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Molecular Detection and Characterization of Rickettsia sp. in Hard Ticks Collected in Nacogdoches, Texas

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By

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ABSTRACT

The number of cases of tick-borne diseases is increasing in the United States. Many efforts to control ticks have been made and are continuously being developed. These methods include surveillance of ticks and detection of the tick-borne pathogens.This study examined hard ticks in Nacogdoches, Texas, to test for the presence of *Rickettsia* sp. 47 ticks were collected from vegetation by flagging and their species, sex, and developmental stage were determined using available keys. Individual ticks were processed for nucleic acid extraction and molecular detection of *Rickettsia* sp. using PCR-based methods. The citrate cynthease gene, 17kDa gene, and outer membrane protein A gene were used as genetic markers. All 47 ticks were tested with these markers and out of 13 *Amblyomma americanum* ticks 4 were positive, 9 *Dermacentor variabillis* ticks 1 was positive, 6 *Amblyomma maculatum* ticks 4 were positive, and out of 19 *Ixodes scapularis* ticks 12 were positive.

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INTRODUCTION

Need for tick surveillance and pathogen detection

Ticks are found across the United States and are of significant medical and veterinary importance. They are vectors for harmful pathogens that cause diseases such as Lyme disease, Rocky Mountain spotted fever, Colorado tick fever, babesiosis, and tularemia (CDC, 2022). In 2019 there were a total of 50,865 cases of tick-borne diseases (TBDs) reported to the Centers for Disease Control (CDC). These numbers were reported by state and local health departments (CDC, 2021). Even though the number of tick-borne diseases reported is high, it is estimated that the total number of tick-borne diseases are underreported every year (Mendell et al., 2019). Misdiagnosis or not seeking medical attention plays a key role in the underreporting of tick-borne diseases. One example of this underreporting can be seen in Lyme disease cases. There are approximately 35,000 cases of Lyme disease reported each year in the United States, but recent studies have found that the true number of annual cases is approximately 300,000 (Tick-borne, 2018). Common symptoms of many of these diseases include fever, headache, muscle aches, and chills. These symptoms are like the flu and many other common infections which leads medical professionals to misdiagnosing patients. Ticks also affect the economy through the cattle industry. The cattle industry is one of the biggest agricultural commodities in the United States and the problems ticks cause can be devastating.

Approximately 80% of the world's cattle population is affected by tick-borne pathogens (TBPs), and the total global cost associated with ticks and tick-borne pathogens is estimated to be around $13.9 billion (Estrada and Salman, 2013). Even with all these consequences there are still only limited control methods. Therefore, there is a need for more tick surveillance and pathogen detection.

Identification is the first step in the battle against tick-borne diseases. The knowledge of where these ticks are located and where specific diseases are prevalent is critical for public health. Eisen and Paddock (2013) explain that the lack of ongoing tick surveillance impedes the development of accurate tick distribution maps. The authors state that often fragments of data can be pulled from journal articles, but critical information is often missing (Eisen and Paddock, 2021). A survey was provided to 140 vector-borne disease professionals (Mader, 2020). Of those 140 professionals only 12% conduct or support tick control efforts, and only 14% said they report their findings to the CDC, which shows that there is a need not only for more surveillance, but for more collaborations across not only Texas but the United States.

Taxonomy and morphology of *Rickettsia* sp.

Rickettsia is in the order Rickettsiales, which consists of two major families, *Rickettsiaceae* and *Anaplasmataceae*. These bacteria are very closely related, and the term rickettsia is used interchangeably between families (Li et al., 2019). This study will focus specifically on the genus *Rickettsia*. There are 25 described species of *Rickettsia*, and the genus is divided into two main groups: the spotted fever group and typhus group

(McGinn and Lamason, 2021*). Rickettsia* cells are very small, approximately 0.3-0.5 X 0.8-2.0 μm. *Rickettsia* are considered pleomorphic which means they can appear in different shapes, although they are typically referred to as coccobacilli. They can be found singly, in pairs, or in short chains (Sunyakumthorn et al., 2008). *Rickettsia* are gram-negative, although typical gram staining does not work for these bacteria. Giemsa stain must be used to obtain the typical red color associated with staining gram-negative bacteria (Rickettsia, 2010). This means they have a thin cell wall that is made up of a single layer of peptidoglycan which is surrounded by an outer membrane. *Rickettisa* also have small genomes ranging from 1.1-1.8 Mbp (El Karkouri et al., 2016). These genomes typically contain numerous protein-coding genes ranging from 902-2309 (Awa et al., 2018). Originally it was thought that these genomes didn't contain plasmids, but in recent studies, there have been 20 plasmids found between 11 species of *Rickettsia* (El Karkouri et al., 2016). Within these plasmids it was determined that there were 260 protein-coding genes (El Karkouri et al., 2016).

