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The Prevalence of the Q-fever Agent Coxiella burnetii in Ticks Collected from an Animal Shelter in Southeast Georgia

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THE PREVALENCE OF THE Q-FEVER AGENT COXIELLA BURNETII IN TICKS COLLECTED FROM AN ANIMAL SHELTER IN SOUTHEAST GEORGIA

by

JOHN H. SMOYER, III

(Under the Direction of Quentin Q. Fang)

ABSTRACT

Q-fever is a zoonosis caused by a worldwide-distributed bacterium *Coxiella burnetii*. Ticks are vectors of the Q-fever agent but play a secondary role in transmission because the agent is also transmitted via aerosols. Most Q-fever studies have focused on farm animals but not ticks collected from dogs in animal shelters. In order to detect the Q-fever agent in these ticks, a nested PCR technique targeting the 16S rDNA of *Coxiella burnetii* was used. A collection of 450 ticks from the animal shelter were screened via nested PCR and 144 (32%) were positives. The positive PCR products were also confirmed by DNA sequencing. This is the first report of the prevalence of the Q-fever agent in ticks from an animal shelter. The results are significant to public health. Highly infected ticks in animal shelters may transmit the Q-fever agent to humans via its feces, excretion, or by biting.

INDEX WORDS: *Coxiella burnetii*, Q fever, ticks, animal shelter

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CHAPTER 1

INTRODUCTION

Importance of Ticks

Ticks are obligate, nonpermanent ectoparasites of terrestrial vertebrates, including mammals, birds, various reptiles and even amphibians. All species of ticks are exclusively hematophagous in all feeding stages. Although only around 890 species of ticks are known worldwide, they can be found in mostly every region of the world. Ticks are classified in the Kingdom Animalia, Phylum Arthropoda, Class Arachnida, Subclass Acari, Suborder Parasitiformes, Superfamily Ixodoidea. Ticks consist of three Families which are Ixodidae (hard ticks), Argasidae (soft ticks), and Nuttalliellidae which contains only a single species. Within arthropods, ticks are the second only to mosquitoes in their economic importance and transmit more kinds of pathogens than any other group of arthropods; for example, ticks are capable as vectors in transmitting various organisms that can cause diseases such as relapsing fever, Lyme disease, Rocky Mountain spotted fever, tularemia, ehrlichiosis, as well as babesiosis and anaplasmosis which are livestock diseases (Sonenshine, 1991).

Characteristics Within the Families of Ixodidae

The three tick families are easily identified because each of them has number of unique characteristics. The family Argasidae (soft ticks) have an integument that is leathery and a body plan that is unique from that of the hard ticks. They have a characteristic capitulum that is subterminal from the front margin of the body in both adults and nymphs. They can be found in dry environments where

relative humidity is low while some may reside in the humid tropics but will still select dry niches in dry habitats. Others may be found secreted into rock crevices or wall crevices as well as being found burrowed into superficial layers of soil (Arthur, 1962).

In the family Ixodidae there is biological uniformity. Members of Ixodidae (hard ticks) are found anywhere in the world where terrestrial vertebrates are found and have a different body design than that of the Argasidae. It includes a dorsal shield (scutum) during all stages of life history, a capitulum which is always visible and anterior from above and a palp whose fourth segment is reduced and contains chemoreceptor sensilla. If the eyes are present they are located laterally on the scutum while the spiracles and pulvilli which are universally present are located posterior to the fourth pair of coxae in nymphs and adults. Their ability to succeed in harsh environments may be due to their high multiplication rate as well as their ability in most genera to withstand wide varieties of temperature and humidity in comparison to other arthropods. These advantages are offset by their wandering habits, dropping from host plus their indiscriminate egg-laying habits (Arthur, 1962).

The family Nuttalliellidae contains only one species, *Nuttalliella namaqua* (Bedford, 1931). This species of tick is rare and has been found only in Little Namaqualand in S.W. Africa. This tick is unique in that it has characteristics that are intermediate between Argasidae and Ixodidae. The ways in which the Nuttalliellidae are similar to Ixodidae is that both of the ticks capitulum and mouth are anteriorly placed while the Nuttalliellidae and Argasidae share the trait of

movable segments of the palp. The scuta do contain an outline but there is no structural difference between the scuta versus the rest of the dorsum. (Arthur,1962).

Development and Reproduction

Ticks undergo four life stages, the egg, larvae, nymph, and adult. After finishing their blood meal the larvae and nymphs drop off their host and seek a microhabitat that serves as a shelter for molting and consist of non-nidicolous and nidicolous species. In non-nidicolous species or those not remaining in a nest, these surroundings could include leaf litter, soil, sand, duff floor of a meadow or forest, or could include other habitats where ticks may quest for hosts. The nidicolous tick species may use as a shelter fibers or similar structures that makeup the nests of their hosts as well as cracks and crevices (Sonenshine, 1991).

When established in the sheltered locations, the ticks become inactive then metamorphose into the next developmental stage which may vary between various species or between various stages of development. In ticks, the molting process is slow as well as temperature dependent. The fed larvae of *Dermacentor andersoni* molt in 10-11 days while the fed nymphs molt in 14-15 days when the temperature is 24ºC (Arthur, 1962). In Sonenshines lab, one hundred *Hyalomma dromedarii* nymphs molted within 19.8± 2.1 days after feeding on rabbits with a controlled temperature at 27ºC ± 2.1ºC and a relative humidity of 92% (LD cycle 16:8). As the temperature is lowered molting becomes

delayed. At temperature below 15ºC or 18ºC respectively no molting will occur for the larvae or nymphs (Sonenshine, 1991).

A factor that may limit the range expansion of ticks into cooler climates may actually be these temperature thresholds for molting. In the American dog tick *Dermacentor variabilis* the larvae and nymphs that had fed molted within 8 and 17 days respectively when temperatures were held at 27ºC (Sonenshine, 1991). The molting increased in the bat tick, *Ornithodoros kelleyi* during each successive developmental stage ranging from the larval through the N1-N4. Molting went from 3.8, 12.1, 15.3, 22.4 and 29 days respectively (Sonenshine and Anastos, 1960). In the argasids molting is affected by host parameters and temperature just as in the ixodid ticks (Sonenshine, 1991). The size of the blood meal also affects molting in ticks. Nymphs of the *Ornithodoros papillipes* which is an argasid tick will molt after feeding on a guinea-pig if consumption reaches at least 30-35% of their average blood volume capacity. The threshold is lower in the *O. tartakovoskyi* at only 20-25% necessary for molting (Balashov, 1972). A reduction in the blood meal volume may on occasion affect the morphology of the upcoming developmental stage such as an example that was noted in the *D. variabilis*. As nymphs having reduced blood meals developed into adults they were abnormally smaller and lacked the characteristic iridescent silver markings normally seen on the scutum (Homsher and Sonenshine, 1970).

A female tick will seek a sheltered environment soon after mating and completion of a blood meal which may closely resemble those used by immatures for molting. These sheltered environments will be the place where the

female will lay her eggs. Pre-oviposition is the timeframe between engorgement and the beginning of ovipositioning. The length varies and is dependent on variables such as the species involved, temperature, diapause as well as other possible variables. Some females may be autogenic thus not requiring a blood meal in order to oviposit while even other females may oviposit without insemination. The females with the greatest egg yields are usually produced only by the females who had previously mated and engorged to repletion. Several factors play a role in the number of eggs produced by female ticks that have mated and are blood-fed. These factors include blood meal volume, egg size, and species. The major factor affecting the number of eggs laid is the volume of the blood meal. Female ticks that intake larger blood meals usually deposit more eggs (Sonenshine, 1991). If a tick produces larger eggs this usually results in a lower number of eggs yielded as opposed to ticks producing a greater number of smaller eggs. A case in point was made with the tick *Ornithodoros moubata* by Oliver (1989).

After each blood meal in the Argasidae small batches of eggs are laid. These ticks have the ability of feeding and ovipositing many times. An example would be their possessing of a multiple gonotrophic cycle (Sonenshine, 1991). Most times the final clutches usually yield fewer eggs than those yielded after the first blood meal (Arthur, 1962). Batches of eggs in *Argas persicus* contain anywhere from 47 to 646 eggs (Arthur, 1962).

In a single gonotrophic cycle in mated females of Ixodidae thousands of eggs were laid. Ixodid ticks have the capability of producing an abundant number of

offspring. In the *D. variabilis* when the temperature is held at 27ºC ovipositioning starts within 3-6 days after feeding (Sonenshine, 1991). Peak egg production is reached on day 4 and continues for an additional 16-17 days. The total egg laying period last a total of 20-21 days. Over 90% of the eggs are layed during the first ten days (Sonenshine, 1970). Ixodid ticks such as *H. impeltatum* are extremely efficient in converting blood meal contents into eggs. In the laboratory these female ticks produced an average of 10,682 ± 303 eggs/female via conversion of an average of 55% of their body weight at repletion into eggs (Logan et al. 1989). Eggs deposited at different times during the egg laying period may vary in weight as well as hatchability. The females stay in one position during ovipositing. This allows the eggs to accumulate in a large quantity in front and at times over the females anterior body part (Sonenshine, 1991).

During the incubation period in some argasid species, females show a pattern of brooding behavior. Either during all or part of the incubation period the female remains over their eggs. In the bat tick *Argas boueti*, the female exibits a unique way in faciliting their young larvae's success at finding a host. The female transports their newly hatched offspring when seeking a new host. This facilitates larval host finding success (Oliver, 1989).

