the acidic environment, enzyme systems and nutrients that are located in the vacuole of the SCV trigger it to differentiate into the LCV [23]. This form is the growing, replicating, and spore-like form also found in the PV [2, 5, 32]. The typical LCV size is 0.05 μm and enters hosts cells by phagocytosis for replication in epithelial cells [2, 23]. In order for the bacteria to replicate, conditions that include the acidic environment, acid hydrolysates, and cationic peptides are required [2]. The low pH protects *C. burnetii* from most antibiotic treatment because antibiotic activity is diminished in acidic pH [1]. This makes it difficult for broad-spectrum antibiotics to

work against *C. burnetii.* The LCV form does not persist in its infectious form extracellularly [23].

Various bacteria possess a dormant, non-reproductive structure to ensure survival in environments known as an endospore. Not all bacteria possess this environmentally stable characteristic, including *C. burnetii*. A typical spore cell wall contains dipicolinic acid and sporulation occurs in seven stages of morphological events. Endospore formation is triggered by the lack of nutrients and typically occurs in Gram-positive bacteria. During the formation of an endospore, the cell divides into a mother cell and a forespore, or a separate compartment. The forespore is engulfed by the mother cell which is destroyed. The endospore is released into the environment to remain dormant until favorable conditions are present.

Sporogenesis of *C. burnetii* is controversial [29]. *Coxiella burnetii* often produces an endospore-like or spore-like particle (SLP) named so because they are not structurally similar to *Bacillus* spores due to the lack of dipicolinic acid [5, 33]. The SLP of *C. burnetii* possesses similarity to the exosporium, forespore membranes, germ cell wall, and coat layers of typical endospores [34]. These forms have not been known to be infectious but, *C. burnetii* lacks sporulation genes that typical endospore-forming organisms possess [5, 29, 33, 35].

The endospore-like properties allow it to stay infectious on wool, meat, and in milk while still being vegetative. An electron-dense polar body resembles an endospore occasionally in LCV. A previous study discovered the LCV containing the endospore deteriorated, after infection, suggesting the endospore is liberated upon lysis of the LCV [5]. *C. burnetii's* development cycle begins in the SCV and differentiates into the

replicating LCV that in turn differentiates back into the SCV during the stationary phase [36] The SCV form is the extracellular survival stage that is metabolically dormant [5, 23]. The SCV hardiness could be due to a cell wall that is analogous to the function of an endospore coat [37, 38]. Structural components that provide the stability of *C. burnetii* in the SCV form are poorly examined [37].

Reservoirs of *C. burnetii*

C. burnetii has an extensive range of reservoir hosts other than humans, including birds, reptiles, domesticated animals, wild animals, ticks, and farm animals [3]. Experimenting with RG3 pathogens like *C. burnetii* can affect individuals who do not work with the pathogen directly, such as janitors, other professors, plumbers, or whoever enters the laboratory without caution [39]. The bacterium can persist for months or years in the environment outside of the host in the SCV form. The same cycle allows the bacteria to survive on wool for up to seven months, fresh meat for up to one month, and 42 months in milk [2]. Ruminants including cattle, sheep, and goats are the main source of human infection and the most frequent reservoirs [2].

Intensive cattle farming at a location with a high prevalence and risk could be a concern for human safety because of the risk due to ruminant reservoirs and multiple possibilities of shedding from livestock animals that can become infectious to humans [40]. The U.S. cattle inventory as of January 1, 2020 totaled 94.4 million head, whereas the inventory of Texas cattle and calves totaled 13.0 million animals. This number leads the U.S. in total number of calves and cattle [41]. Because *C. burnetii* can survive for long periods of time in the environment, dry climates with little precipitation and open

landscapes with higher wind gusts favor the transmission of *C. burnetii* by contributing to airborne transmission [42].

Shedding of *C. burnetii*

Shedding and excretion of *C. burnetii* occurs in a variety of ways, including through animals' milk, feces, urine, and most commonly through birth or abortion tissues, and lasts for several months [43]. This bacterium is shed through vaginal mucus for up to 14 days in smaller livestock like goats and 71 days in livestock such as lambs. In manure, *C. burnetii* has been shown to be viable for up to 20 days in goats and 14 days in cattle [44]. Goats primarily shed this bacterium through milk but it has also been discovered in vaginal mucus [2]. The variety of shedding routes of this RG3 pathogen has led to a public health concern and higher risk for dairy farmers, veterinarians, packing plant workers, zoo workers, or anyone who interacts with animals [45].

The affected animals' fetuses, placental fluids, and placental membranes contain the highest concentration of *C. burnetii* [6]. Previous studies have shown that this bacterium can replicate to as many as 1.0×10^9 organisms per gram of tissue in placental tissues in smaller livestock [24]. The increase of organisms centralized in this location can lead to abortion, stillbirth, infertility, metritis and endometritis in animals [43]. The bacteria can then persist and stay throughout the whole pregnancy, or even after the pregnancy and replicate during future pregnancies [46]. This could increase the risk for abortion, stillbirth, or metritis and endometritis for a second time [46]. Chronic infections in animals result in abortion rates of 5 to 91% in small ruminant flocks [3]. This can be detrimental for industries and operations who rely on the birth of healthy animals and as a result have large economic losses in the meat and agricultural industry [47].

Transmission of *C. burnetii*

Inter-herd transmission of *C. burnetii* by wind and sales are poorly understood [48]. It is known that livestock acquire coxiellosis through inhalation of *C. burnetii* [48]. Transporting cattle long distances can increase the risk of infection by passive transport through aerosols [49]. Contaminated particles have been reported to travel as far as 11 miles and can remain infective in dust for up to 120 days [2]. Therefore, while infected livestock are being transported, the bacteria have the potential to be transmitted to other hosts, including humans and other animals, thus increasing the risk for their infection.

Because of the long distance *C. burnetii* can travel, individuals can become infected without having any direct contact with animals [2]. This is problematic for individuals who live in areas close to animal facilities or operations without direct association with them on a daily basis as they can be at risk for coming into contact with the pathogen. The longer an individual is exposed to any pathogen, the higher the risk of becoming infected [6]. This can potentially contribute to the number of Q fever cases.

A study was performed researching the inter-herd spread of *C. burnetii* among dairy cattle [48]. This study included 2,799 dairy cattle that were tested for antibodies against *C. burnetii* in bulk tank milk using an ELISA test kit. Herds that tested negative in May of 2012 were retested in May of 2013. Data on the cattles' individual movement between herds starting in May 2013 was also collected. The first round of testing reported that 1,941 or 69.3% of the dairy cattle were seropositive. Of the 858 that were negative in May of 2012, only 826 were retested in May of 2013. Of these, 306 tested positive for *C. burnetii*. This study showed that new inter-herd movement of cattle introduced the pathogen to the rest of the herd and animal trade did result in a small way to introducing

the new infection. They reported that among the new herd, infections of *C. burnetii*, 92%, was due to airborne transmission and the remaining 8% was from cattle trade [48].

Ticks can serve as a vector of transmission of many strains of *Coxiella* to humans and animals. Although they are not the main transmission route for the pathogen, they do play a role. Over 40 species of ticks have been found to harbor *C. burnetii* [50]. The first experiment examining ticks as vectors for *C. burnetii* was performed by D. J. W. Smith in *Haemaphysalis humerosa* and *Haemaphysalis bispinosa* [51-53]. It is known that *C. burnetii* replicates in the intestinal cells of ticks at a rapid rate and then is shed through tick feces and saliva onto the host during feeding [54-57]. The feces can contain up to 1.0 x 10^9 organisms and can be excreted for up to 635 days [51, 58, 59]. Being bitten by the infected tick, inhaling excreta, and crushing a tick with hands (creating direct contact with the infected tick) increase the risk for transmission [60]. In Northern Ireland, a nurse groomed a sheep dog at a research station that had pieces of sheep placenta in the wool. Two to three weeks later she developed Q fever which was linked back to the dog, on whom infected ticks had fed [61, 62].

Animal-to-animal transmission of *C. burnetii* has been recorded in many species. One example involved a pet dog that gave birth to four puppies and all four died shortly post-partum. The mother of the puppies was taken to the vet to be tested and but turned out to be seropositive for *C. burnetii* due to eating deer liver [63]. Two human family members were also serologically diagnosed with Q fever [63]. One member tested positive due to having contact with the dog during parturition. The other was exposed to both the deer liver and the dog, making both animals a possible route of transmission [63].

In humans, transmission could occur by direct contact from contaminated wool, birth tissue or birthing fluids that are released into the environment, consuming contaminated milk, conducting necropsies and autopsies, blood transfusions, bone marrow transplantation, tick bites, or most commonly by inhalation of dust particles contaminated with urine or feces, similar to animals [64].

Human-to-human transmission of *C. burnetii* is uncommon but possible. After a 32-year-old woman was admitted to the hospital due to a high-risk pregnancy and experiencing chronic placental abruption, she serologically tested positive for Q fever. The patient disclosed that she had assisted in the delivery of her dog's puppies. The dog was also brought in for testing and was seropositive [65]. It was implied that the dog's placenta and birthing fluids were the reservoir for the bacteria that was transmitted to the patient by inhalation. A second 32-year-old woman was admitted to the same hospital due to premature labor and was rooming with the patient mentioned above. After the second patient delivered her baby, she came back with pneumonia and blood titers that were associated with acute Q fever. It was discovered that both women sharing the same toilet transmitted of the pathogen by vaginal excretions, urine and feces contaminating air particles due to aerosols created during flushing [65].