Reproduction and dissemination of *Rickettsia*

Rickettsia are obligate intracellular pathogens, which means they depend on entry into the host cell cytoplasm for survival (Radulovic et al., 2002). To ensure survival the bacteria developed a specific mechanism of infection. They use surface proteins OmpA and OmpB to attach to the membrane of the host endothelial cells. OmpA binds to α2β1 integrin which triggers entry into the host cells by phagocytosis (David, 2007; Hillman et al., 2013). This is when the plasma membrane of the host cell engulfs the bacterium and

forms a phagosome membrane surrounding the bacterium. To continue the process of infection and replication the bacterium must escape the phagosome. *Rickettsia* does this by secreting phospholipase D and hemolysin C (Sahni, 2013). These secretions disrupt the membrane of the phagosome and allow the rickettsia to escape into the cytosol of the host cell. Once in the cytosol of the host cell, the infective mechanism begins to differ between the spotted fever group and the typhus group. The spotted fever group develops an actin tail by polymerization of the host's cell actin molecules (Van, 2000). With the formation of this tail, the bacterium is propelled into the cell membrane which creates cell membrane protrusions. These protrusions are then engulfed by the neighboring host cells and a new life cycle can be initiated. This type of spread is considered cell-to-cell spreading because the bacteria infect neighboring cells through the cell-cell junctions (David, 2007). However, the typhus group does not form an actin tail and therefore must lyse the host cell to gain access to neighboring cells. Once *Rickettsia* are in the neighboring cell's cytoplasm they are free to reinitiate their life cycle. *Rickettsia* do this by reproducing via transverse binary fission (McGinn and Lamason, 2021).The *Rickettsia* will continue the cycle of replication within the cell's cytoplasm and move to neighbor cells causing infection dissemination (Figure I) (Proietti, 2017).

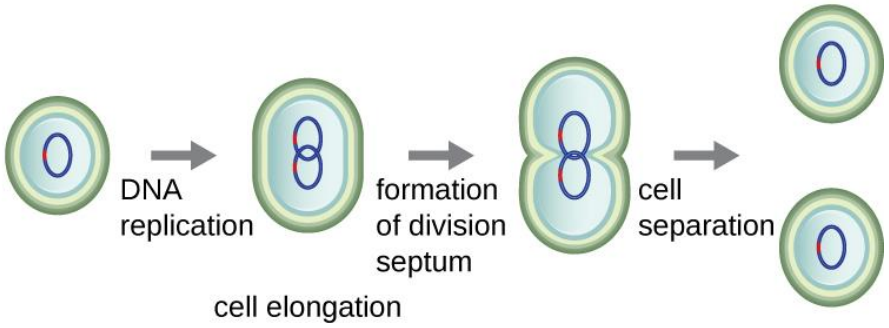


Figure I. - *Rickettsia* reproduction via binary fission (Parker et al., 2016)

Transmission cycle

Rickettsia are characterized as being transmitted by ectoparasites such as ticks, mites, fleas, and lice (McGinn and Lamason, 2021). These are typically referred to as vectors because although they harbor these pathogens, they do not show signs of illness. There are multiple ways a vector, specifically ticks, can become infected with *Rickettsia* (Figure 2). One of the ways is through transovarial transmission (Gibson, 2007). This is when the infected female tick passes the bacteria to the eggs. The eggs will then hatch into infected larvae and take their first blood meal. During this blood meal, the infected larvae can pass the pathogen to the second host. After this blood meal, the larvae will morph into infected nymphs. The nymphs will take a second blood meal, and can also pass the bacteria to the third host. Once the second blood meal has been taken the tick becomes an infected adult and the cycle will continue (Gibson, 2007). It is important to note that although the female tick is infected this does not mean that 100% of her eggs

will be infected with *Rickettsia.* The uninfected eggs can remain uninfected throughout their whole life cycle. However, the second way a tick can become infected with *Rickettsia* is through concurrent feeding (Gibson, 2007). This occurs when an uninfected larva takes its first blood meal, and either feeds at the same time as an infected tick or chooses a host that already has the pathogen from a previous blood meal with an infected tick. If the tick becomes infected with *Rickettsia* during these blood meals, it will remain infected throughout its life cycle as previously described.