History and Life Cycles

The four stages exhibited in all ticks include the embryonated egg with the other three stages being active stages which include the larva, nymphal, and adult stages. Only in the adult stage is sexual dimorphism evident. Automatic implication to the individual tick as an adult is when it is referenced as either male

or female. Most species of ticks have a three host life cycle in which each active stage attempts to find a host, feed, and then drop off followed by development in its environment (Sonenshine, 1991). Development is more gradual with the Argasidae. These ticks possess multiple nymphal stages prior to becoming an adult. This type of developmental pattern is known as the multi-host cycle. This pattern includes a larva, 3 nymphal stages and the adult (Woolley, 1988). Ovipositing in mated females of the argasids occurs after each blood meal. These ticks have multiple gonotrophic cycles. In contrast, the ticks in the Ixodidae have only a single stage which directly matures into an adult (Sonenshine, 1991). In ticks known as either 2 hosts or 1 host, juveniles that have fed will remain on the host during development. Females take in enormous blood meal which is followed by the laying of thousands of eggs and then death. This is an example of a tick with a single gonotrophic cycle. All species of ticks lay eggs in which the young hatch outside of the body (Sonenshine, 1991).

Seasonal Activities of Ticks

As it became clearer of the role that ticks play in the transmission of human and animal diseases it became obvious of the necessity of becoming aware and more knowledgeable of their seasonal activity so that effective control measures could become instituted. Tick activity is used in the context as it was by Milne (1945) as a timeframe of tick infestation of sheep as well as other host which goes along with the periods on which it is usually found on the tips of vegetation. In determining the population of ticks on the vegetation a white woolen blanket or a white flag made of the same material is dragged over the same surface to

which the ticks that are active at that particular time will attach. The number of attached ticks counted on the blanket will best give a quantitative analysis of ground populations over a period of time. *Ixodes ricinus* feeds either during March to May (spring activity) or from September to October (autumn activity) (Arthur, 1962). Details relating to the seasonal incidence of *I. ricinus* in the locales of northern England as well as Scotland has been reported by Macleod (1939) and Milne (1945).

Seasonal activity and host associations of the *I. scapularis* (Acari: Ixodidae) has been documented closer to home in Southeastern Missouri. Seasonal incidence for the adult blacklegged tick, *I. scapularis* lasted from October thru May. Bimodal activity occurred in adults with November being the time higher peak and February being the time with the lower peak. Immature *I. scapularis* activity exhibited the basic pattern found in the northeast with July being the time of peak activity for the larva which followed the peaking time of the nymphs which occurred May and June (Kollars et al. 1999). Vertebrates playing important roles as hosts of *I. scapularis* include Odocoileus virginianus (Zimmerman) "white-tailed deer", and Canis latrans "coyotes". The main hosts of nymphal *I. scapularis* includes *Eumeces laticeps* (broad-headed skinks), and *Sceloporus undulatus* (Eastern fence lizards). The main host of the larval *I. scapularis* includes *Eueces jasciatus* (broad-headed skink, 5 lined skink), and *Thryothorus iydovicians* (Lathum) (Cardinal Wren). Important host for the immature *I. scapularis* were the homeotherms (warm blooded). Nymphs found on the homeotherms accounted for 30% of the total number of nymphs collected and

the number of larvae collected from these hosts accounted for 39% of the total larvae collected (Kollars et al. 1999). Another necessary behavior important for the tick's survival is diapause which helps save energy until conditions improve. Characteristics of diapause include ticks becoming inactive, a reduction in metabolic rates, and the tick's lack of feeding on host even if given the opportunity (Kollars et al. 1999).

Prevalence of Ticks Found in Georgia

A study conducted in Southeastern Georgia on ticks parasitizing domestic dogs by Wells et al. (2004) discusses the frequency of recovered tick species collected from family-owned dogs as opposed to tick species collected from shelter-maintained dogs. From the family-owned dogs in Bulloch County, 2,098 ticks were collected which included 1,980 adults, 46 nymphs, and 72 larvae. These ticks were collected from September 1996 through November 2003. There were 368 ticks that had identified from shelter-maintained dogs of the Swainsboro Animal Shelter in Emanuel County at the time the data was compiled for this paper. Of those 386 ticks 355 were adults with the remaining 13 being nymphs. Ticks collected from the animal shelter were done within the months of August and September of 2002 as well as 2003.

Ticks collected from the family-owned dogs consisted of the following distribution as far as different tick species collected is concerned. Of the 2,098 ticks collected 1,146 (54.6%) of the ticks were *Ixodes scapularis*, 582 (27.7%) were *Dermacentor variabilis*, 170 (8.1%) were *Amblyomma maculatum*, 109 (5.2%) were *Amblyomma americanum*, 72 (3.4 %) were Amblyomma

tubercalatum, 14 (0.7%) were *Ixodes affinis*, 3 (0.1%) were *Rhipicephalus sanguineus*, 1 (<0.1%) were *Haemaphysalis leporispalustris*, and 1 (<0.1%) were *Ixodes cookei* (Wells, et al. 2004).

In another study conducted in Georgia and South Carolina, 913 ticks were removed from 460 patients from 1990 through 1995 (Felz et al. 1996). Only 6 (0.7%) of the 913 ticks collected and identified were *R. sanguineus*. This information has epidemiological importance being that these tick species are vectors of zoonotic pathogens or cause tick paralysis in human (Felz et al. 1996). In a survey in Northwestern Georgia conducted between January 1998 through September 1999, 324 dogs in three northwestern Georgia counties were examined (Goldberg, 2002). The three most commonly collected were *D. variabilis* or the American Dog tick, the brown dog tick *R. sanguineus*, and the lone star tick (Goldberg M, 2002).

Studies conducted in Georgia primarily dealt with the prevalence of the formerly mentioned ticks in the wild. These ticks can be found on ranging domestic dogs as well as those confined to animal shelters. According to a survey conducted by Wells et al. (2004) the ticks that were recovered from dogs in an animal shelter included *A. americanum*, *A. maculatum*, *D. variabilis*, *I. scapularis*, and *R. sanguineus*.

Rhipicephalus sanguineus feeds primarily on dogs throughout all stages of its life (Walker, et al. 2000) but may also feed on a wide range of wildlife as well as humans. (Arthur, 1962). The immature stages of these ticks primarily attach themselves on the legs, chest, and belly of the dog while the nymphs may also

be found on the ears. Adult *R. sanguineus* ticks can be found attached mainly on the ears, head and neck (Walker, 2000). These ticks have been known as a common household pest throughout the world with dog kennels serving as breeding grounds as well (Arthur, 1962). Houses may become infested as dogs are allowed indoors. Summer appears to be the peak time for seasonal activity but these peaks may continue during the year when heated homes are inhabited. (Sonenshine, 1991).

R. sanguineus is capable of transmitting pathogenic agents such as *Babesia canis* (Shortt, 1973), *Ehrlichia* canis (Groves et al. 1975 : Walker et al. 2000), *Rickettsia* rickettsii (Greene and Breitschwerdt, 1990), and *Coxiella burnetii* (Bernasconi et al. 2002). *R. sanguineus* can also transmit the pathogen responsible for *Babesia canis* either transovarially and transstadially (Shortt, 1973). The causative agent for canine ehrlichiosis, *E. canis*, was first described in Algeria (Donatien and Lestoquard, 1935). Later findings of this pathogen included other parts of the world including the Middle East, the Orient, and other areas (Ewing, 1972). All stages of the brown dog tick is capable of transmitting *E. canis* (Groves et al., 1975 , Walker et al., 2000). Canine ehrlichiosis is the most commonly reported infectious canine disease in the United States wherever the brown tick occurs (Matthew et al. 1996). *R. rickettsii* is the causative agent for Rocky Mountain spotted fever it is not certain of the significance of the role that the brown dog tick in transmittance of this pathogen (Greene and Breitschwerdt, 1990).

The American dog tick *D. variabilis* is a major pest in areas including southeastern Canada as well as the eastern and south central United States and act as vectors for several pathogenic agents. The larvae and nymphs parasitize and feed on small mammals while the adults feed on dogs as well as medium sized mammals. Livestock as well as humans may also be attacked by these small parasites. Late spring and early summer are the active periods for the larvae and nymphs while spring and early summer are the periods in which the adults are most abundant. In the eastern United States this tick is a major vector of *R. rickettsii* which is the agent of Rocky Mountain spotted fever. The agents of anaplasmosis as well as tularemia are transmitted by the American dog tick (Markowitz et al. 1985; Sonenshine et al. 2002). This genus of ticks has also tested positive for the agent of Q-fever (*C. burnetii*). A study conducted in Germany by Sting et al. (2004) found the infectious agent responsible for Q-fever (*C. burnetii*) in one non-engorged tick of the genus *Dermacentor* as well as one sample of tick excrement from a herd of sheep.

The blacklegged tick (*I. scapularis*) can be found in large areas of eastern, south central as well as in the Midwestern United States and are capable as well as transmitting various pathogenic agents to its host. The adult blacklegged tick most commonly are found on white-tailed deer while the immature stages of this tick feed on small mammals, lizards, and birds. Any of the stages will bite humans. The Lyme disease spirochetes *B. burgdorferi* are most likely transmitted by the nymphal stage of this tick which is active in the late spring and early summer as well as by the adults which are active in the fall and early spring. The

adults also are capable of transmitting the protozoan *Babesia microti* which causes human babeiosis. Also included is the agent for human granulocytic ehrlichiosis known as *Ehrlichia phagocytophila* (Sonenshine et al. 2002) now known as *Anaplasma phagocytophilum* (Dumler et al. 2001). Infected *I. scapularis* nymphs have demonstrated the ability of transmitting this pathogen (des Vignes et al. 2001).

The lone star tick (*A. americanum*) can be found along the Atlantic coast from New York to Florida extending out west into Oklahoma and Texas and are capable of transmitting various pathogenic agents to its host. This tick in any stage of its life ranging from larvae to adult may readily attack humans and their companion animals as well as livestock and wildlife. Since deer serve as the primary hosts for the lone star adult tick, these ticks are found in abundance in areas with large populations of deer. Emergence from diapause for the nymphs and adults occurs in late spring in the southeastern United States followed by host-seeking. This tick has been implicated as a vector for the agent of human monocytic ehrlichiosis, *E. chaffeensis* as well as being suspected of transmitting *E. ewingii* to humans (Stromdahl et al. 2000; Sonenshine, 2002; Shulze et al. 2005).