Although not much information is provided, another case of human-to human transmission was reported after infected aerosols were spread during an autopsy of a patient who died of Q fever [66]. After the Q fever outbreak in The Netherlands, there was a concern about transmission via donated tissues [67]. Postmortem tests were performed on 1,033 tissue donors that included corneas, heart valves, skin, and bone marrow for possible presence of *C. burnetii*. Tests were conducted using ELISA for IgG

antibodies against Phase II *C. burnetii* and the results showed 3% tested positive, indicating that *C. burnetii* can be transmitted via donated tissues [67].

Sexual transmission of *C. burnetii*, though rare, has been documented for both humans and animals. A case of sexual transmission of *C. burnetii* in humans was documented in Spain. Nine patients from Poland traveling to Spain were employed to shear sheep from March through June. At the end of June the men returned home to their wives because of fever, fatigue, and muscle pains they were experiencing [68]. The symptoms began while they were living in Spain and all nine patients and their spouses, children, parents and siblings were tested for Q fever. Antibodies to *C. burnetii* antigens were not detected in all the family members' urine and semen samples. The individuals who traveled to Poland did have *C. burnetii* organisms in their semen. Antibodies to Phase I and II antigens were present in their wives. Because other members of the family tested negative they were excluded. The study concluded that *C. burnetii* was transmitted by sexual contact [68]. The observations from the study suggested the bacteria were attached to spermatozoa.

Huebner and Jellison discovered that pasteurizing milk and other dairy products could prevent *C. burnetii* infections in individuals who otherwise consume raw milk [2]. Areas of the world where pasteurization does not occur at all, or as often, have a higher risk of contracting an infection when compared to other parts of the world. Some of these areas with unpasteurized products include France, Germany, Denmark, Italy, The Netherlands, Czech Republic, Austria and Ireland where unpasteurized milk is sold in vending machines on a daily basis for consumption [2, 69].

Pathogenesis of *C. burnetii*

There are two forms of Q fever that are reported in humans, chronic and acute. Similarly, there are two antigenic forms of *C. burnetii* that are used to determine if the patient has the acute or chronic form. These antigenic forms are Phase I and Phase II. Phase I can be isolated from both animals and humans and is considered to be the more virulent form. Phase II can be obtained from tissue cultures or embryonic eggs [70]. The two antigen phases cause two different types of antibodies to appear in response to the antigen. Phase II antibodies appear earlier in the infection while Phase I appear later [71]. Additionally, there is no direct relationship between Phase 1 or Phase II antibodies with SCV or LCV.

The diagnosis of Q fever is also based on the patient's immune status as well as their overall condition rather than just as acute or chronic [32]. Children and the elderly are more likely to show fewer symptoms [39, 72]. When children do show symptoms, they are consistent with adult symptoms associated with acute and chronic Q fever [66]. The human primary infection lasts between seven and 32 days once the individual is exposed to the pathogen [73] .

Mortality resulting from acute Q fever is less than 2% of those infected [72]. The virulence and pathogenesis of *C. burnetii* are still not fully understood [74, 75]. In an acute Q fever infection, any organ can be affected, but the most common are the lungs and liver because it is the main portal of entry. When infection occurs via the respiratory route, the targeted cells are the alveolar macrophage cells located in the lungs. As a defense mechanism, the mobile lung macrophages may try to clear the airway by the mucociliary process of transporting the bacteria from the lung to the tracheobronchial

lymph nodes, causing spread of the infection even more [32]. In the case that the bacteria travel to the liver, the target cells for infection are the Kupffer cells resulting in potential spread into the bloodstream [3]. If the bacteria get into the bloodstream and the patient is or remains asymptomatic and donates blood, the donor could transmit this pathogen to the recipient. The Kupffer cells becoming infected could trigger local inflammation and the initiation of granuloma formation [3]. This is a structure consisting of monocytes that differentiate into macrophage cells to form a protective response, preventing the granuloma as being detected as foreign [76]. Other roles of the macrophage cells include assisting with tissue repair, and intracellularly killing the foreign pathogen by phagocytosis. Virulent *C. burnetii* bacteria kill THP-1 cells, a human monocytic cell line, preventing phagosome maturation [77]. The bacteria are unable to bind to and fuse with the lysosome because of the lack of cathepsin D. The monocytes'-activation by IFN-gamma stimulates the killing of *C. burnetii* organisms by phagosome maturation and phagosome alkalization [77].

Mortality does occur in chronic Q fever patients also and is much higher than acute Q fever [78]. A study was done with 439 individuals in which 166 showed complications. Of those patients, the mortality rate was 38% for chronic Q fever patients [79]. In this version of Q fever, IL-10 is overproduced because the cytokine network is altered, causing a block of *C. burnetii* maturation [80, 81]. IL-10 is produced by macrophages, monocytes, dendritic cells, lymphocytes, B cells, mast cells, eosinophils, and CD4 T cells [82].

Symptomatology

Acute Q fever is typically misdiagnosed because of its similarities to symptoms of influenza [83]. Influenza has symptoms that include headaches, sore throat, cough, fever, and body aches. The common symptoms between influenza and acute Q fever are the length of illness, fever, and headaches [84]. Every case of acute Q fever is different because clinical symptoms and signs among patients vary [4]. Acute Q fever symptoms include headache, fever, pneumonia, and granulomatous lymphadenitis [83, 85].

Acute Q fever has two main clinical forms of infection, pneumonia and hepatitis. The route of infection can influence which form of disease occurs [3]. Ingesting raw milk results mainly in hepatitis, which could cause enlargement of the liver [3]. Pneumonia can result in symptoms including a mild cough, chest pain and headache that 40.5% of infected patients reported to be most severe pain they ever had [72, 86]. Acute Q fever patients can also develop myocarditis and other neurological complications because of inflammation of the heart muscle [2]. When inflammation of the lining of the heart valves occurs, it can damage the tissues and heart valves, causing the circulation of blood to the lungs to be limited. *C. burnetii* has been found to survive in granulomas of the heart and cardiac valve tissues for years [87]. Limited blood flow to these areas can result in fluid buildup in the heart and lungs.

Chronic Q fever can require antibiotic therapy and have a high mortality rate if left untreated [88]. This rarer version of Q fever can develop between two months and up to two years after the initial symptomatic, asymptomatic or subclinical infection [87]. Chronic Q fever occurs in 5% of all Q fever infections and lasts around six months after initial onset [4].

Heart valve lesions, immunosuppression, pregnancy, and vascular abnormalities are all predisposing conditions that can increase the severity of chronic Q fever [89]. The uterus and mammary glands are the primary sites of chronic *C. burnetii* infections [3]. There is still an open question as to why *C. burnetii* replicates preferentially in animal and human reproductive tissues [27].

Placental tissue consists of trophoblastic cells and inflammatory or immunomodulatory cells that could be mobilized in response to an infection [90]. Previous studies have shown a positive antigen in the trophoblast along the chronic villi and intervillous spaces [91]. A previous study concluded that *C. burnetii* did infect and replicate in BeWo, which are derived from human trophoblast, and the intact trophoblast can become swollen. The findings also demonstrated that the bacteria caused an inflammatory response similar to the function of tumor necrosis factor (TNF), causing pregnancy complications from Q fever [90]. Swelling of the trophoblast can also cause premature birth. Infected placentas show intercotyledonary thickening that causes an inflammatory response that is more severe than normal. Tumor necrosis factor plays an important role during the fetal and developmental stages by preventing the offspring from developing anomalies, stimulates urine activity, and balances trophoblastic cell turnover [92]. Too much TNF can lead to apoptosis of the trophoblast, causing human chronic gonadotropin (HCG) and trophoblastic fusion. This also leads to movement of *C. burnetii,* causing spontaneous abortion and premature labor [92].

Endocarditis is the most common manifestation resulting from chronic Q fever. It has a spontaneous mortality rate of 65% and will result in death without antibiotic therapy [3]. Endocarditis was such a reoccurring effect of Q fever that chronic Q fever

and Q fever endocarditis were used synonymously to describe the same illness [93, 94]. Once the bacteria enter the bloodstream, they attach to the heart, damaging the valves, and preventing the blood from being pumped. The second most common manifestation of chronic Q fever is vascular infection [89]. Symptoms include fatigue, myalgia, and sleep disturbance [95]. Surgery being performed on vascular Q fever infections due to aneurysm or graft can lead to complications such as vascular ruptures, paravascular abscesses, and pseudo-aneurysm formation on top of the weakness from the infection before surgery [96]. There are currently no antibiotic treatments for vascular Q-fever patients [96].

Immunity

There are two types of immunity the human body uses for protection against a foreign pathogen, innate and adaptive. Humans and animals do not have natural immunity against *C. burnetii* [24]. The initial contact the host has with the pathogen is the entry site of the body, specifically the respiratory mucosa. This triggers the cellular and humoral response [97]. The body's adaptive immunity is built up after exposure and recovery from acute Q fever. Antibodies have been detected in infected individual's bone marrow for up to 5 years after initial illness with acute Q fever [98]. Chronic Q fever patients with endocarditis do not develop sufficient immunity, allowing the chronic infection to persist [24].