Information regarding *Rickettsia* – arthropod interaction is lacking. *Rickettsia* are intracellular not only in their mammalian hosts but also arthropod vectors, such as ticks. Thepparit et al. (2010) reported that the outer membrane protein (OmpB) of *Rickettsia felis* interacts with the tick histone H2B, and that this complex formation is important for the adherence of bacteria to the surface of the tick cell in culture. It is proposed that *Rickettsia* utilize a phagocytosis-mediated entry mechanism as previously described (Thepparit et al., 2010).

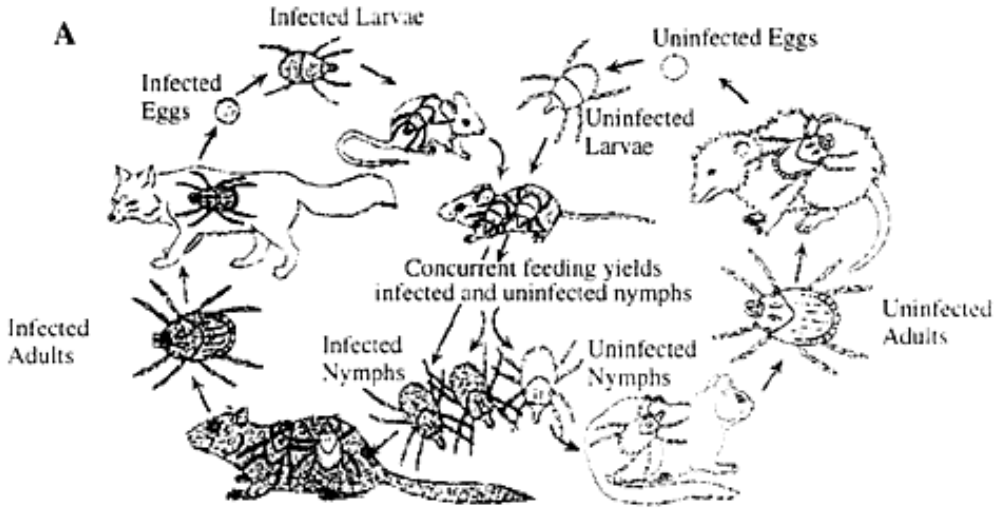


Figure II. – The transmission cycle of *Rickettsia* (Gibson, 2007)

Typhus group

As previously mentioned, the genus *Rickettsia* can be divided into two main groups: the spotted fever group and typhus group. Certain aspects set these groups apart such as vector species, diseases they cause, and mode of host infection. The typhus group consists of species such as *R. prowazekii*, *R. typhi*, and *R. felis* and are typically transmitted by fleas and lice (Typhus, 2016). *R. prowazekii* is an interesting species because humans are the reservoir host. This species is transmitted by *Pediculus humanus corporis,* also known as body louse, and they are the primary vector in human-human transmission (Typhus, 2016). The lice become infected when they feed on an infected person, and they defecate when they feed on a new host releasing the *R. prowazekii* in the feces. Transmission occurs when the feces is rubbed into bite wounds or other breaks in

the skin. They also can be transmitted by inhalation or coming in contact with the membranes of the mouth and eyes (Typhus, 2016). Although *R. typhi* is in the same group as *R. prowazekii*, its vectors, host, and transmission vary. *R. typhi* can have multiple intermediate hosts such as rats, cats, and opossums. This species is typically transmitted by fleas from these small mammals. One vector species is *Xenopsylla cheopis*, also known as rat flea (Flea-Borne, 2020). The fleas become infected when they bite an infected animal. The flea then can bite a human, and this initiates infection. When the flea feeds it defecates and this can then be rubbed into the bite or other wounds on the skin. The person could also encounter the flea feces and rub their eyes causing infection. One major difference between *R. typhi* and *R. prowazekii* is that there is no human-to-human transmission in the *R. typhi* species (Flea-Borne, 2020).

Spotted fever group

The spotted fever group causes many infections in humans, and they are commonly referred to as spotted fever group rickettsioses. Over the past twenty years the number of spotted fever rickettsiosis cases has increased in the United States from 495 cases in the year 2000 to 6,248 cases in the year 2017 (Epidemiology, 2021). Spotted fever rickettsiosis is transmitted by ticks belonging to the family *Ixodidae* (Proietti, 2017). Their transmission cycle is characteristic since there is transovarial transmission. This means that if the female tick is carrying *Rickettsia*, it can pass the pathogen to laid eggs (Walker, 2015). This group can also exhibit transmission through mating, meaning an infected male can pass the pathogen to a female during the mating process. Other than

these differences their transmission cycle to humans remains like that of other rickettsial groups. The reservoirs for this group transmission cycle are deer, mice, dogs, etc. Since the transmission cycle remains the same for most species within the spotted fever group, the diseases they cause are very similar (Proietti, 2017).