Another important tick found in the southeastern United States as well as the south central U.S. and Mexico is the Gulf Coast Tick (*A. maculatum*)(Sonenshine et al. 2002) which are capable of transmitting the pathogen *Rickettsia parkeri* which is categorized in the spotted fever group (Paddock et al. 2004). The adult Gulf Coast ticks primarily feed on ruminants where as the larvae and nymphs

attack a wide range of birds and mammals. The Gulf Coast tick feeds on the head and ears of its host. Severe injury to the skin or secondary infections caused by these tick bites to the livestock may render the hides useless (Sonenshine et al. 2002).

*Rickettsia*e Transmitted by Ticks

Some of the oldest known arthropod-borne diseases are rickettsioses which are caused by obligate intracellular bacteria belonging to the genus *Rickettsia*. This genus is made up of two groups which include the spotted fever group (SFG) *Rickettsia*e as well as the typhus group (TG) *Rickettsia*e. The rickettsioses categorized within the SFG are associated with arthropods which mostly included ticks, mites, and fleas (Raoult et al. 1997) and can cause disease in man which includes a rash that occurs over most of the body and is a characteristic of the disease. This is how the disease got the name spotted fever. In this way, spotted fevers are like those pathogens within the TG that cause epidemic typhus, murine typhus, and scrub typhus which are also characterized by a conspicuous complete body rash. The genus *Rickettsia* received its name in honor of H.T. Ricketts who discovered the organisms in the tissues and eggs of ticks and provided evidence in favor of transmission via biting ticks through critical experimentation (Ricketts, 1909; Sonenshine, 1993).

These *Rickettsia*e which are responsible for the spotted fevers multiply in the tick's body tissues and are then passed on to their offspring which then introduce the Rickettsiae into the vertebrates as the ticks feed. The *Rickettsia*e of spotted fever are zoonic but also may be passed around sometimes between the wild

vertebrates and their tick vectors in epizootic cycles as well as enzootically (Sonenshine, 1993). Humans are not necessary for the upkeep of the tick-borne rickettsiosis and are considered a sort of a "dead end host which plays no role in maintenance of the organism in nature" (Walker and Fishbein, 1992). The spotted fever is generally distributed globally in every continent except Antarctica with the only tick-borne spotted fever disease of man in the western hemisphere being Rocky Mountain spotted fever with the causative agent being R. rickettsii (Kettle, 1992; Sonenshine, 1993). This pathogen is mostly found in the United States. The major spotted fever found in Europe, Asia, and Africa is known as Boutonneuse fever whose causative agent is *Rickettsia conorii*. This disease is known in various regions by a variety of names such as Mediterranean spotted fever, South African tick typhus, and Indian tick typhus (Sonenshine, 1993). Other spotted fever organisms that are tick-borne include *Rickettsia sibirica* which is the causative agent of North Asian tick typhus (Hoogstral, 1967; Kettle, 1992; Sonenshine, 1993) and *Rickettsia australis* which is the causative agent of Queensland tick typhus as well others. (Andrew et al. 1946; Arthur, 1962; Sonenshine, 1993).

Ticks have been known to harbor some species belonging to *Rickettsia* which have not been implicated in any known human illnesses as well as some *Rickettsia*-like symbionts of *Wolbachia* species. The *Wolbachia* species are sometimes confused with the disease-producing Rickettsial agents. *Wolbachia* species have been known to be deadly to some insects such as mosquitoes but are not known to kill ticks. Other significant *Rickettsia*l infections capable of

causing fatal diseases include Heartwater, Anaplasmosis, Ehrlichiosis, and certain fowl diseases (Sonenshine, 1993). Heartwater is a disease affecting ungulates living in Africa as well as the Caribbean region and is caused by *Cowdria* ruminantium (Moulder, 1974; Sonenshine 1993).

Most of these organisms develop within the cytoplasm in membrane inclusion vacuoles of infected cells. The *Rickettsia* spp. remains free in the nucleus or cytoplasm of their host. Ticks are able to transmit the causative agent of Q-fever *C. burnetii* but transmission of this pathogen is not accomplished exclusively via ticks but they are considered the primary vectors in natural cycles (Babudieri, 1959; Lang, 1990; Sonenshine, 1993).

There are two broad categories in which tick-borne *Rickettsia*e can be grouped. The first would include those pathogens with the capability of freely multiplying within the cytoplasm of the host cells as well as in the nucleus on occasion within the host cell which would include *Rickettsia* and *Wolbachia*. The second would include those that are found within the intracytoplasmic membrane bound inclusion (parasitophorous vacuoles) only as a colony which includes *Cowdria*, *Anaplasma*, *Ehrlichia*, *Aegyptianella*, and *Coxiella* (Kocan and Bezuidenhout, 1987; Gothe and Kreier, 1977). Most *Rickettsia*e are only able to multiply within living cells although they can for varying periods survive outside (Tigertt et al. 1961; Sonenshine, 1993).

What is Q-fever?

Q-fever is a zoonosis that is caused by *C. burnetii* and has a worldwide distribution (Fournier et al. 1998, Maurin et al. 1999). Q-fever has a lengthy

incubation period lasting on average of 20 days followed by distinguishable symptoms including a sudden onset of fever ranging between 38º to 40ºC. The other symptoms include chills, sweats, diarrhea, sore throat, photophobia, myalgia, and severe frontal headache along with other symptoms. Characteristically the fever is remittent with wide fluctuations lasting 5-14 days with frequent drops in fever. Frequently pneumonitis is present along with a mild cough, chest pain as well as other symptoms associated. Fatigue, as well as enlargement of the liver beyond its normal size (hepatomegaly) are common symptoms (Pellegrin et al. 1980; Qizilbash, 1983; Srigley et al. 1985; Sonenshine, 1993). A flat red rash which may include pimples or a spot or blotch on occasions may occur over the shoulders and trunk but usually a rash will not be present. Another important sign of acute Q-fever in some parts of the world includes pneumonia but not in all areas of the world. (Sonenshine, 1993).

Even though mortality rates are generally below 1%, Q-fever should be thought of as a severe debilitating condition. Q-fever may last from months or up to years persisting as a chronic illness (Raoult, 1993; Sonenshine, 1993). Endocarditis may become an important clinical condition of this disease if Q-fever becomes chronic. Endocarditis may also occur without manifesting characteristic clinical symptoms thus may only be detectable when electrocardiograms or other routine cardiac tests are done (Rehacek and Tarasevich, 1988; Marrie, 1990).

Etiologic Agent of Q-fever

In 1935 the Q-fever agent was first recognized by Edward Holbroock Derrick in febrile livestock handlers in Australia (Derrick, 1937). The "nine mile agent" later identified as the same organism as the Q-fever agent in Australia was isolated by a worker in 1936 in the United States at the Rocky Mountain laboratory in Montana and is most likely distributed world-wide. In 1938 it was reported that transmission may occur via ticks (Rehacek and Tarasevich, 1988; McDade, 1990). This pathogen formerly referred to as *Rickettsia burnetii* was classified into a new genus by Philip (1948) known as *Coxiella*, named after Herald R. Cox who isolated the organism first in the United States. This pathogen is a gram-negative, obligate intracellular bacterium that reside inside large replication vacuoles (Baca et al.1994) that have phagolysosomal characteristics which include low pH with the presense of lysosomal hydrolases and glycoproteins with RAB7 on their membranes (Maurin et al. 1992, Beron et al. 2002, Heinzen et al. 1999). The average size of these *Rickettsia*e ranges from 0.2-0.4 x 0.4-1.0 um and are smaller than those of the genus *Rickettsia* and their appearance in form ranges from coccoid, rods, or diplobacilli. Within this group of organisms there appears to be a variety of two cell sizes that are known. The first includes the small cell variants ranging in size from 0.25-0.5 um while the second includes the large cell variants ranging in size from 0.4-0.8 um (Sonenshine, 1993; Maurin and Raoult, 1999; Heinzen et al. 1999).

C. burnetii seem to have the ability to survive under harsh conditions. In susceptible cells, *Rickettsia*e develop in the phagolysosomes of the cytoplasm

(Rehacek and Tarasevich, 1988). Even with the presence of lysosomal enzymes the *C. burnetii* are able to survive and replicate. This success may be due either to their exceptional ability of replication or their resistance. In cells that would ordinarily destroy other *Rickettsia*, *C. burnetii* seem to be able to survive and thus replicate. The parasites multiplication seems to be activated by the pH of the phagolysosome. Spore-like bodies as well as small cell variants in this environment are formed. The small cell variants show resistance to sonic disruption as well as osmotic pressure changes. It is not known why but *C. burnetii* is highly resistant to chemical agents and has the ability to survive dry atmospheric conditions. Its survivability may be because of its endospore-like forms while the other possibility may be due to the small cell variants of the cell envelopes structure (Tigertt et al. 1961; Sonenshine, 1993).These organisms are able to survive in dried tick feces, hair, dry sand, dried or frozen tissues, and soil, which would be areas and conditions that would be lethal to other *Rickettsia*e. (Tigertt et al.1961; Sonenshine, 1993).

In the laboratory, a variety of cell systems can be used to cultivate *C. burnetii*. The best environment for growth includes yolk sacs of embryonated hen eggs. Tissue cultures such as with the cells of mice, guinea-pigs, and other sources provide a good environment for the *C. burnetii* to grow in (Rehacek and Tarasevich, 1988). The microbes multiply abundantly in the vacuoles and phagolysosomes during intracellular growth until virtually the entire cytoplasm is changed into one large vacuole (Kettle, 1992; Sonenshine, 1993).