The host's immune response is triggered by the dendritic cells that first detect the pathogen's presence [99]. *C. burnetii's* Phase I is highly infectious and can often infect and grow in the dendritic cells without causing the induction of inflammatory cytokine production [100]. A positive titer from diagnosis does not indicate shedding or disease,

just current or previous infection [6]. Phase II bacteria have the opposite effect, and induce inflammatory cytokine production because of the truncated lipopolysaccharide (LPS) [100]. The Phase I LPS has a carbohydrate structure that blocks antibodies from reaching the *C. burnetii* surface proteins, allowing the bacteria to persist at other unknown sites [101]. The Phase I lipopolysaccharide is involved with phagocytosis of *C. burnetii* by macrophages through toll-like receptor 4 (TLR4), but does not influence *C. burnetii's* survival [102]. The LPS is the only verified component that is different between Phase I and Phase II *C. burnetii* [32].

Cell-mediated immunity prevents Q fever from reactivating [100]. The body's immunity and elimination of pathogens relies on the activation of specific T- cells with some TH1 cells [100]. Infected macrophages release pro-inflammatory cytokines such as interleukin 1 (IL-1) that produces anti-microbial proteins, tumor necrosis factor (TNF), and interleukin 12 (IL-12) to control the cellular immune response [32]. Specific cells including CD8+ T cells, Interferon-γ, and TNF are all sent to sites of infection to help control it by stimulating an antimicrobial response [100]. Increased TNF levels in the patient is an assumption for chronic Q fever that results in an increase of TNF receptor type II. There is also an increase in IL-1 receptor antagonist that leads to block the activity of IL-1. This results in a decrease in resistance to the bacterial infection [81]. Cytokines released by T cells stimulate an antimicrobial response in infected cells by production of reactive oxygen (ROS) and nitrogen (RNS). The ROS and RNS also control the growth and replication of *C. burnetii* [100, 103, 104]. This is an important role of cell-mediated immunity to have the ability to control and prevent the reactivation of the bacteria [30, 103].

Humoral immunity is known as antibody-mediated immunity for protecting against extracellular pathogens. One of the main roles of the humoral barriers is auto-recruitment of phagocytic cells. Antibodies in the body are not able to eliminate all of *C. burnetii,* but are aided by complement activation and toxin neutralization to direct the different bactericidal activities [32, 100]. These antibodies develop three to four weeks after acute fever symptoms occur. In humans, an acute infection is suggested when Phase II antibody titers are higher than Phase I titers [6, 72]. Typical mononuclear phagocytes are responsible for phagocytosis, killing the infected pathogen, however, *C. burnetii* resides in the phagolysosomes themselves, making it more difficult to kill [100]. Antibody opsonization of Phase I bacteria increases the phagocytosis of *C. burnetii* [105].

Pathogenesis of Coxiellosis

Pathogenesis of coxiellosis in larger livestock animals is not as well documented as it is in humans. Once an animal inhales *C. burnetii,* it localizes in the tracheobronchial lymph nodes. The macrophages in the lungs aid in spreading the bacteria by keeping them in their intracellular acidic vesicles [32]. *C. burnetii* then localizes in the mammary gland and then the placenta or fetus of the animal [106, 107]. The abortion rate in cattle for various reasons, including pathogens, ranges between 2-5%, causing an economic and production problem worldwide [108, 109]. The animal can also have a higher risk of producing underweight offspring. Abortions in cattle due to *C. burnetii* are not typically diagnosed in regions where the infection rate is higher but depends on the location and whether it is a required reportable disease [110, 111]. Most of the time the causes for abortion are not examined in great detail. The mother cow is normally taken to the processing plant because of abortion or miscarriage and not researched.

Diagnosis of *C. burnetii*

Humans

A confirmed acute case of Q fever must have laboratory evidence and must be clinically compatible or linked to another confirmed case. This means that the case is confirmed by a method listed for reporting purposes such as serology, persistent antibodies, or PCR [112]. A probable acute Q fever case must be clinically compatible with supportive evidence from the laboratory [113]. A confirmed chronic Q fever case must have laboratory confirmed evidence and also be clinically compatible. The most common method of detection in humans is by serologic detection of specific antibodies [114]. Having more than one test done only further confirms or denies the infection.

Phase I is the virulent form and Phase II is the long-term form that emerges with a low virulence in animal infections [115]. For humans, there are many tests that can be done for Q fever diagnosis. When determining if an individual has an acute or chronic disease, serological testing is done to measure the antibodies with an ELISA test. Individuals who have Immunoglobulin G (IgG) and immunoglobulin M (IgM) antibody titers to Phase II antigen that are higher than those of Phase I have acute Q fever [116]. It is not uncommon for Phase I titers to appear later in the infection. To solve this issue a second sample is needed two weeks following the first test for repeat [116]. In chronic Q fever, an individual must have Immunoglobulin A (IgA) or IgM and have higher Phase I antibody titer with lower Phase II antibodies [116]. Chronic infection is consistent with Phase I antibody titers of <1:16. However, seroconversion, which is the time when an antibody can develop and become detectable in blood, occurs 7-15 days after the symptoms appear, and Phase II antibodies do not appear until the second week because

they are time-dependent. If blood is drawn too early, the test could miss any detectable antibody titer, resulting a false negative test [72, 116].

DNA detection of Q fever in humans can be carried out in many ways, including from blood, milk, and placenta, fetal tissue from abortion, bone marrow, vascular graft, or bone biopsy [4]. PCR-based methods, including conventional PCR and qPCR, are frequently used more to detect specific organisms like *C. burnetii* [114]. Both methods can detect the bacterial DNA before the antibody response required for testing using ELISA or other serological methods [117]. PCR has been shown to be useful when detecting *C. burnetii* infections in feces, milk, vaginal mucus, and sometimes tissue fluids and is also the most useful tool for detecting the bacteria in aborted bovine fetuses [118, 119]. PCR can detect the bacteria regardless of whether it is dead or alive. When conducting either PCR or qPCR, the IS1111 insertion sequence is the primary target for detecting *C. burnetii*. This sequence has multiple copies per bacterial cell which allows for increased sensitivity [120]. If an individual tests positive with PCR but has a negative serology result, they are diagnosed with acute Q fever [121].

Animals

Diagnosing coxiellosis in animals is similar to the testing done in humans. Tissue smears/impressions or frozen tissue from placental membranes including cotyledons, fetal tissue, and vaginal mucous can be obtained and Giemsa stained for visual detection of *C. burnetii* [84]. This method is typically confirmed by further immunohistological testing [122]. Similar to *C. burnetii* testing in humans, PCR can be used to detect DNA from live or dead bacteria, contamination, or an infection using samples from tissues, milk, feces, vaginal mucous, and soil [123]. Another diagnostic test that is rarely

performed because of risk to human health is obtaining a positive culture of bacteria from these samples, which is a procedure that must be done in a Biosafety Level 3 laboratory.

If screening a large group of livestock, the ELISA test is the preferred diagnostic method [6, 122, 124]. This is because it is convenient for large scale screening without taking each animal to the veterinarian. There are commercial kits that can be purchased to detect Anti-Phase I and II antibodies. These tests are also the most commonly available antibody test for humans [6]. However, it is important to note that seronegative animals can still be infected and actively shedding the organism [125].

Phase-specific antibody testing in livestock is still poorly characterized. In livestock, Phase II antibodies are associated with acute coxiellosis infection while Phase I resembles the chronic form [126]. Specific antibodies can be used to detect an immune response but not before 2-3 weeks of exposure and infection [24]. Previous studies have reported that the antibodies diminish after parturition due to the loss of the colostrum [126, 127]. When testing aborted bovine calves for *C. burnetii,* one would typically use PCR or qPCR of vaginal excretions if less than 14 days after abortion, and blood tests [128]. PCR-methods are reported to be less reliable for determining the cause of the abortion, because it could be positive for vaginal *C. burnetii* excretion during the postpartum period instead of *C. burnetii* infection at the time of abortion [128].

Bringing any new animal into a herd can pass along numerous different bacteria from the previous location. If the new animal was infected with *C. burnetii*, it can transmit and shed the bacterium resulting in an epidemic for the herd. *C. burnetii* testing of livestock before being introduced to a new herd is not routinely performed at this time but should be. Screening animals for bacteria and diseases could decrease the risk of

spreading coxiellosis to a whole herd of livestock if caught early. After screening, culling the livestock that are shedding the bacteria would potentially increase farm profitability by preventing the loss of the animal and others infected [129].

Prevention of *C. burnetii* infection

The best way to control the spread of infection and transmission of most infectious diseases is by vaccination [130]. There are several different types of vaccines for bacterial infections such as live-attenuated, inactivated, toxoid, subunit. Live-attenuated vaccines use a weakened form of the bacteria. This live form is the closest to a natural infection. By using a weakened whole bacterial cell, it reduces the virulence in the host while still provoking the immune response. The inactivated vaccine, also known as a bacterin vaccine, aids in fighting bacterial infections though the use of inactivated or killed bacteria. This type of vaccine includes the killed version of the agent causing the disease. This does not typically provide immunity as strong as a live vaccine. Toxoid vaccines are used to prevent diseases caused by bacteria that produce toxins. This is because an inactivated toxin can induce an antibody response. Toxoid vaccines are made by purifying the bacterial toxin. The toxicity of the toxin is suppressed or inactivated by heat or with formaldehyde to form the toxoid [131]. The immunity produced is in response to the toxin instead of the whole cell. A subunit vaccine only includes parts of the infectious bacteria that contain only the antigenic parts to the pathogen instead of the whole cell. These vaccines may lack pathogen-associated molecular patterns (PAMPs) that are required for antigen recognition by the immune system [132]. The conjugate vaccine is a combination of pathogens to increase immune response.