Tick-borne *Rickettsia* globally

Many *Rickettsia* species are present all over the world and on several different continents, and their distribution is related to their vectors. These vectors are typically ticks belonging to the family *Ixodidae*, and while some *Rickettsia* species are only associated with one tick species others can occur in multiple species (Parola et al., 2013). As of 2020, there are twenty-five known pathogenic species belonging to the genus *Rickettsia* around the world (Table I) (Piotrowski and Rymaszewska, 2020). Some of these species include, *R. rickettsii*, *R. conorii*, *R. japonica*, and *R. australis*. Although most of these pathogenic species were discovered in ticks, there was a gap in linking these species to diseases in humans. For example, *R. parkeri* was identified in 1937 in *Amblyomma maculatum* ticks but the first infection wasn't described until almost 70 years later (Piotrowski and Rymaszewska, 2020). Developments in molecular techniques has furthered our understanding and identification of new and previously recognized rickettsial species. Some species that have been considered nonpathogenic have now been linked to diseases in humans (Piotrowski and Rymaszewska, 2020). Also in recent years, there have been more collaborations within the field between clinicians, scientists, veterinarians, and field investigators. Approximately 300 experts gathered from five

continents to present their work on *Rickettsia* in 2011. From this meeting in Greece many original articles were published, furthering the understanding of rickettsia globally (Parola et al., 2013).

Table I. – List of currently known species of ticks transmitting pathogenic bacterial species of the genus Rickettsia (Piotrowski and Rymaszewska, 2020)

Pathogenic Species *Rickettsia*	**Tick Species Identified as a Vector**
Rickettsia aeschlimannii	*Amblyomma tigrinum, A. variegatum,* *Dermacentor marginatus, D. nuttalli,* *Haemaphysalis concinna, H. japonica, H. punctata,* *Hyalomma aegyptium, H. anatolicum excavatum, H. detritum detritum, H. detritum, H. dromedari, H. excavatum, H. impeltatum, H. lusitanicum, H. marginatum marginatum, H. marginatum rufipes, H. marginatum, H. truncatum,* *Ixodes ricinus,* *Rhipicephalus annulatus, R. appendiculatus, R. bursa, R. evertsi evertsi, R. sanguineus*
Rickettsia africae	*Amblyomma cohaerens, A. compressum, A. eburneum, A. gemma, A. hebraeum, A. lepidum, A. loculosum, A. ovale, A. variegatum,* *Hyalomma aegyptium, H. dromedarii, H. impeltatum, H. marginatum rufipes,* *Rhipicephalus annulatus, R. decoloratus, R. evertsi, R. geigyi, R. sanguineus*
Rickettsia australis	*Ixodes cornuatus, I. holocyclus, I. tasmani*
Rickettsia conorii	*Haemaphysalis leachi, H. punctaleachi,* *Rhipicephalus bursa, R. evertsi evertsi, R. mushamae, R. pumilio, R. sanguineus, R. simus, R. turanicus*

Table I (Continued)

Rickettsia conorii subsp. *indica*	*Rhipicephalus sanguineus, R. turanicus*
Rickettsia conorii subsp. *israelensis*	*Rhipicephalus sanguineus*
Rickettsia conorii subsp. *caspia*	*Rhipicephalus pumilio, R. sanguineus*
Rickettsia heilongjiangensis	*Dermacentor niveus, D. nuttalli, D. silvarum,* *Haemaphysalis concinna, H. flava, H. japonica douglasi, H. japonica, H. longicornis, H.verticalis, Hyalomma asiaticum kozlovi,* *Ixodes persulcatus,* *Rhipicephalus pumilio,*
Rickettsia helvetica	*Dermacentor reticulatus,* *Ixodes arboricola, I. festai, I. hexagonus, I. persulcatus, I. ricinus, I. trianguliceps*
Rickettsia honei	*Bothriocroton hydrosauri,* *Haemaphysalis novaeguineae,* *Ixodes sp.*
Rickettsia honei strain *marmionii*	*Haemaphysalis novaeguineae*
Rickettsia japonica	*Dermacentor taiwanensis,* *Haemaphysalis cornigera, H. flava, H. formosensis, H. hystricis, H. longicornis,* *Ixodes ovatus*

Table 1 (Continued)