Ecology and Epidemiology of Q-fever Agent

Q-fever may be as opposed to the other tick-borne spotted fever group rickettsioses epidemic as well as endemic in which infection may occur in a number of possible ways. Animal handlers are the most likely to represent Q-fever cases. Respiratory infections of *C. burnetii* may be acquired while coming in contact with infected animals as well as their products. High concentration of *C. burnetii* may be found in any of the products of these infected animals such as (aerosols) fine mist or spray from afterbirth membranes, amniotic, as well as other fluids during the birthing process. Blood, urine, feces, nasopharyngeal discharges, and milk may also contain high concentrations of this pathogen (Tigertt et al. 1961; Sonenshine, 1993). Potentially these *Rickettsia*e will spread along with dust and debris within the animal stalls, barns as well as such other similar facility. The problem may be worsened in facilities where ventilation is poor which may make the spread of the disease to the human workers easier (Sonenshine, 1993; Schulz et al. 2005). Workers that come into contact with animal products that are contaminated such as meats (Kettle 1992), milk (Benson et al. 1963) etc., are at risk of being exposed. High rates of exposure have been manifested via antibodies against *C. burnetii* (Sonenshine,1993; Schulz et al. 2005). Since this pathogen has the ability to survive in the soil and dust for a period of time the potential for infection may still be possible long after the removal of the domestic animals from an area of exposure to *C. burnetii*. The primary reservoirs for Q-fever include cattle, sheep and goats (Babudieri, 1959). Farms as well as farming communities are the most common sites for epidemic

Q-fever. This is especially true during periods of time when animal handling is high such as during wool sheering, slaughtering, calving, etc. (Sonenshine, 1993). In southern California *C. burnetii* was isolated from the air in buildings housing cattle, goats, and sheep (Sonenshine, 1993). Another group liable to be affected includes certain professional groups such as veterinarians who work with animals on a regular basis. When a building is introduced with contaminated aerosols there is a potential that infections could quickly reach epidemic proportions. At a veterinary school in Munich an outbreak of Q-fever involving 249 people occurred. Factories have also reported epidemic outbreaks (Rehacek and Tarasevich, 1988).

An important way in spreading *C. burnetii* is via milk since *Rickettsia*e are shed by animals into their milk (Benson et al. 1963, Biberstein et al. 1974). Sour milk kills *Rickettsia*e quickly but Rickettsiae are able to exist in milk and butter that is refrigerated for up to 3 months. Pasteurization kills these organisms. In the epidemiology of Q-fever, ticks play a secondary role but still an important role. The organism is capable of being harbored in the ixodid and argasid ticks while also passing it in their feces. Invasion of the organism into the midgut epithelium occurs after ingestion of a blood meal that is contaminated. Here they multiply strongly. Swelling of the infected cells occurs followed by rupturing and the releasing of masses of *Rickettsia*e into the hemolymph or gut to infect other tissues including the salivary glands (Sutakova and Rehacek, 1990; Sonenshine, 1993).

Transmission of the *Rickettsia*e is accomplished in tick feces, in eggs transovarilly prior to shell formation and to a new host when fed on by an infected tick (Parker and Davis, 1938; Arthur, 1962; Sonenshine, 1993). Feces from ticks play an important role in the spread of *C. burnetii*. This pathogen persists in contaminated tick feces. Tick species have been reported to have been contaminated for up to six years (Sonenshine, 1993). Ticks feces may form part of the dust in a local environment once they have dried and become powdered. This makes the spreading to humans or domestic animals of this infectious organism thereby easier (Arthur, 1962; Sonenshine, 1993).

C. burnetii is spread in the coxal fluids of argasid ticks. An infection from these pathogens usually does not cause any symptoms to be exhibited in mammals and has no clear course but infections from this pathogen may cause abortions. Infection existing but inactive before pregnancy could develop quickly thus causing a large numbers of *Rickettsia*e to multiply rapidly in the placenta, amniotic fluid as well as other fluids or secretions that are released during birthing (Tigertt, 1961; Sonenshine, 1993). The infection is carried by small mammals (Sonenshine, 1993) such as rabbits (Marrie et al. 1986) as they seem to play an important role in its spreading. An important tie between the wild, untamed animal and the domestic cycles of *C. burnetii* may be the small infected mammals in and around the agricultural communities. Important rickettsemias are developed and shed by these animals via their feces for weeks after infection even when there is no apparent illness. Q-fever can be found mostly world wide. Outbreaks are also known as the "Balkan grippe" since World War II in Europe

with reports of Q-fever outbreaks having come from all of the continents in the world except Antarctica (Sonenshine, 1993).

C. burnetii is also classified as a category B bioterrorism agent due to its ability to survive in the environment as well as its high infective ability when aerosolized (Seshadri, et al. 2003; Marrie, 2004). *C. burnetii* has demonstrated high resistance to chemical and physical agents, which makes it possible for the organism to remain infectious after years outside the host cell (Aitken et al. 1987). The manifestation of this disease can either be in the form of an acute or chronic illness with symptoms such as debilitating headache and cyclic fever (Maurin et al. 1999). The acute Q fever in immunocompetent hosts is usually self-limiting, whereas development of the chronic form of the disease occurs in the individuals defective in cell-mediated immunity (Maurin et al. 1999; Marrie, 2004).

Phases of *Coxiella burnetii*

A description of the *Coxiella* bacterian includes the highly virulent phase (phase 1) which are found in infected hosts and insect vectors and the less virulent phase (phase 2) bacteria. The conversion of phase I to Phase II can occur via serial passage in nonimmunologically competent hosts which may include embryonated eggs or tissue culture (Baca et al. 1983; Bobb et al. 1962; Fiset, 1957). Although the phase 2 organisms are avirulent for mammals, these organisms effectively infect and grow in cultured host cells (Baca et al. 1994, Maurin et al. 1992, Baca et al. 1983). The phase 2 *C. burnetii* of the Nine Mile strain clone 4 has a 26-kb deletion in the genome, which codes for a group if

lipopolysaccharide biosynthetic genes (Hoover et al. 2002). This feature gives genetic support for the reported differences in the composition of LPS from *C. burnetii* phase 1 and 2 (Amano et al. 1984, Schramek et al. 1982). The phase 1 C. burnetti agent is the etiological agent of Q fever; this disease is one of worldwide distribution (Maurin et al. 1999; Norlander, 2000; Williams et al. 1991).

This difference in features of the Lipopolysaccharides (LPS) between the two phases may be the main components that appear to be structurally as well as antigenically unique between the two phases of *C. burnetii* (Hackstadt et al., 1985). Hackstadt et al. (1985) shows additional evidence that *C. burnetii* phage variation is similar to the smooth-to-rough LPS variation of gram-negative enteric bacteria in which he compares phase I LPS with that of smooth LPS and phase II to rough LPS. There is actually a third variant in which its LPS chemotype has an intermediate structural complexity that is between phase I and Phase II LPS's (Hackstadt et al. 1885).

C. burnetii experiences phase variation because of a partial loss of its LPS causing an antigenic shift. During acute Q-fever the antibodies to phase II antigens predominate and have a higher titer than the phase I antibody. The IgM antibodies are mostly the first to appear just as with many other infectious diseases. Between 7 and 15 days after the onset of clinical symptoms phase II antigen-specific IgM appear in the circulation if early antibiotic therapy is not induced and reaches its maximum titer in 4 to 8 weeks. These particular antibodies can be detected by IFS for mostly short periods, which may vary with the infection or the individual host (Dupuis et al.1985, Hunt et al. 1983). Over the

12 months subsequent to infection the titers usually decrease slowly. The IgG for phase II appears later but may continue for years or even for life. In the diagnosis for acute Q-fever it is recommended that the cutoff values in the IFA for titters for phase II IgG of > 1:200 and titers of phase II IgM of > 1:50 (Tissot-Dupont et al., 1994). It should be noted that in chronic forms of the disease, mostly with endocarditis, elevated levels of phase I antibodies were detected. Cutoff values in these cases of > 1:800 for phase I IgG are used as a diagnosis of chronic Q-fever. A strong correlation with chronic Q-fever, especially endocarditis (Peacock et al. 1983, Raoult et al. 1987, Worswick et al. 1985), is the presence of IgA to phase I antigen. For this reason IgA has been considered a diagnostic of chronic forms of Q-fever (Peacock et al.1983). It has recently been shown that anti-phase I antigen IgA titers do not contribute to the diagnosis of chronic Q-fever (Tissot-Dupont et al. 1994). These though may be helpful for the follow-up of treated patients (Raoult et al. 1999).

Acute and Chronic Q-fever

Acute

There are three distinct clinical syndromes of the acute form of the disease. These include nonspecific febrile illness (fever), pneumonia (Maurin and Raoult, 1999), and hepatitis (Marrie et al. 2003), and meningoenchaliitis although to a lesser degree (Raoult et al. 1995). In the acute phase, recovery usually occurs within one to two week period, and the use of antibiotics such as tetracycline may accelerate the convalescence period (Marrie et al. 1997, Yeaman et al. 1990). Acute infections are usually diagnosed too late for tetracyclines to be effective in

treatments. If not diagnosed appropriately, inappropriate treatment or lack of treatment may occur (Marrie et al. 1997, Yeaman et al. 1990).