All of the vaccines created against *C. burnetii* are inactivated vaccines. However, there is currently no FDA-approved vaccine for *C. burnetii* available in the U.S. for humans or cattle [100]. Several vaccines use the Phase 1 Henzerling strain because of its demonstrated protection against infection from Phase 1 *C. burnetii* [133]. A previous study found that unlike the Phase 1 cellular vaccine, the Henzerling strain could be administered safely in a booster regimen and produce significant T-cell production and antigen-specific antibodies [133]. A separate study examined differences between strains of *C. burnetii.* Based on the 10 strains that were used in vaccine trials, the Nine Mile strain used in the Phase 1 Coxevac vaccine in Europe was the most desirable and exhibited the greatest protection [134].

The U.S. did have a Q fever vaccine from the Special Immunizations Program of the U.S. Army Medical Research Institute for Infectious Disease (USAMRID). For individuals to receive the investigational new drug (IND), a skin test using 0.02 mg of a formalin-inactivated form of the Phase 1 Henzerling strain of *C. burnetii* vaccine is administered to determine prior exposure [135]. The vaccine was placed on hold after skin testing resulted in issues that have not been disclosed. However, the investigational new drug vaccine (IND) is available in the U.S. on an investigational basis only [136].

Q-Vax is a whole cell formalin-inactivated vaccine from the Phase 1 Henzerling strain with a 97% efficacy rate, but is licensed only in Australia for protection against *C. burnetii* in humans for up to five years [117, 137, 138]. Abattoirs in Australia pay for their employees to be vaccinated with Q-Vax because Australia is ranked as having one of the highest reported rates of Q fever infections in the world. Before vaccination, skin and serum antibody tests of each employee are needed to determine whether an

individual is already immune or currently infected. The skin test injects 0.1mL of Q-Vax into the forearm. After 7 days the individual is regarded as positive for immunity if the injection site is lumpy [121]. A positive skin or antibody test shows that a person has been in contact with the bacteria at some point by assessing for sensitization to the bacteria's antigens [139]. If both tests are determined to be negative, then the individual can receive the Q-Vax vaccine. If one has previously been vaccinated against Q fever, they can have hypersensitivity and severe side effects that have not been disclosed if vaccinated a second time. A study in Australia examined 827 vaccinated individuals. They reported that females showed stronger cell mediated immunity (CMI) responses with a lower number of bacteria after infection. The results indicated that the immune response to the *C. burnetii* vaccine is influenced by sex and the modulated genes after infection are sex-dependent [139-141].

Phase I Coxevac is a vaccine licensed in Europe, and has been found to induce active immunity against *C. burnetii* in cattle and goats [130]. It has also been shown to reduce the number of abortions in livestock and lower excretion of the bacteria in milk, vaginal mucus, and feces if it is administered to non-infected, non-pregnant animals [130]. The vaccine was initially used under exceptional circumstances because of the incomplete information known about Coxevac at the time of authorization [142]. However, in 2014 it was re-evaluated and received full approval status. This vaccine is a Phase I vaccine targeted to *C. burnetii* composed of inactivated *C. burnetii* Nine Mile strain [143]. The American Nine Mile Strain was sequenced in 2003 with 1,500,000 to 2,400,000 base pairs [33]. The Nine Mile strain is the only *C. burnetii* strain that has been sequenced [33]. Because this vaccine contains Phase I *C. burnetii*, it increases the active

immunity against Q fever in both cattle and goats. The Nine Mile RSA439 strain is a Phase II strain derived from the Nine Mile Strain that was originally isolated from a tick in Montana in 1935 [144]. Once the bacteria pass through multiple generations *in vitro*, the Phase I isolates are converted into Phase II bacteria with truncated lipopolysaccharides, leading to a loss of virulence [145, 146]. The Nine Mile Strain II has not been sequenced so the Phase I strain is used for reference [33]. It is common to see reactions at the injection site in animals after vaccine administration. In cattle, a palpable reaction of 9-10 cm at the injection site can last up to 17 days, and the duration of immunity is 280 days after vaccination. In goats, the reaction can last up to 6 days with the palpable reaction being 3-4 cm [142]. The duration of immunity in goats is one year after vaccination. Trials of this vaccine were conducted over 4 breeding seasons with a flock of sheep [147]. The control group was not vaccinated and served as an open reservoir for *C. burnetii.* Vaginal swabs, fecal samples, and air were sampled 30 days post lambing each breeding season. Positive air samples were detected in the second and beginning of the third breeding season. By the end of the third breeding season they could not detect shedding of *C. burnetii* in the ewes in the vaginal mucus or milk, but it was still in the environment because of the spore-like form the bacteria produces and the obstacles it takes to kill it in the environment [130].

Antibiotics for *C. burnetii* infection

Human

Antibiotic selection depends on the type of Q fever, but they are recommended for both acute and chronic cases. No antibiotic has been shown to have a bactericidal effect [2]. One must keep in mind that *Coxiella burnetii* is less susceptible to most antibiotics

because it replicates inside the host's phagolysosomes with an acidic pH that often prevents bactericidal activity [46].

Erythromycin, a macrolide, and doxycycline, a tetracycline have been used for treating Q fever. Erythromycin is mainly used for treating patients with *C. burnetii*-associated pneumonia and is bacteriostatic to prevent replication [148]. Currently, there are not as many studies with erythromycin as with doxycycline. The ones that have been reported have indicated doxycycline is the preferred drug when compared to erythromycin. Once erythromycin is consumed orally, it is mainly metabolized by the liver by undergoing demethylation by the hepatic enzyme CYP3A4 [149]. Erythromycin is easily absorbed in the gastrointestinal system and diffused into tissues and phagocytes. The phagocytes circulate through the blood and begin phagocytosis of *C. burnetii [149]*. Erythromycin inhibits protein synthesis by binding to the 23S ribosomal RNA molecule. This blocks elongation of the peptide chain during bacterial translation [149]. Q fever patients have had erythromycin resistant infections. If the drug cannot bind to the ribosome, the bacteria can continue with protein synthesis [150, 151]. A study was conducted in Spain with 11 acute Q fever patients who were prescribed erythromycin. The results showed that all 11 patients had broken their fever by the fourth day but does not include information on the infection [148]. Other patients from the same study were given other antibiotics. None of those individuals had responded to the antibiotics, suggesting that erythromycin is a good treatment for Q fever pneumonia [148]. Another experiment in Spain treated 25 patients with acute Q fever pneumonia with erythromycin at 500 mg every 6 hours for 10 days. The patients recovered, but not as fast as 23 who

were treated with doxycycline at 100 mg twice a day [152]. This shows that erythromycin is effective, but not as effective when compared to doxycycline.

Doxycycline, a tetracycline drug, has been shown to be effective in some cases against *C. burnetii*, but some strains have acquired resistance if extended treatment is taken [153]. By itself it is the first choice of treatment of acute Q fever. In adults it is recommended to take 100 mg twice a day for two weeks [46, 117]. It is the preferred drug because the efficacy and record of success in treatment for *C. burnetii* infections is higher than any other drug [153]. This drug has an effect as fast as two days after the start of treatment [152]. Doxycycline inhibits *C. burnetii* mitochondrial protein synthesis by binding to the 30S ribosomal subunit causing it to have a bacteriostatic effect [154]. There are strains that have developed resistance to doxycycline [2]. A study was done examining the effects of doxycycline in Q fever-positive patients compared to those who were not treated. The results showed that those that took doxycycline broke their fever in less than 2 days but the study did not discuss the effects on the infection. The untreated patients' fever broke after 3.3 days [155]. Three cases were reported of isolates with doxycycline's minimum inhibitory concentration greater than 8µl/mg [156].

The combination of doxycycline and hydroxychloroquine are the first choice of anti-microbial drugs for chronic Q fever [46, 157]. The hydroxychloroquine raises the pH of the lysosomal compartments to allow the doxycycline to prevent further growth of *C. burnetii* [72]. A long-term study of the combined drugs was done in France from 1983 to 2006. This study discovered that the mortality rate for Q fever and the need for heart valve replacement decreased when this combination was used [153]. These are both common effects due to chronic Q fever rather than acute. The recommended dosage for

the combination of these drugs is 100 mg doxycycline twice a day with 200 mg hydroxychloroquine three times a day for up to 18 months [46, 72, 157].