Chronic

Chronic Q-fever is a deadly form of the disease which often requires prolonged antibiotic therapy which may become a result of the infection (Kimbrough et al. 1979, Raoult et al. 1988), and which can relapse even though years of antibiotic treatments have been administered. Recently the concept of reoccurring or chronic Q-fever in humans has been enlarged by the detection of the *C. burnetii* pathogen's genome by PCR as well as by culture of infections in the testis and the male genital tract (Kruszewska et al. 1996, Milazzo et al. 2001), infections of the bone (Cottalorda et al. 1995, Poujol et al. 1998), lung, pleura (Whittick, 1950; Vila et al. 1996), and liver (Peacock et al.1983, Yebra et al. 1988), or soft tissue (Ellis et al. 1983). Recently, an additional sequela to Q-fever has been described in Australia namely, a long lasting often highly disabling post-infection fatigue syndrome (Q-fever fatigue syndrome) in approximately 10% of infected individuals (Marmion et al. 1996). In this report the QFS was viewed as a failure in some genetically predisposed individuals to 'switch off' residual elements of the cytokine mediated acute-phase reaction in the face of the persistence of *C. burnetii* cells after initial attack (Penttila et al. 1998). *C. burnetii* has also been implicated in long term complications including vascular disease (Lovey et al.1999). To this end the chronic form of Q-fever is almost always manifested as endocarditis (Raoult et al. 1995), but sometimes it is manifested as granulamatous hepatitis (Raoult et al. 1990), osteomyelitis or endovascular

infection (Marrie et al. 2003). Among those that had been acutely infected, the 12 year risk of endocarditis or venous thromboembolic disease was not increased (Lovey et al. 1999). However, there was a significantly higher 12 year risk of arterial disease among those who had been acutely infected (7%) as compared with those who had never been infected (4%) in addition to an increased risk of developing a cerebrovascular accident (CVA) and cardiac ischaemia (Lovey et al. 1999). In the 411 people who had been acutely infected in 1983, the 12 year mortality was significantly higher at 9.7% as compared with the 1247 participants who had remained serologically negative in 1983 at 7.0% (Lovey et al. 1999). Even though clinical manifestations for chronic Q-fever mainly includes endocarditis, reports from different countries indicate that epidemiological and clinical features of the disease may vary from one part of the world to another (Tselentis et al. 1995, Coyle et al. 1983, Haldane et al. 1983) (Marrie et al. 1988, Kimbrough et al. 1979, Dupont et al. 1992).

According to Spyridaki et al. (2002) the detection, isolation and subsequent identification and categorization of *Coxiella burnetii* strains into pathogenic or nonpathogenic is of critical importance to the understanding of epidermiology, natural history, and potential threat to human health by these bacteria. In a study by Yuasa et al. (1996), it was suggested that Q-fever should be part of the differential diagnosis of acute flu-like illness, pneumonia, endocarditis, chronic bone and liver diseases. There are only around 20 to 60 Q fever cases reported annually in the U.S., but the true incidence may be greatly underestimated (Rolain et al. 2001). In Southern France 5% to 8% of endocarditis cases are due

to *C. burnetii*, and the prevalence of acute Q-fever in Southeast France is 50 per 100,000 inhabitants (Tissot-Dupont et al. 1992). Regarding pneumonia, it can range from very mild-to-severe form and in some cases require assisted ventilation (Marrie et al. 2003). A common clinical manifestation of the effect of the disease on respiratory tissue is multiple round opacities, as evidenced by chest radiographs. (Marrie et al. 2003). Doxycycline or a fluoroquinolone is a preferred method of treatment (Marrie et al. 2003).

Detection and Diagnosis of *Coxiella burnetii*

Serological Diagnosis of Q-fever

Due to the fact that clinical diagnosis of this disease is difficult serology has been relied upon for diagnosis. Various methods employed include microagglutination (Fiset et al. 1969), complement fixation (Herr et al. 1985), radioimmunoassay (Doller et al. 1984), indirect immunofluorescence antibody tests (IFA) (Field et al. 1983), indirect haemolysis test (Tokarevich et al. 1990), or enzyme-linked immunosorbent fluorescence assay (ELISA) (Kovacova et al. 1987; Schmeer et al. 1988), dot immunoblotting, and Western immunoblotting (Blondeau et al. 1990). In choosing a diagnostic test to employ certain criteria needs to be taken into account such as specificity, sensitivity, positive predictive value, cost, and the amount of antigen required. The methods that are most commonly used and reliable include indirect immunofluorescence, complement fixation, ELISA, and microagglutination. The first two are the only ones that are commercially available (Fournier et al. 1998).

Coxiella burnetii grows slowly and can be cultivated in eukaryotic cell cultures or embryonated eggs in a bio-safety level 3 lab which may be time consuming. The drawback in the detection of bacteria by the use of either ELISA or direct immunofluorescence is its difficulty in performing along with its high detection limits. Another disadvantage in the use of serological methods like indirect immunofluorescence, complement fixation or ELISA is that there is a delay in diagnosis because it takes one to two weeks after infection before specific antibodies appear (Fournier et al. 1998, Klee et al. 2006).

DNA Amplification

The identification of *C. burnetii* via polymerase chain reaction (PCR) has been used successfully in cell cultures as well as clinical samples (Stein et al. 1992). Primers which are derived from genes specific to *C. burnetii* are readily available and allow a reliable and yet simple method for the detection of this bacterium (Stein et al.1992, Stein et al. 1992). For retrospective diagnosis, PCR has proven to be more sensitive than standard culture techniques with frozen samples, as well as for the follow-up patients treated for chronic Q-fever (Stein et al. 1992). If the specimen is maintained at -80° C, they are suitable for years for analysis via PCR (Fournier et al.1998). It has been shown by Fritz et al. (1995) that the amount of *C. burnetii* in tissue can be quantified by PCR. Thus, this technique (PCR) was the one chosen in our study using specially designed primers.

Treatment, Prevention, and Control of Q-fever

Several drugs can be used in the treatment of acute Q-fever while some promise is being shown with potential vaccines which may protect from infection

of this disease. A group of drugs used widely for treatment are the Quinolones. Treatment lasts at least 2 weeks but may last longer than 2 weeks in chronic cases (Marrie, 1990). Some apparent success has been accomplished in an effort to prepare vaccines against Q-fever. High risk groups such as animal handlers need this protection. Within a 10-15 day period after an inoculation with a Formalin-killed vaccine which was developed in Australia bestowed protection against the disease. Immunity with this vaccine lasted at least 5 years (Sonenshine, 1993). Enhancement of lymphocyte stimulation via a whole cell vaccine has been developed and is undergoing trials (Ormsbee and Marmion, 1990).

Objectives of Research

Dog shelters are facilities that provide a limited amount of room to a large dog population. In some cases 2 to 6 dogs were placed into one cage. Staff members working in these facilities are constantly handling and cleaning up after these animals. These duties may include cleaning up the feces and excretion left in the cage by these dogs. Ectoparasites such as ticks can be found on dogs in these facilities and may play a role in transmitting disease to these animals which in some cases may be transmitted to those working in the facility either directly or indirectly depending on the pathogen. Little has been done in way of a survey in determining the prevalence and types of pathogens that are present in these ticks at these shelters. A test was conducted by Kordick et al. (1999) in a dog pound setting which examined the coinfection of multiple tick-borne pathogens in dogs but no test were conducted to determine the prevalence of these pathogens

in the ticks. These species included *Ehrlichia*, *Babesia*, *Rickettsia*, and *Bartonella*. At first our study was mostly dealing with the prevalence of *Ehrlichia* canis in ticks collected from an animal shelter. Several studies have been conducted in relation to the relationship between this pathogen and dogs but virtually nothing has been done in the area concerning this pathogen, which is a tick borne disease agent and its relationship with the ticks residing in these animal shelters where the dogs reside. The objective of this study is: 1) to determine the species of ticks found in the animal shelter, 2) determine the overall prevalence of *Coxiella burnetii* in the general tick population in the shelter, and 3) determine the prevalence within each tick species and sex. This information may give us a general idea of how widespread the disease is within the population of ticks residing in animal shelters. This information could also give us an indication as to which species of ticks would probably be the most abundant as well as which species would have the highest incidence of infection. This information could be important to those working in animal shelters as well as veterinarians who work closely with dogs who may not be aware of the prevalence of this pathogen in the tick parasites that infest some of the dogs they treat and care for as well as the ease of transmission. This information may also be important to the public who may be interested in buying an animal from these types of facilities and help them become more aware of some of the risks involved.

CHAPTER 2

METHODS AND MATERIALS

Tick Collection

Ticks were collected from dogs at an animal shelter in Swainsboro Georgia by the use of forceps, with the ticks being placed in vials. Six to eight dogs were chosen at random per visit and collected from with an average of around 6 ticks collected from each dog. Specimen were kept alive and brought back to the lab in a vial and stored in a vial at -70°C. The ticks were later identified to species and sex.

Isolation of Nucleic Acids

An individual tick from each vial was placed into a 1.5 ml microcentrifuge tube followed by the addition of 300 ul of lysis solution,containing one microliter of Proteinase K (Epicenter Technologies, Madison Wisconsin) (50 µg/ul) The tick samples were then homogenized in the lysis solution with a pestel. The mixed samples were then incubated at 65°C for 15 minutes. Samples were then placed on ice for 3-5 minutes. To precipitate the protein component, 150 µl of MPC Protein Precipitation Reagent (Epicenter Technologies, Madison Wisconsin) was added to each tube and vortexed. The samples were then pelletted by centrifugation at 4°C for 10 minutes at 10,500 g. If any of the pellets became loose, dislodged, clear, or small then an additional 25 µl of MPC Protein Precipitation was added, mixed, and then pelletted again. The supernatant containing the nuclear DNA was transferred to a clean microcentrifuge tube with the pellet being discarded. We added 500 µl of Isopropanol to the recovered

supernatant followed by inversion 30-40 times with the supernatant being stored at -20°C for 30 minutes.

The precipitated DNA was again pelletted by centrifugation at 4°C for 10 minutes at >11,500 rpm, with the isopropanol being discarded without disturbing the pellet. Each DNA pellet was washed with 1 ml of 75% ethanol followed by removal of the alcohol. This wash step was repeated twice and the pellet was allowed to air dry. Each pellet was then resuspended in 35µl of TRIS-HCl (10mM) and stored at -80°C (Epicenter Technologies, Madison WI). An aliquot from each sample was then gel electrophoresed in order to verify the presence of the high molecular weight DNA (Whitlock et al. 2000).

Polymerase Chain Reaction

In detecting the *Coxiella burnetii* agent in this study a nested PCR which is specific for the 16S rRNA gene was used. Genomic DNA was used as a template for the nested PCR for the outer reaction and the PCR products from the outer reaction are be used as the template for the inner reaction (Fang, et. al. 2002). Pairs of primers were developed initially for nested PCR amplification of *Rickettsia*l 16S rDNA sequences from tick samples. Over 50 sequences of *Rickettsia*les were downloaded from GenBank and aligned using software Genetic Data Environment (GDE) (Smith et al. 1994). The published primers targeting 16S rDNA are mapped on to the alignment. New primers were designed based on the alignment with references of the published primers. The primers used for outer reaction of the nested PCR were Ehr26F (5'-TTACACATGCAAGTCGRACG) and Ehr1324R (5'-

CTGTGTGTACAAGGCCCGAGA). The inner primers were designed using the same strategy as used in the outer-primers, but each primer pair targets only one species in the *Rickettsia*les. The tick samples were first screened with the outer primers. Positive PCR products were then screened with each of the inner primers. A large number of the positive were observed using outer primer pairs but no DNA bands were produced using any inner PCR primers. Therefore, positives of the outer primers were sequenced. After BLAST searching in GenBank, the DNA sequenced from the positives using the outer primers had a very high match with the Q-fever agent, *C. burnetii* in GenBank. Then, the outer primers and the inner primers were redesigned from the alignment of *Coxiella* burnettii 16S rDNA sequences. The outer primers were: Cub_16SoutF (5'-ATTCTGGCTCAGATTGAACGC) and Cbu_16SoutR (5'-AAGGTTAGCCTACCCGCTTCT). The inner primers were: Cbu_16SinnF (5'-AAACTCGGGCTAATACCGCAT) and Cbu_16SinnR, (5'-TTCGCGCCTCAGTGTCAGTAT). All tick samples then were rescreened using both outer and inner primers designed from *Coxiella* burnettii alignment.

The PCR reactions were done with the use of a Perkin-Elmer 9600 Thermal Cycler with GeneAmp reagents (Perkin-Elmer, Foster City, CA). The DNA was amplified in a 25 µl solution for 40 cycles (Fang et al. 2002). The outer reaction included a denaturation at 94°C for 35s followed by an annealing process at 55°C for 45s, and an extension at 72°C for 2 minutes (Fang et al. 2002).The inner reaction included a denaturation at 94°C for a period of 40 seconds and annealing for 45 seconds at 55°C and an extension period of 2 minutes at 72°C

(Fang et al. 2002). The mixture for the PCR reaction will include 1x DNA polymerase buffer (10mM Tris-HCl, pH 8.5, 50 mM KCl), 2.5 mM MgCl2, 200 uM of each dNTP, 2.5 U/100ul of DNA polymerase, 0.2 of each primer, and 0.5 ul of DNA template (Fang et al. 2002). Sterile water was used in place of the template for negative controls and DNA from cultured Human monocytic ehrlichiosis (HME) agents were run in our positive controls and will be ran with each PCR reaction. The PCR product was visualized on a 1% agarose gel containing the standard 1 kb DNA ladder (Fang et al. 2002).

Positive Verification

Samples suspected as being positive for the *C. burnetii* were rescreened from the original template. Samples producing identical results to that of the initial screening were counted as positives (Fang et al. 2002). Five positive samples were randomly selected to be sent off for sequencing. The QIA quick PCR (Qiagen) purification kits were used to clean up the samples for sequencing. The purified DNA was sequenced with the 3730 DNA sequencer by Applied Biosystem at the University of Maryland's DNA sequencing facility (Center for Biosystems Research). Sequence contigs were assembled from the forward and reverse sequences, using the STADEN softward package (Staden et al. 1998). The DNA sequences transferred back to the lab were subjected to NCBI's nucleotide-nucleotide BLAST searches on GenBank to determine the most homologous sequences.

CHAPTER 3

RESULTS

A total of 450 ticks collected from the Swainsboro Animal Shelter in Swainsboro Georgia were tested and surveyed within this study. Five species were identified and included *Rhipicephalus sanguineus* (360/450 = 80.0%), *Amblyomma maculatum* (42/450 ~ 9.3%), *Dermacentor variabilis* (44/450 = 9.8%), *Amblyomma americanum* (3/450 ~ 0.7%), and *Ixodes scapularis* (1/450 = 0.2%) as shown in Table 3.1.

Table 3.1

Tick species collected from dogs at the Swainsboro Animal Shelter, Emanuel County, Georgia 2002-2003.

Tick Species	Total Collected	% of Ticks
Amblyomma americanum	3	0.7
Amblyomma maculatum	42	9.3
Dermacentor variabilis	44	9.8
Ixodes scapularis	1	0.2
Rhipicephalus sanguineus	360	80.0
Total	450	

The nucleic acids of each of these tick samples were successfully isolated which was confirmed via gel electrophoresis which verified the presence of the high molecular weight DNA of ticks (Figure 3.1). Each of these tick samples were then tested for the presence of *Rickettsia*-like pathogens using PCR technique. Confirmation of PCR products was done via gel electrophoresis. Outer primer pair (Ehrl26F/Ehrl1340), which target *Rickettsia*l like bacteria, produces a positive DNA band around 1.3 kb in size on agarose gel. The outer primers used are very conserved and are able to amplify *Rickettsia*, *Wolbachia*, or *Ehrlichia*-like pathogens. Of the 450 ticks tested with these outer primers, 198 tested positive (44%).

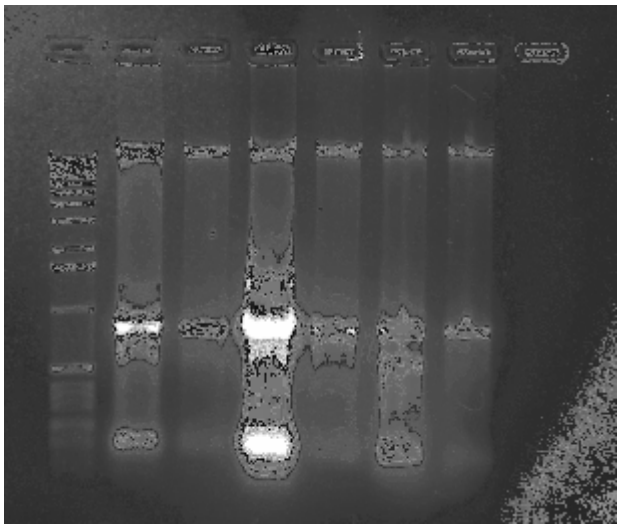

Figure 3.1

The successful extraction of DNA from the tick samples was confirmed via gel electrophoresis. The DNA was viewed by placing the gel containing the DNA under a UV light in which a picture was taken as shown above.

These 198 samples were then tested with the *Coxiella* Cbu_16SinnF/Cbu_16SinnR nested inner primers. These inner primers are specific to the *Coxiella* agent. The inner primer pair produces a PCR product around 608 base pairs in length. Of the 198 outer primer positive samples, 144 (72.8%) were positive in nested inner primer amplification. Figure 3.2 shows some of the positives on agarose gel. Fifty-four samples that had previously tested positive with the outer primer pair tested negative with the inner primer pair. Of the 450 total ticks tested, 144 (32.0%) tested positive. Of the 144 positive samples in nested reaction, 140 (97.2%) were from the tick species *R. sanguineus*, 3 (2.1%) came from *A. maculatum*, and 1 (.7%) came from *D. variabilis*. From the 450 samples of ticks tested with the *Coxiella* 16S rDNA inner primer, the following is a breakdown of the proportions of each species collected that tested positive. Of the 360 *R. sanguineus* collected, 140/360 (38.9%) tested positive, 3/42 (7.1%) *A. maculatum*, and 1/44 (2.3%) *D. variabilis* as shown in Table 3.2. Out of the 144 positive samples 5 were sent off for sequencing which included samples 5001, 5004, 5012, 5307 and 5308. The sequences were compared with homologous sequences with the use of NCBI's nucleotide-nucleotide BLAST program. The BLAST search results showed that the sequenced positives produced significant alignments with the *Coxiella spp.*16S rDNA-positive samples. The closely related strains were scored in bits and percentages of matched sequences. Bit scores ranged from 821 to 1059. The most similar strain had a bit score of 1059 with a (558/562) 99% sequence similarity between the agent isolated and the *Coxiella* strain (*Rhipicephalus*

sanguineus symbiont). The second closest strains included the *Coxiella burnetii* strains VR145, nine mile, and S4. Each scored 955 bits and a 96% sequence similarity.

Accession numbers for the *Coxiella* strain sequences downloaded from GenBank are *Coxiella spp*. (*Rhipicephalus sanguineus* symbiont)(D84559.1); *C. burnetii* strain VR145 (AY342037); *C. burnetii* nine mile strain (Y11502.1); *C. burnetii* strain S4 (Y11501.1); *C. burnetii* RSA 493 (AE016828.2); *C. burnetii* (Y11500.1); *C. burnetii* (D89800.1); *C. burnetii* (D89798.1); *C. burnetii* (D89797.1); *C. burnetii* (89792.1); *C. burnetii* (89796.1); *C.burnetii* (D89795.1); *C. burnetii* (D89799.1); *C. burnetii* (D89791.1); *C. burnetii* (M21291.1) and *Coxiella* symbiont of Carios capens (DQ100452.1).

GeneBank accession numbers for non-*Coxiella* species are *Ornithodoros moubata* symbiont (AB001521.1); *Haemaphysalis longicornis* symbiont (AY342035.1); *H. longicornis* symbiont (AB001519.1); *H. longicornis* symbiont (AY342036.1); *Legionella* sp. (AB058918.1); *Legionella* sp. (AB058917.1); *Legionella* sp. (AB058916.1); *Legionella* sp. (AB058915.1); *Legionella* sp. (AB058914.1); *Legionella* sp. (AB058911.1); *Legionella* sp. (AB058910.1); *Legionella* sp. (AB058907.1); *Legionella* sp. (AB058909.1); and *Legionella* (AB058908.1).