Pregnant women with *C. burnetii* infection are to be treated with co-trimoxazole, a mixture of trimethoprim and sulfamethoxazole, until the last six weeks of pregnancy and then switch to doxycycline with hydroxychloroquine once they have given birth [46, 72]. Side effects for co-trimoxazole alone are rare but do include chest pain, dark urine, abdominal pain, nausea and pale skin [158]. These drugs were paired because doxycycline by itself can potentially become a risk to the fetus if taken during pregnancy, and it is a category D drug [46]. Doxycycline is contraindicated for use during pregnancy because it is a member of the tetracycline class, which has been linked to permanent tooth staining, hepatoxicity, decreases in bone growth, and premature labor [159-161]. Co-trimoxazole is a category C drug, meaning adverse effects have been documented in animal studies, but none have been reported for humans [46]. Co-trimoxazole is bactericidal and blocks folic acid synthesis, which is necessary for nucleic acid synthesis [162]. Sulfamethoxazole inhibits the formation of dihydrofolic acid by inhibiting the incorporation of para-aminobenzoic acid. Trimethoprim further inhibits formation of dihydrofolic acid by inhibiting dihydrofolate reductase [162]. The combination of both drugs causes the bacterial cell not to continue with synthesis of essential nucleic acids, of which folic acid is a precursor. Patients who are pregnant with Q fever are administered 320 mg of trimethoprim and 1600 mg of sulfamethoxazole daily for up to 5 weeks [163]. After delivery, the patient is given 200 mg of doxycycline and 600 mg of hydroxychloroquine for up to a year [163]. Children under 8 years old are also advised to take this drug, instead of doxycycline, if infected with Q fever due to risk of dental

staining [46]. The recommended regimen for children is a mixture of trimethoprim and sulfamethoxazole at 1:5 ratio [72].

Animals

There is little knowledge on antibiotics for the treatment of coxiellosis and even less evidence to suggest that antibiotics are beneficial in animals [6]. If antibiotics are going to be used, tetracycline is the preferred drug for infected herds especially during abortion storms [6]. In-feed antimicrobial drugs are not recommended because they do not reach the target sites (reproductive tissues or fetus) in the concentration that is needed [6, 164]. Oxytetracycline has been tested with various animals including sheep, goats, and cattle but had no effect on the bacteria [130, 147, 165].

Precautions against *C. burnetii* infection

Human

Protection from all potential routes of exposure to *C. burnetii* is difficult to control regardless of the precautions taken, but can be managed to an extent. For humans, there are many precautions that can be taken. One is to not consume unpasteurized milk. Not only can *C. burnetii* be transmitted by consuming contaminated milk, but also other pathogens of concern such as *Salmonella*, *Listeria*, and *Campylobacter* [166]. When working with a herd that could be infected, hands are to be washed frequently and clothes should be changed and washed before leaving the premises. Because this is a RG3 pathogen, one should not culture it unless in the appropriate locations designed for these experiments (BSL 3 labs) [167]. In the lab, tables and areas must be disinfected with 95% alcohol before and after using the areas [167]. If one does experiment with this bacterium

or come in contact with it, wastes should be autoclaved at 131 °C for 15 minutes [2, 24]. If no autoclave is available, items can be soaked in 95% alcohol or 10% bleach for 24 hours [167]. Diluted bleach, UV radiation, and autoclaving has no effect *on C. burnetii* with exposure times less than 10 minutes [167].

Animal

Animals, with the help of their caretakers, can prevent the transmission and potential of becoming infected with *C. burnetii.* The best prevention and precaution is to vaccinate livestock with a Phase 1 antigen vaccine with killed bacteria such as Coxevac [6]. This could decrease the amount of bacteria the animal is shedding with a potential to also decrease the number of abortions. Screening livestock animals with the ELISA test before herd introduction or at first signs or symptoms should be performed and transmission controlled by treating the ones who are positive and separating them from the rest of the herd [168]. This could prevent a herd epidemic that would potentially affect the caretakers, and individuals who live in the general vicinity who could inhale in the contaminated particles. Most *C. burnetii* infection precautions for livestock animals are necessary during parturition. Animals expected to give birth should be segregated from others to decrease the potential exposure to the rest of the herd. Placentas left on the ground to decompose are a source of *C. burnetii* transmission [169]. Aborted fetuses and remaining placentas should be discarded by incineration or closed composting, away from other animals' reach, so they do not consume the potentially contaminated tissues and become infected themselves [6]. While discarding the fetus the individual should wear personal protective equipment that includes goggles and a face mask [6]. Adding an enclosed area of any kind with controlled airflow can decrease the wind transmission and

lower the potential infection rate to humans and other livestock [6]. Because transmission of *C. burnetii* is mainly by aerosol, wetting down environments such as soil on dry days can also prevent the spread of *C. burnetii* by reducing the opportunity to travel [6, 170]. It is known that contaminated feces is a transmitter and therefore manure should not be spread across the operation if herd animals are infected during windy conditions [2].

CHAPTER 3: MATERIALS AND METHODS

Sample collection

Placentomes and placentas (n=286) were collected from one local beef processor and three live operations willing to participate in the study during the fall of 2019 and spring of 2020. A total of 136 dairy cattle and 150 beef cattle samples were collected. Samples were sourced from local beef processors and were extracted after the animal's death. Locations were not traceable for every sample due to the limited ownership information. Some vendors at beef processors were under the individuals name while others were under a business operation name that could be traced. The 286 cotyledons originated from 73 different locations. The cull cow beef processor was chosen due to the association between abortion and herd culling. Samples from the beef processors were collected at random and were traced in order to determine whether the samples were from beef or dairy cattle (along with breed) as well as origin and vendor. Three placentomes were extracted from each placenta, placed on ice for transport to the laboratory and then stored at -80 °C for further processing. Full placentas were collected in the field at private operations, placed on ice, and transported to the laboratory where the cotyledons were extracted and stored at -80 °C for further processing. Some private owners cut cotyledons from placentas upon calving and placed them on ice or at -20 °C for pickup. Once obtained, samples were transported to the laboratory on ice and stored at -80 °C until further analyzed.

This project originally began with the idea of collecting equal numbers of both aborted and non-aborted fetuses only in the Texas Panhandle to investigate differences in prevalence of *C. burnetii* in these in the Texas Panhandle. However, we quickly discovered that obtaining fresh aborted samples was almost impossible, and the abortion rate of 2-3% would not have provided an adequate sample size for analysis. After collecting predominantly non-aborted placentas it was decided to instead look at non-aborted samples from beef and dairy cattle in our general geographic location while not limiting samples to only the Texas Panhandle.

Sample preparation

All samples were placed at 4 °C, allowed to fully thaw, and were rinsed with deionized (DI) water prior to further processing. Placentomes were split into the caruncle (maternal components) and the cotyledon (fetal components). Placental tissue (250 mg sections) were cut from each cotyledon for DNA extraction. The remaining cotyledons and caruncles were placed in 50 ml conical tubes and stored at -80 °C for preservation for any potential further analysis.

DNA extraction

In DNA extraction, several steps are needed to be able to break open the cells, remove lipid membranes, and separate DNA from proteins. There are three stages to DNA extraction: cell lysis, precipitation, and purification. The nucleic acid should be free of protein, carbohydrate, and lipid contaminants. For this study, total DNA was extracted from cotyledon tissue using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's guidelines. Tissue (25 mg) was placed in a 1.5 ml tube along with 180 µl

of buffer ATL and 20 µl of 20 mg/ml proteinase K. Tubes were placed in a dry bath at 56 °C with occasional vortexing until fully lysed (~4 hr). Buffer ATL is a detergent-based cell lysis buffer and proteinase K degrades proteins that are present in the tissues, cells and solution as well as protecting nucleic acids from nuclease attack.

Once a homogenous solution was obtained indicating tissue and cell lysis, 200 µl of buffer AL was added to the solution and vortexed for 10 sec. Ethanol, 200 µl of 95%, was added and the sample was vortexed again for 10 sec to mix. The combination of buffer AL and ethanol helps to remove salts and increases aggregation and precipitation of DNA in preparation for binding to a filter and washing away other contaminates such as lipids and proteins.

The entire solution was then placed into DNeasy Mini Spin Column and centrifuged at >10,000 × g for 1 min. At this point the aggregated DNA has bound to a filter in the spin column, along with some proteins and lipids, whereas most of the salts and other contaminates passed through the filter and have been removed. Buffer AW, 500 µl, was added to the spin column and centrifuged at >10,000 × g for 1 min. An additional 500 µl of buffer AW2 was added to the spin column and centrifuged at >10,000 × g for 3 min. Buffers AW1 and AW2 are acidic-based wash solutions that further remove proteins, lipids and other contaminates from the spin column filter while leaving the pure DNA bound to the filter. Finally, 200 µl of buffer AE was added to the spin column and after sitting for 1 min to absorb into the filter was centrifuged at >10,000 × g for 1 min. Buffer AE is a slightly basic elution buffer that releases the DNA from the filter. Extracted DNA was stored at -80 °C until used for qPCR detection.

qPCR detection

Detection of *C. burnetii* targets the DNA gyrase subunit A that encodes 97 kDa *gyrA* gene [171]. A reaction mix of 20 µl was prepared using 10 µl of iTaq Universal Probe Supermix (BioRad), 1 µl of *C. burnetii* primers/probes, 1 µl of the internal extraction control primer/probe mix, 3 µl of water, and 5 µl of template DNA. Traditional PCR is conducted starting with enzyme activation for 2 minutes at 95°C. After enzyme activation, the DNA denatures for 10 seconds at 95°C. Then annealing and extending occurred for one minute at 60°C; this process occurred for 50 cycles.

Bacterial quantification

Six 1:10 dilutions of the control DNA were made and represented in Figure 2, ranging from 200,000 to 2 cells/ul, to create the standard curve required for bacterial quantification. The Y-axis on the qPCR standard curve was from the average calculated from the triplicates for each of the six dilutions. The X-axis of the standard curve was generated from the log concentration. The standard curve was created using GraphPad Prism 8.4.3 referenced in Figure 3. For bacterial quantification, samples of *C. burnetii* genomic DNA controls (provided with the Genesig Advanced Kit) were used to establish a standard curve with qPCR. The reaction mix was composed of 10 µl of iTaq probe super mix, 1 µl of *C. burnetii* primer/probe mix, 4 µl of water, and 5 µl of positive control dilution. It was then vortexed and centrifuged before pipetting 15 µl into each well. The endogenous control reaction was not used because it was for human samples only. After the C_T values from the Bio-Rad CFX96 were generated, the value was converted to determine the number of bacterial cells per gram of tissue.

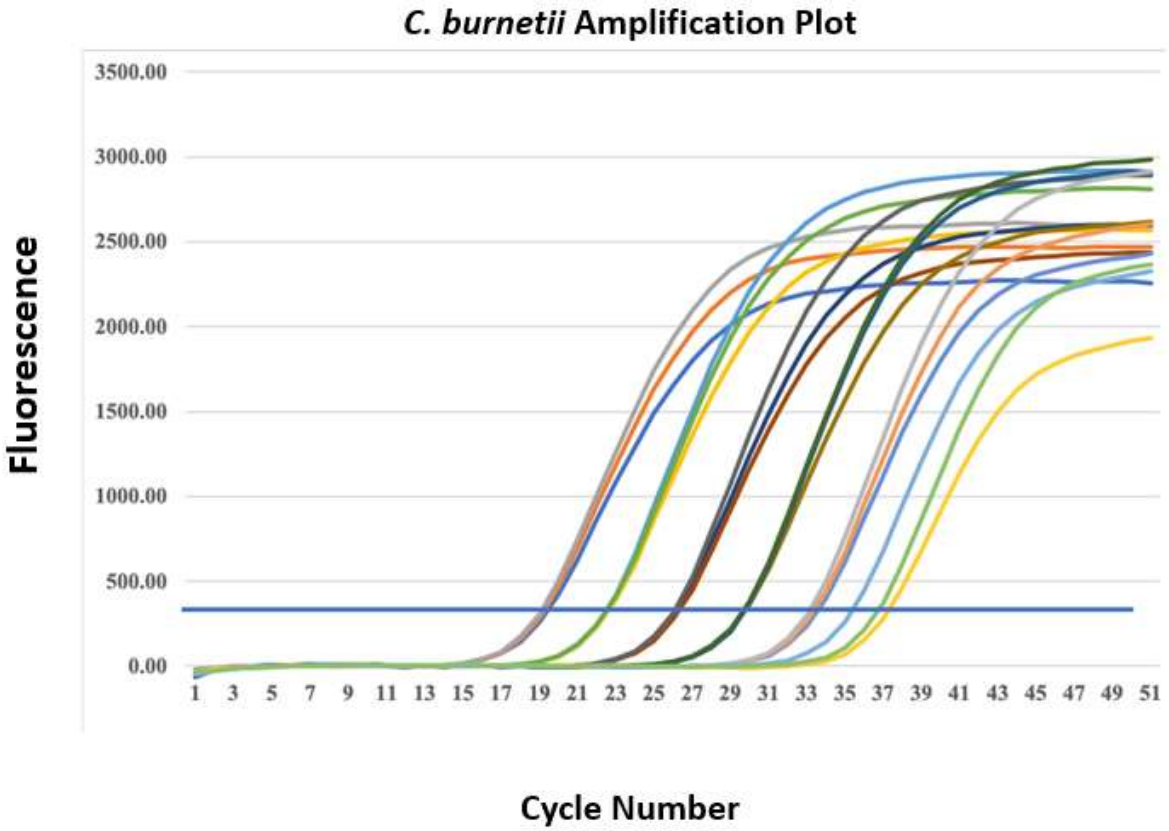

Figure 2. Standard curve quantification amplification of *C. burnetii* DNA. Six 1:10 dilutions were made from a *Coxiella burnetii* positive control, representing a range from 200,000 to 2 cells/ ul. Triplicate qPCR reactions containing 5 ul each of diluted DNA were performed. Critical threshold values obtained were used to create a standard curve for bacterial quantification.

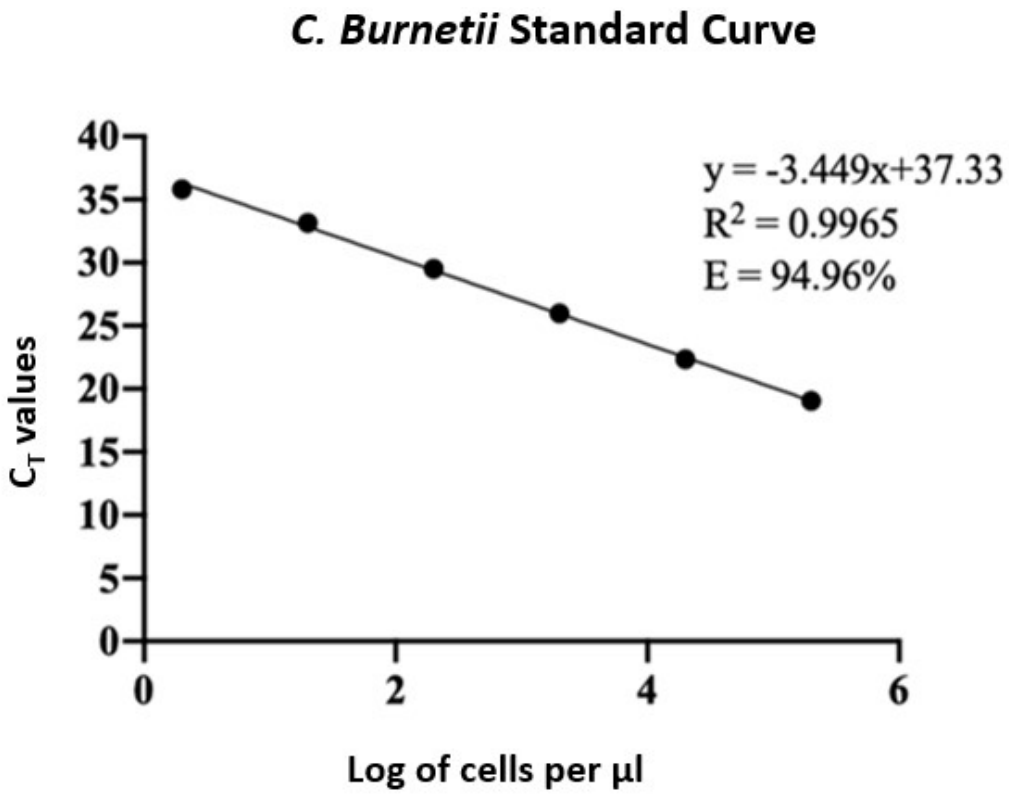

Figure 3. *Coxiella burnetii* standard curve. The standard curve was generated from critical threshold (C_T) values obtained from six 1:10 dilutions of the *C. burnetii* positive control. Bacterial concentrations are plotted as the log concentration. Linear regression analysis was performed to obtain the best fit line and equation used for quantifying bacteria in positive samples.

CHAPTER 4: RESULTS AND CONCLUSIONS

This study examined 286 cotyledons from one beef processing plant and three live operations to compare the prevalence of *C. burnetii* in the cotyledon tissue in beef and dairy cattle. Samples originated from 73 different locations found in Texas, Oklahoma, New Mexico, Arizona, Kansas, and Nebraska. Out of the 73 sites, the specific location was not known for 30. Figure 4 illustrates the positive sample locations in black and negative sample locations collected in gray.

To determine the number of bacterial cells per gram of tissue, a standard curve needed to be generated. Figure 3 describes the six 1:10 dilutions ranging from 200,000 cells to 2 cells per µl. The diluted samples were amplified three times and had a count for each of the three cycles as shown in Figure 2 and Table 1. The average of the three cycles were used to generate the Y-axis of the qPCR standard curve in Table 1. The zero to six log concentrations were used to generate the X-axis of the qPCR standard curve in Table 1. The efficiency was calculated by using the formula $E= -1+10 (^{-1/slope})$ [172]. Reaching 100% for the number of molecules of the target sequence as possible is ideal and is acceptable between 90-105% to leave a difference for errors and indicating that the polymerase enzyme is properly working at its maximum capacity. If the efficiency is over 100% this means it has polymerase inhibition and excessive amounts of DNA/RNA are in the sample causing a lower slope.