The prevalence of C. *burnetii* varied between male and female adults as well as nymphs between the species collected. Out of the 450 ticks surveyed in this study only 420 were positively identified as male, female or nymph (developmental stage). The following data will take this number into

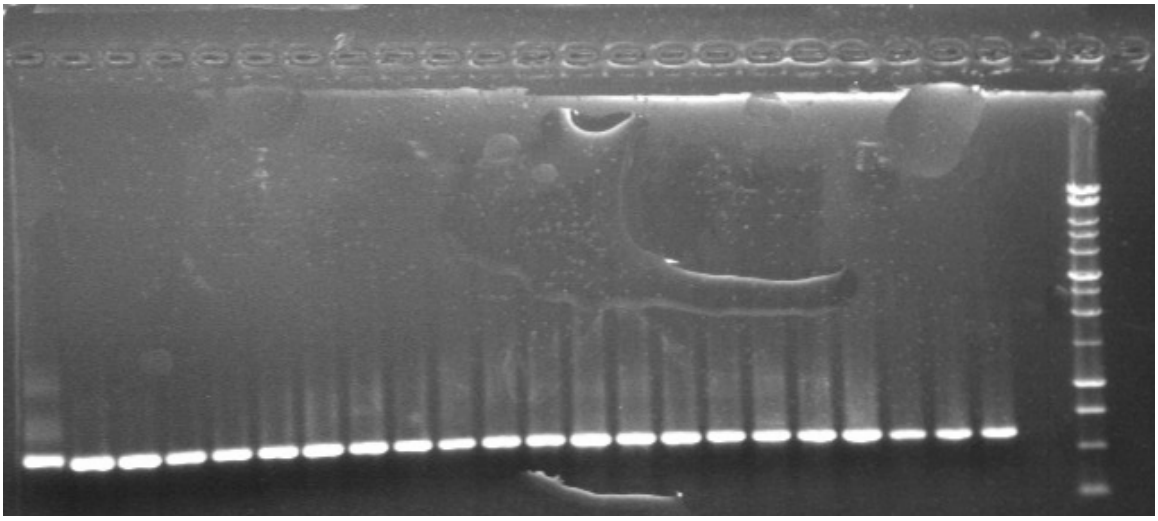

Figure 3.2

Results from samples tested via nested PCR techniques using nested primers designed from *Coxiella burnetii* alignment. The expected 608 base pair fragment were detected after the samples were loaded onto a gel, ran, and then visualized under UV light.

consideration with the following results. Within the *R. sanguineus* species 83 of the 146 males collected (56.8%) tested positive for C. *burnetii*. In the females of this tick species 46 of the 168 females (27.4%) tested positive while 4 of the 20 nymphs collected (20.0%) did as well as shown in Table 3.3. There were 360 ticks screened from the species *R. sanguineus* but only 334 of these ticks were recorded as male, female, or nymph. A comparison in Table 3.3 gives a breakdown on the prevalence of *C. burnetii* within each of the tick species collected from the animal shelter.

There were 133 ticks out of the 334 ticks that were clearly identified by gender or developmental stage within the tick species *Rhipicephalus sanguineus* that

Table 3.2

Prevalence of *Coxiella burnetii* infection found in each of the tick species collected. Each of the positive samples collected produced the 608 bp 16S rRNA gene fragment that is characteristic of the agent of *Coxiella burnetii* after PCR amplification.

Tick	No. ticks screened	No. positive	Prevalence/ (95% CI)
Amblymoma americanum	3	0	0.0
Amblyomma maculatum	42	3	7.1 (0.0-15.1)
Dermacentor variabilis	44	1	2.3 (0.0-6.9)
Ixodes scapularis	1	0	0.0
Rhipicephalus sanguineus	360	140	38.9 (34.6-45.0)

tested positive for *Coxiella burnetii*. Of those 133 ticks within this species that tested positive 83 were (62.4%) were comprised of males while 46 of the 133 (34.6%) samples were comprised of females and 4 of the 133 positive samples (3.0) were comprised of nymphs as seen in Table 3.4. This table also gives a summary in reference to the infection rate of *Coxiella burnetii* in the other tick species collected from the animal shelter. The number of ticks representing the other species was small.

As previously stated a total of 450 ticks were tested for *Coxiella burnetii* but the genders of only 420 of these ticks were recorded. Of the 420 ticks where gender is recorded 199 were male while 200 were recorded as female and a total

Table 3.3

The prevalence of *Coxiella burnetii* among the adult and nymphs within various species of ticks collected from the Swainsboro Animal Shelter.

Tick	Adult Gender Or Nymph	No. of Ticks Screened	No. Positive	Prevalence/ (95% CI)
Amblyomma americanum	Male	1	0	0.0
Amblyomma americanum	Female	2	0	0.0
Amblyomma americanum	Nymph	0	0	Ø
Amblyomma maculatum	Male	25	0	0.0
Amblyomma maculatum	Female	13	2	15.4 (0.0-36.3)
Amblyomma maculatum	Nymph	1	0	0.0
Dermacentor variabilis	Male	27	1	3.7 (0.0-11.1)
Dermacentor variabilis	Female	16	0	0.0
Dermacentor variabilis	Nymph	0	0	Ø
Ixodes scapularis	Male	0	0	Ø
Ixodes scapularis	Female	1	0	0.0
Ixodes scapularis	Nymphs	0	0	Ø
Rhipicephalus sanguineus	Male	146	83	56.8 (48.6-65.0)
Rhipicephalus sanguineus	Female	168	46	27.4 (20.4-34.4)
Rhipicephalus sanguineus	Nymphs	20	4	20.0 (15.0-38.5)

Table 3.4

The distribution of *Coxiella burnetii* within the genders of infected adult ticks or nymphs collected from the Swainsboro Animal Shelter testing positive.

Tick	Sex	Total No. Positive in Each Species	No. Within Gender Testing Positive	% of Positive Ticks Within Each Gender/(95% CI)
Amblyomma americanum	♂	(0)	0	Ø
Amblyomma americanum	♀		0	Ø
Amblyomma americanum	N		0	Ø
Amblyomma maculatum	♂		0	0.0
Amblyomma maculatum	♀	(2)	2	100.0
Amblyomma maculatum	N		0	0.0
Dermacentor variabilis	♂	(1)	1	100.0
Dermacentor variabilis	♀		0	0.0
Dermacentor variabilis	N		0	0.0
Ixodes scapularis	♂	(0)	0	Ø
Ixodes scapularis	♀		0	Ø
Ixodes scapularis	N		0	Ø
Rhipicephalus sanguineus	♂	(133)	83	62.4 (54.0 70.8)
Rhipicephalus sanguineus	♀		46	34.6 (26.4-42.8)
Rhipicephalus sanguineus	N		4	3.0 (0.0-6.2)

♂= male ♀= female N=nymph

of 21 as nymphs. A total of 84 of the 199 males (42.21%) tested positive for *Coxiella burnetii* while 48 of the 200 females (24.00%) and 4 of the 21 nymphs (19.05%) did likewise (See Table 3.5).

Table 3.5

The distribution of *Coxiella burnetii* within the male, female, and nymph ticks collected from the Swainsboro Animal Shelter.

Adult Gender Or Nymphs	No. Collected	No. Positive	% Positive For *Coxiella burnetii/* (95% CI)
Male	199	84	42.21 (35.2-49.2)
Female	200	48	24.00 (18.0-30.0)
Nymphs	21	4	19.05 (14.0-36.8)

There was a wide distribution of ticks collected from the animal shelter based on species and gender or developmental stage as seen in Table 3.6. There were 420 ticks that were identified altogether (according to gender or developmental stage) covering 5 species. The species *Rhipicephalus sanguieneus* comprised 334 of the 420 (79.52%) ticks identified. Of those 420 identified 34.76% (146/420) were comprised of male *Rhipicephalus sanguineus* while the females of this species comprised 40.00% (168/420) of the ticks of this survey and the nymphs of this species comprised 4.76% (20/420). The other species contributed

Table 3.6

The distribution of adults and nymphs from each species collected from the Swainsboro Animal Shelter. Thirty-two percent of the total tick samples (144/450) tested positive.

Tick	Sex	No. Collected	Total Ticks Collected	% of Total Ticks Collected
Amblyomma americanum	♂	1	420	.24
Amblyomma americanum	♀	2	420	.48
Amblyomma americanum	N	0	420	0.00
Amblyomma maculatum	♂	25	420	5.95
Amblyomma maculatum	♀	13	420	3.10
Amblyomma maculatum	N	1	420	.24
Dermacentor variabilis	♂	27	420	6.43
Dermacentor variabilis	♀	16	420	3.80
Dermacentor variabilis	N	0	420	0.00
Ixodes scapularis	♂	0	420	0.00
Ixodes scapularis	♀	1	420	0.24
Ixodes scapularis	N	0	420	0.00
Rhipicephalus sanguineus	♂	146	420	34.76
Rhipicephalus sanguineus	♀	168	420	40.00
Rhipicephalus sanguineus	N	20	420	4.76

♂= male ♀= female N=nymph

less in number to the survey of ticks collected in comparison to *Rhipicephalus sanguineus* and can be compared to one another in Table 3.6.

CHAPTER 4

DISCUSSION

C. burnetii is an obligate intracellular parasite (Spyridaki et al.2002). *C. burnetii* is the only species in the genera *Coxiella* (Kettle, 1992) as this pathogen was formerly known as *Rickettsia burnetii* and was later placed into a new genera known as *Coxiella* because of cultural and biochemical characteristics. The new genus *Coxiella* was a new name given after the man who first isolated this microorganism in the United States Herald R. Cox (Philip, 1948). *Coxiella* was also partially named after Sir McFarlane Burnet who was an Australian Nobel Prize winner in Medicine who also is credited as having been the first person to have studied the agent *Coxiella burnetii* (Kettle, 1992).