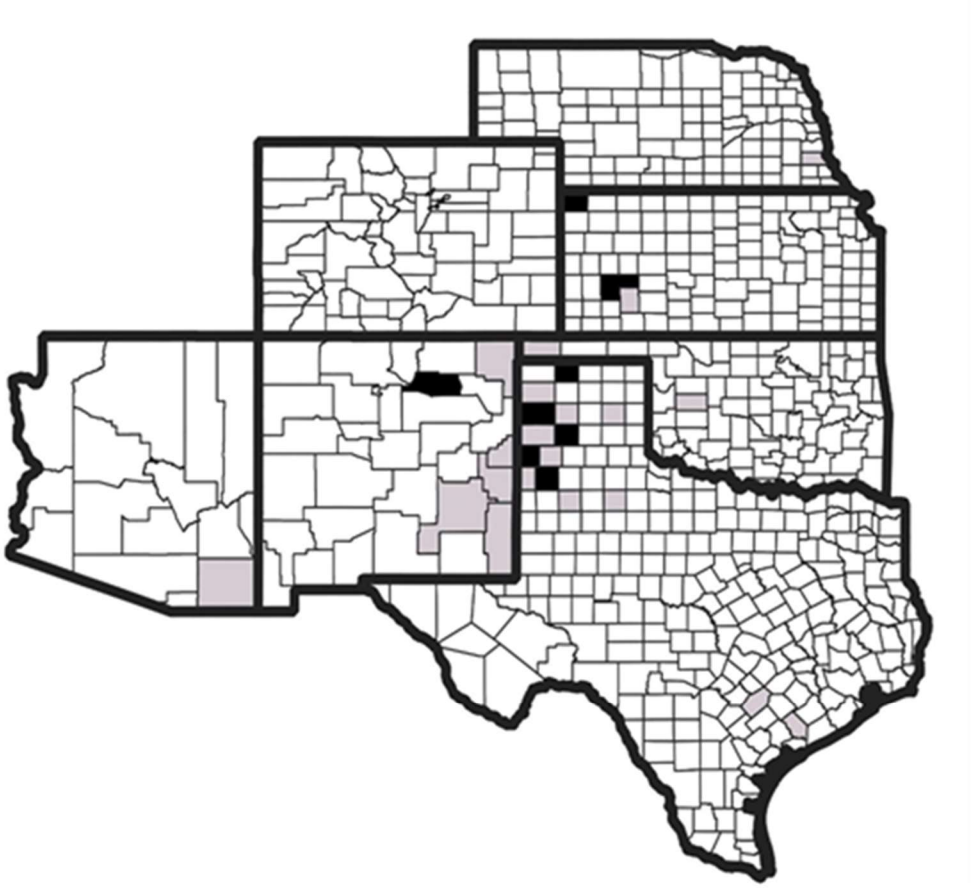

Figure 4. *C. burnetii* sample county locations collected from beef and dairy cotyledons. The black represents a positive sample obtained from that county. The gray represents negative samples obtained from that county.

Table 1. Generation of *Coxiella burnetii* standard curve.

	Log Concentration	C_T Value for Reaction 1	C_T Value for Reaction 2	C_T Value for Reaction 3	Average	Standard Deviation
200,000	5	19.19954064	19.04239771	18.90320196	19.0483801	0.148259895
20,000	4	22.39824968	22.29363083	22.31716018	22.3363469	0.054885096
2,000	3	25.99058936	26.09142863	25.85936259	25.98046019	0.116364132
200	2	29.54357825	29.54774998	29.46494803	29.51875876	0.046648113
20	1	33.35281553	33.17285059	32.97350872	33.16639161	0.189735877
2	0	37.08539323	35.1639513	36.50321771	36.25085408	0.985266624

If inhibitors are in the samples more cycles are needed for them to cross over the threshold. If the number is less than 100% it is most commonly due to bad primers or non-optimal reagent concentrations [172]. Our efficiency was 94.96%, meaning the polymerase was working properly at an acceptable rate and at a good efficiency.

Table 2 shows the source of each positive *C. burnetii* sample (if able to be tracked) with the number of bacterial cells per gram of cotyledon tissue as quantified by using the standard curve. A total of 286 samples were tested, comprised of 150 beef and 136 dairy cattle. Of the 286 samples, a total of 16 (5.6%) were positive for *C. burnetii*. Nine of the 150 (6.0%) beef samples were positive, whereas seven of the 136 (5.1%) dairy samples were positive. Only two known samples were positive from the same location (Mora, NM). One reason may have been because we only collected tagged fetuses from the gut table of the processing plant. Therefore, we could have only obtained one sample from that location that had a tag on it, making it incomparable to another. If we did obtain more than one per location and only one was positive, it could have been because *C. burnetii* had not reached the placental tissue at time of slaughter. The amount of transmission on live operations may have been low, decreasing the chance of transmission or presence of the bacteria at the location, therefore not causing an infection to the rest of the herd. The average numerically of bacterial cells in beef ($2.7 \text{ X } 10^3$) and in dairy ($1.7 \text{ x } 10^3$) were not statistically different (Table 2 and Figure 5).

Table 2. *C. burnetii* quantification of positive beef and dairy cattle samples including county of origin (if known).

County (State)	Beef/Dairy	Bacterial Cells / g
Unknown	Beef	7.9×10^3
Mora (NM)	Beef	6.3×10^3
Oldham (TX)	Beef	3.3×10^3
Unknown	Beef	2.0×10^3
Unknown	Beef	1.2×10^3
Randall (TX)	Beef	1.0×10^3
Unknown	Beef	9.3×10^2
Unknown	Beef	8.8×10^2
Mora (NM)	Beef	8.6×10^2
	Beef Average	**2.7×10^3**
Lamb (TX)	Dairy	3.5×10^3
Finney (KS)	Dairy	2.9×10^3
Parmer (TX)	Dairy	2.2×10^3
Unknown	Dairy	1.3×10^3
Unknown	Dairy	1.0×10^3
Sherman (TX)	Dairy	5.1×10^2
Cheyenne (KS)	Dairy	3.6×10^2
	Dairy Average	**1.7×10^3**
	Combined Average	**2.3×10^3**

Two statistical tests were performed including Chi-square and t-test. Chi-square analysis was used for comparing beef and dairy positive and negative outcomes to determine if there was a difference in prevalence of positive beef and dairy samples. The null hypothesis for the Chi-square test is that there was no difference in the prevalence of *C. burnetii* in beef and dairy cattle.

H_0: There is no difference in the prevalence of *C. burnetii* in beef and dairy cattle ($\mu_1 = \mu_2$).

H_1: There is a difference in the prevalence of *C. burnetii* in beef and dairy cattle ($\mu_1 \neq \mu_2$).

Chi-square results in Table 3 indicates there was no difference (P =0.75) in *Coxiella burnetii* prevalence between beef and dairy samples.

The T-test analysis was conducted to determine if there was a difference between the two means of bacterial cells per gram of cotyledonary tissue. The null and alternative hypothesis of this t- test are

H_0: The two means are not different ($\mu_1 = \mu_2$).

H_1: The two means are different ($\mu_1 \neq \mu_2$).

A Box-and-Whisker plot represented in Figure 5 illustrates there was no significant difference in the means between beef and dairy cattle (P= 0.3638).

Table 3. Contingency table of negative and positive results of *C. burnetii* in beef and dairy cattle. Chi-square analyses showed no difference ($P = 0.75$)

	Negative	Positive	Total
Beef	141	9	150
Dairy	129	7	136
Total	270	16	286

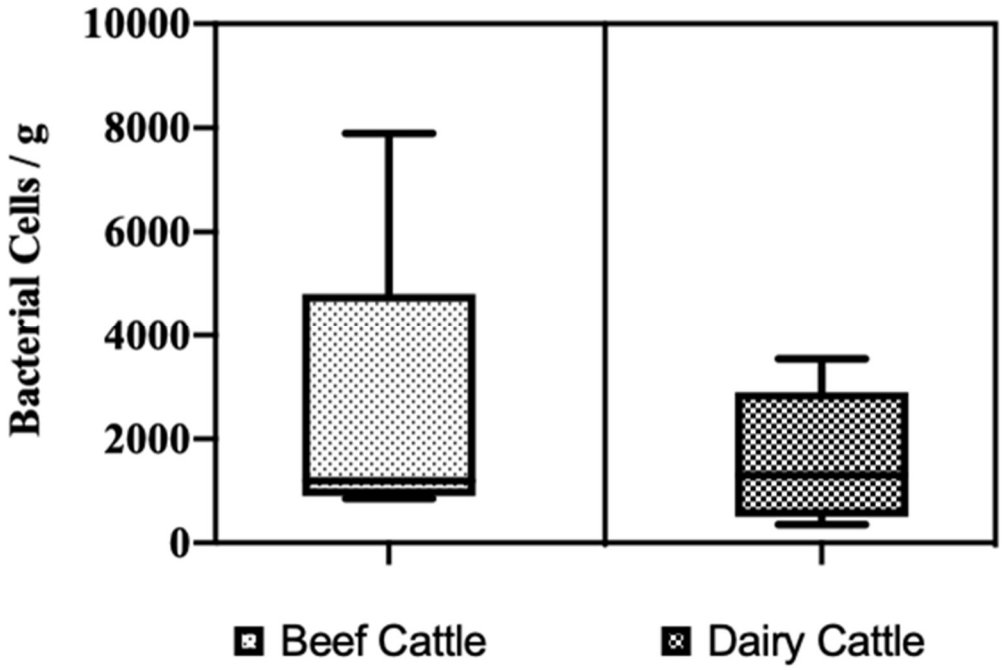

Figure 5. Box plot of *C. burnetii*-positive beef and dairy cattle bacterial cells/ g cotyledon tissue. The center horizontal line represents the mean. Unpaired t-test analysis showed no significant difference in the means between beef and dairy cattle (t = 0.9386, 14 df, *P*= 0.36).

Although different tissues have been tested, this is the first study in the United States, to our knowledge, that examined the prevalence of *C. burnetii* in the cotyledons of beef and dairy cattle. Overall, the prevalence of *C. burnetii* in the 286 samples collected was 5.6%. This included nine positive beef and seven positive dairy samples allowing us to speculate there is no preference of prevalence in beef or dairy. We anticipated a higher overall prevalence due to the ease of transmission though feces, urine, milk, and placental tissue.