Ticks and vertebrates like rodents play key roles in the enzootic cycle with ticks playing an important role in maintaining the viability of this parasite in the environment (Aitken, 1987). *C. burnetii* can grow to high titers in ticks and can remain viable during its entire life in which the pathogen can also be transmitted to the new generation transovarially (Walker and Fishbein, 1991). In an area which may be endemic for Q fever, each tick species that preys upon a susceptible host has a potential to harbor and spread *C. burnetii*. *C. burnetii* is transmitted to domestic animals in nature indirectly through contact with infected excreta or by tick bites (Aitken, 1987).

(Bernasconi et al.2002) did a survery in southern Switzerland in reference to pathogens harbored by the (brown dog tick) *R. sanguineus* as well as *Rhipicephalus turanicus*. These ticks may act as a vector of various pathogens

such as *Rickettsia conorii* and *C. burnetii*. (Bernasconi et al. 2002) was able to identify the occurrence of the *Coxiella* sp. in both tick species by using the Cbu_16Sinner primers. The *R. sanguineus* tick had been imported into areas such as kennels which provide an appropriate condition and blood source necessary for their survival (Bernasconi et al. 2002).

The prevalence of the Brown Dog Tick (*R. sanguineus*) was the most abundant in terms of ticks collected from the animal shelter consisting of 80% of the total ticks collected which may be a reason why the majority of the positive samples (97.2%) came from this tick species. *C. burnetii* has been detected in these ticks in past studies via nested PCR techniques as well as PCR-restriction fragment length polymorphism analyses (Spyridaki et al. 2002). PCR was the preferred method of choice in testing these ticks for the prevalence *C. burnetii* because it is easy, accurate, and cost efficient. In this study 144 out of the total 450 collected (32%) tested positive for *C. burnetii*. A total of 140/360 (38.9%) of the *R. sanguineus* ticks tested positive with the suspected *C. burnetii* pathogen according to the PCR results using Cub_16S inner primers. According to the NCBI blast *C. burnetii* is the most likely pathogen identified using this primer and copying the expected 608 base pair sequence belonging to this pathogen. In order to determine as best as possible the pathogens most likely identity random samples were sent off for sequencing and then compared to other samples using NCBI's blast program. The most likely candidate from our results was the pathogen *C. burnetii*.

Next in abundance for ticks collected in this study was *D. variabilis* consisting of (44/450) 9.8% (see Table 1) of the ticks collected in this study. *C. burnetii* has been isolated from various *Dermacentor* species such as *D. andersoni* that were collected from Montana (Davis et al. 1938) and *D. marginatus* in the Southwest part of Germany (Kettle, 1992). *C. burnetii* has been present in the haemocytes of naturally infected *D. marginatus* (Kettle, 1992). In this study only (1/44) 2.3% of the *D. variabilis* ticks collected tested positive for *C. burnetii*. This is somewhat significant because of the basic lack of information published in reference to a relationship between this pathogen and the *D. variabilis* (American Dog tick).

Over 40 species of ticks are naturally infected with *C. burnetii* including one from a study involving a tick from the genus *Amblyomma* known as *Amblyomma triguttatum* (Pope et al. 1960). While it has been detected in the kangaroo tick (*A. triguttatum*) there is virtually no mention of this pathogen being found in the tick *A. maculatum* which accounted for (42/450) 9.3% of the ticks collected in this study. In this study (3/42) 7.1% of these ticks collected tested positive which may be somewhat significant because of the basic lack of information published in reference to a relationship between this pathogen and the *A. maculatum* tick. A study within itself could be conducted to test for the prevalence of *C. burnetii* in *A. maculatum* ticks collected from dog shelters. *A. americanum* ticks contributed to (3/450) .7% of the total amount of ticks collected but none of the 3 collected tested positive for the *C. burnetii* pathogen.

I. scapularis accounted for (1/450) .2% of the total amount of ticks collected. There have been documented cases of a couple of *Ixodes* species

being infected with *Coxiella burnetii* such as was the case with it being isolated from *Ixodes holocyclus* in Queensland (Carley and Pope, 1953). *Ixodes ricinis* is considered as a vector Q-fever (Kettle, 1992). In a study comparing the virulence of *C. burnetii* isolates from ticks versus bovine milk, laboratory animals were infected with isolates of *C. burnetii* received from ticks of *I. ricinus* and the ticks of bovine milk. In comparison, the fever in guinea pigs infected with isolates from milk was less evident than in guinea pigs infected with isolates from ticks (Kocianova et al. 2001).Virtually no study can be found indicating that this pathogen has been isolated from *I. scapularis* and in this study the single *I. scapularis* tick tested for *C. burnetii* tested negative.

The prevalence of this pathogen in ticks collected from animal shelters in S.E. Georgia is unknown. This was the first study conducted of this sort in this area. In the final analysis involving all the ticks collected 144 of the 450 surveyed (32%) tested positive for *C. burnetii*. This high rate of infection (32%) in the overall population of ticks collected from the animal shelter may have been due in large part to a large dog and tick population confined to a small area. The ticks could possibly become infected by coming into contact with contaminated dog urine and feces (Tigertt et al. 1961; Sonenshine, 1993). Many of the dogs had multiple (2-20) ticks feeding simultaneously on them at once. If this dog is infected with *C. burnetii* than these ticks when feeding again may pass the infection to another dog. If another dog becomes infected than there is a potential for other ticks as they feed on this dog to become infected thus spreading the disease easily and rapidly in this limited and small environment. These ticks as

earlier stated can pass this pathogen (*C. burnetii*) to the next generation transovarilly (Sonenshine,1993) which means that new ticks born to these infected parents will potentially increase the population of infected ticks which in turn may potentially cause an increase in the number of infected host (dogs). This may increase the risk of more dogs becoming infected with this pathogen as they are fed upon by potentially infected ticks. If more dogs become infected than this may cause a potential problem with disease control since *C. burnetii* may be spread via excretion or tick bites (Aitken et al. 1987). This is potentially troubling since 2-6 dogs may be placed together in a pen as observed at the site of study. This could potentially affect those working in these areas that are exposed to excreta from the dogs as well as occasional tick bites encountered from tick exposure. This could be a problem for those who purchase their pets from these facilities as the potential to becoming exposed to this pathogen is possible just by exposure from the excreta given off by the pet since it is possible that the potential pets may lack any symptoms.

An interesting result from this survey showed that the prevalence of male infection among all the ticks collected in which sex was determined was 42.21%. This was almost twice as high as female infection rate of 24.00% with the nymphs not far behind at 19.05%. Why was the male incidence of infection that much more than the females or nymphs? The total number of females collected (200) was pretty even with the number of males collected (199). This may be an area that needs more attention and observation. Within the species *R. sanguineus* 146 males were identified by sex and 83 (56.8%) tested positive for

C. burnetii. For the females 168 were identified by sex and only 46 (27.4%) tested positive with 4 in 20 (20.0%) of nymphs testing the same. Within this species there were 334 ticks tested with 133 testing positive. Out of those 133 ticks within this species testing positive males contributed to 83 (62.4%) of the infected ticks. Females contributed to 46 (34.6%) of the 133 positive samples and nymphs 4 (3.0%) of the positive samples. This is mostly consistent with the overall results in reference to male, female, and nymph infection ratio.

The number of female to male to nymph infection was documented. Within the species *A. maculatum* 100% of the infections within this species were seen in the females. Two ticks collected from this species tested positive and both were female. The significance of this is hard to determine because of the small numbers (13) of females within this species that were collected and tested. Within the species *D. variabilis* 100% of the infected samples for *C. burnetii* came from male ticks. Again only 1 tick from this species tested positive and the significance of this is hard to determine because of the small number (44) of this species collected and tested(see Table 1). The reason that fewer of these ticks were tested may have been due to the fact that ticks in this survey were collected randomly and those predominantly found in the animal shelter were *R. sanguineus*. The significance of the pathogen in reference to the male, female, and nymph ratio is concerned within the other species other than *R. sanguineus* (i.e. *D. variabilis* or *A. maculatum*) could be better determined effectively only if these ticks are collected and tested and a comparison made between the male, female, and nymphs within these species.

In a previously mentioned study conducted at a dog shelter in North Carolina dogs were tested for coinfection for several different pathogens species of *Ehrlichia*, *Babesia*, *Bartonella*, and *Rickettsia* (Kordrick et al. 1998). This study dealt with the prevalence of *C. burnetii* within the tick population in a similar setting. A future study could be more inclusive by combining the ideas from both studies which would include testing for the prevalence of *C. burnetti* in both the dogs and the ticks in an animal shelter setting. This would provide a more thorough picture in relation to the widespread distribution of this pathogen in the overall community of the animal shelter. It would also be useful if the dogs as well as the tick parasites were tested for this pathogen when first entering the facility. The dogs would be tested again along with their newly acquired ticks (Those acquired since entering the facility) prior to leaving. This could give us an idea on when the dogs most likely acquired the *C. burnetii* pathogen as well as possibly who transmitted it to whom. Since this pathogen can be transmitted in so many different ways such as through the feces and excreta of the other dogs, every dog would need to be tested for this *C. burnetii* at the beginning of the study. Another necessary component in this future study would be to test those if possible working in the animal shelter to see if they are infected as well with this pathogen.

In summary, this study examines the potentially high prevalence of a very infective pathogen in a small limited enclosed environment which is potentially important to both human and veterinary medicine. As mentioned in a study about tick borne pathogens in a kennel setting in North Carolina that private and public

health providers, in particular veterinarians and physicians, should be aware of the increased potential for vector-borne illnesses in both humans and dogs, especially when large numbers of dogs with extensive tick exposure are maintained for recreational purposes.(Kordick et al.1999).

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