We did not examine management practices, but it may be possible that the number of positive cases were low due to proper management. Livestock owners could be properly cleaning the pens or disposing of feces or birthing tissues that are left on the ground. This could eliminate inter-herd transmission to prevent the cattle from inhaling infectious urine, birthing tissue and fluids, or feces through the wind. Morbid or open cows are often culled, decreasing the probability of transmission. Albeit, *C. burnetii* could stay in the soil or be transmitted by wind.

Similar studies outside of the U.S. recorded in Table 4 have examined the prevalence of *C. burnetii* using placental tissue from beef and dairy cattle. A separate study detected *C. burnetii* placental samples with qPCR and reported a prevalence of 5.37%, [173] similar to our outcome and detection method.

Many studies outside the United States also detected the prevalence of *C. burnetii* in samples other than placental tissue (Table 4). Multiple studies used milk samples to detect *C. burnetii* [174-176]. The lowest prevalence was 8.2% for cattle, detected by

ELISA [174]. Differently, the other studies had a higher prevalence but also detected *C. burnetii* by ELISA [175, 176]. Analysis from the Denmark study reported that there was no indication of a relationship between herd density and the prevalence of the herds that tested positive [175]. Our study agrees with this statement because collection of multiple tissue samples from different cattle on the same property did not show a relationship. Meaning, *C. burnetii* may be present but not be transmitted to other animals in the herd based on herd size. Two other studies used blood to detect *C. burnetii* antibodies in cattle [171, 177]. Neither had results in a similar range when compared to our data, with one having 0 positive samples and the other having 19.5% by using different PCR methods. Another study mentioned in Table 4 that used milk for their sample method was indirectly similar to ours due to the prevalence detected at 5.06% and sample size of 238 [178]. Collection samples included milk, dough, yogurt, cheese, and ice cream, all of which were unpasteurized. DNA was extracted and PCR was used to examine prevalence.

Other studies outside the United States used placental tissue from other cloven-hooved animals to detect *C. burnetii*. Both studies had a 8.6-9% positive for sheep and goats similarly detected through PCR methods. A higher overall prevalence was reported by one of the studies at 92% that included cattle, sheep and goats. This could have been due to a population of known infected animals. Inside the United States a study tested environmental samples such as soil and dried goat birthing tissues and found 3.92% were positive [179].

Multiple studies conducted in the United States described in Table 5 also reported the prevalence of *C. burnetii* in beef and dairy cattle using samples other than placental tissue [180-186]. Each study collected various samples such as blood, milk, feces, vaginal swabs, and environmental samples. Multiple studies reported a similar prevalence to our study using immunofluorescence assay, complement fixation, and a PCR method [180, 181]. The 2016 study used milk and vaginal swabs discovering a 2 and 7% prevalence. The Northwest Texas study sampled blood from 421 beef and dairy cattle and found 5.23% had a positive result. Many studies reported a higher seropositivity and prevalence of *C. burnetii* than this project [182, 184, 186, 187].

In a related study, *C. burnetii* was detected on 7 separate goat farms and also detected more than 50 miles away [188]. This illustrates a human risk because of how far this bacterium can travel by wind. Other researchers note that if *C. burnetii* is shed in milk it is likely to be shed in feces, urine, and birthing tissues [43, 64]. These illustrate potential risk to humans by inhaling infectious particles through the air even though they might not be in contact with livestock on a daily basis or at all [189].

Certain occupations can increase the risk of *C. burnetii* infection. The reason these individuals have a higher risk of being infected is because they are not only around animals every day, but specifically ruminant livestock. A study identified the prevalence of *C. burnetii* specifically with individuals who work in the agricultural field around livestock and applied it to a population [190]. They reported that agricultural employees are six times more likely to have serological evidence of *C. burnetii* infection when compared to other non-agricultural occupations. Separate but relevant, a study with

humans using placental tissue and the cream layer of breast milk to detect *C. burnetii* in 12 women [191]. All subjects were farmers or veterinarians who underwent a total of 19 pregnancies during a 5 year period. Nine of the 12 pregnancies tested were positive for *C. burnetii* antibodies. The lack of *C. burnetii* in the placenta of 7 women could be due to treatment [191].

A study performed on Texas Panhandle residents, using blood, indicated a *C. burnetii* prevalence of 10.7%. Among these positive tests, most were in an Ag-related profession such as animal transport, animal husbandry, private slaughter, manure scraping, hauling and loading [192].

Something that could have done differently is to increase our sample size. The sample size is not sufficient to translate our data into population estimates with the millions of cattle that are available in the Texas Panhandle. Additionally, we could have collected different tissues for samples such as blood, feces, urine, or vaginal swabs. Previous studies that included more than one type of tissue found different prevalence for each. By speculating, this could be due to the bacteria not having progressed to multiple locations in the animal. Testing multiple tissues from the same animal could more accurately confirm an infection. If we had collected blood, urine, or feces we could have included males into this study.

Table 4. *Coxiella burnetii* reported in countries other than the United States.

Species	Location	Sample	Test	Results	Reference(s)
Cattle Caprine Sheep	Britain	Cotyledon tissue Fetal tissue	qPCR	Cattle cotyledons: 92% Cattle fetal tissue: 33% Caprine: 10.75% Sheep: 8.6%	[173]
Cattle Humans	Columbia	Blood	qPCR	Cattle: 19.5% Humans: 25.9%	[177]
Humans	Denmark	Placental tissue Breast milk Urine Bone marrow	IFA PCR	63%	[191]
Cattle	Denmark	Milk	ELISA	59%	[176]
Cattle	Denmark	Milk	ELISA	59% of bulk tank milk	[175]
Cattle Goats Sheep Buffalo	India	Blood	ELISA PCR	Cattle: 0.97% Goats: 5.64% Sheep: 1.85% Buffalo: 1.06%	[171]
Humans	Iran	Blood	ELISA	32.42%	[193]
Cattle	Iran	Unpasteurized dairy products	PCR	8.4%	[178]

(Table 4 cont'd)

Species	Location	Sample	Test	Results	Reference(s)
Cattle Goats Sheep	Sweden	Milk Blood Vaginal swabs	ELISA qPCR	Cattle: 7.9% Goats: 1.7% Sheep: 0.4%	[174]
Goats Sheep	West Africa	Milk	ELISA qPCR	Goats: 18.5% Sheep: 24.2%	[194]
Goats	31 countries	Blood	ELISA	8.0%	[195]

Table 5. *Coxiella burnetii* reported in the United States

Species	Location	Sample	Test	Results	Reference(s)
Sheep	California	Blood	CF	10.07%	[196]
Sheep	California	Placental tissue	CF	29%	[197]
Humans	Denver, Colorado conference attendees	Blood	ELISA	6.0%	[198]
Humans	Colorado	Blood Environmental samples	qPCR ELSA	Humans: 53% Environmental: 75%	[179]
Humans	Honolulu, Hawaii conference attendees	Blood	ELISA	22.2%	[199]
Cattle	Indiana	Milk	qPCR ELISA	61.1%	[184]
Cattle Goats Sheep	Iowa	Feces	qPCR	0%	[183]
Humans	Michigan	Milk	IFA	Humans: 100% (linked to one dairy)	[200]
Cattle Goats Humans	Missouri	Blood Milk Vaginal swabs Fecal material Environmental samples	IFA qPCR	Cow milk: 2% Cow vaginal swabs: 7% Goat milk: 17% Goat vaginal swabs: 26% Goat feces: 0% Humans: 81%	[180]
Humans	North Dakota	Blood	ELISA	3.4%	[201]
Cattle	Texas	Blood	CF	5.23%	[181]
Cattle	18 states in the United States	Milk	qPCR	In 2002: 28% In 2003: 23.5% In 2004: 31.3%	[182]

(Table 5 cont'd)

Species	Location	Sample	Test	Results	Reference(s)
Humans Goats	Washington Montana	Milk Vaginal mucus Feces Blood	ELISA qPCR IFA	Humans: 19.2% Goats: 100%	[202]
Goats	Washington Montana Oregon	Environmental samples	qPCR	70.1%	[188]
Goats	Washington Montana Oregon	Blood Vaginal mucus Milk Feces	ELISA	Blood: 12% Vaginal mucus: 29% Milk: 40% Feces: 11%	[203]
Goats	Washington Montana Oregon	Blood Feces Vaginal mucus Milk	PCR ELISA	PCR: 24% ELISA: 21%	[204]
Sheep	Wyoming	Blood	IFA	7.4%	[205]
Cattle Goats	10 states in the United States	Milk	qPCR	Cattle:14.3% Goats: 57.1%	[186]
Cattle Caprine	39 states in the United States	Milk	qPCR	Cattle: 5.0% in Michigan; 95.5% in other states	[187]
Humans	41 states in the United States	Blood	IFA	17.0%	[206]
Humans	United States	Blood	ELISA	2.43%	[190]

A future study that could be performed is to examine breeds, sex, and age of the animal. For example, a study examined *C. burnetii* infection in humans with an average age of reported Q fever infection of 56 years [207]. A study could be done to look at ages of cattle and see if older animals are more susceptible to *C. burnetii* infection or if sex could influence the outcome. A study mentioned previously examined cattle from auction and researched the seroprevalence among dairy and beef breeds. They reported the highest prevalence by blood testing was Holstein and Red Poll [181]. For future studies it would be interesting to attempt detection of *C. burnetii* on a larger scale, comparing more breeds. Additionally, the influence of livestock diet could be examined.

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