Rickettsia massiliae	*Amblyomma sylvaticum,* *Dermacentor variabilis,* *Haemaphysalis paraleachi,* *Ixodes ricinus,* *Rhipicephalus bursa, R. evertsi, R. guilhoni, R. lunulatus, R. muhsamae, R. praetextatus, R. pusillus, R. sanguineus, R. senegalensis, R. sulcatus, R. turanicus*
Rickettsia monacensis	*Amblyomma dissimile,* *Dermacentor variabilis,* *Ixodes boliviensis, I. persulcatus, I. ricinus, I. sinensis,* *Rhipicephalus sanguineus*
Rickettsia montanensis	*Amblyomma americanum,* *Dermacentor andersoni, D. variabilis*
Rickettsia parkeri	*Amblyomma americanum, A. maculatum, A. tigrinum, A. triste, A. debitatum,* *Dermacentor variabilis*
Rickettsia philipii	*Dermacentor occidentalis*
Rickettsia raoultii	*Amblyomma testudinarium,* *Dermacentor everstanii, D. marginatus, D. niveus, D. nuttalli, D. reticulatus, D. silvarum,* *Haemaphysalis concinna, H. japonica, H. lagrangei, H. longicornis, H. ornithophila, H. shimoga, H. verticalis,* *Hyalomma asiaticum, H. marginatum,* *Ixodes persulcatus, I. ricinus,* *Rhipicephalus sanguinaus*
Rickettsia rickettsii	*Amblyomma americanum, A. aureolatum, A. cajennense, A. imitator,* *Dermacentor andersoni, D. nitens, D. occidentalis, D. variabilis,* *Haemaphysalis leporispalustris,* *Rhipicephalus sanguineus*
Rickettsia sibirica subsp. *mongolitimonae*	*Dermacentor marginatus, D. nuttalli, D. reticulatus, D. silvarum, D. sinicus,* *Haemaphysalis concinna, H. erinacei, H. yeni,* *Hyalomma aegyptium, H. anatolicum excavatum, H. asiaticum, H. truncatum,* *Ixodes persulcatus,* *Rhipicephalus pusillus, R. turanicus*

Table I (Continued)

Rickettsia sibirica subsp. *sibirica*	*Dermacentor nuttalli, D. silvarum, D. sinicus* *Ixodes kaiseri*
Rickettsia slovaca	*Dermacentor marginatus, D. nuttalli, D. reticulatus,* *Hyalomma aegyptium,* *Rhipicephalus sanguineus*
Rickettsia tamurae	*Amblyomma testudinarium*
Rickettsia sp. strain Atlantic rainforest (lub strain Bahia)	*Amblyomma aureolatum, A. dubitatum, A. ovale,* *Rhipicephalus sanguineus*
Candidatus *Rickettsia tarasevichiae*	*Dermacentor reticulates,* *Haemaphysalis concinna* *Ixodes persulcatus, I. ricinus*

Tick-borne *Rickettsia* in the United States

In the United States, there are roughly 4,000-6,000 cases of tick-borne rickettsiosis reported each year (CDC, 2022). Spotted fever rickettsiosis has been reported in all of the lower forty-eight states, with more than 60% of the cases reported in five states, North Carolina, Tennessee, Oklahoma, Arkansas, and Missouri (RMSF, 2019). The most common spotted fever rickettsiosis reported in the United States is Rocky Mountain spotted fever, which is caused by *R. rickettsii.* This species is typically transmitted by *Dermacentor variabilis* or *Dermacentor andersoni* which can be found across North America (Figures III and IV) (RMSF, 2019). Another spotted fever rickettsiosis disease commonly reported in the United States is tidewater fever. This disease is caused by the species *R. parkeri,* and its only known vector is *Amblyomma maculatum.* This tick species has been reported in twelve states across the U.S., including Texas (Wright et al., 2011). One study conducted in southeastern Virginia from May to September 2010, collected *A. maculatum* ticks and tested them for *R. parkeri* (Wright et al., 2011). They had three collection sites and a total of 65 adult and 6 nymph *A. maculatum* ticks were collected. These ticks were subjected to PCR detection methods and of the 65 adult ticks collected 28 were positive for *R. parkeri,* and of the 6 nymphs collected 4 were positive for *R. parkeri*. These numbers of infected ticks showed that the *R. parkeri* species is highly established in Virginia when it was previously thought to not be (Wright et al., 2011).

Sumner et al. (2007) also investigated the frequency of *R. parkeri* in *A. maculatum* ticks. They collected ticks over a nine-year period from various locations across Florida, Kentucky, Mississippi, Oklahoma, Georgia, and South Carolina. Most ticks were collected via the dragging method, but a few non-attached ticks were collected from animals. PCR methods revealed that 21 of 182 collected ticks were positive for the *R. parkeri* pathogen. Researcher's concluded that a person exposed to habitats where *A. maculatum* ticks reside could potentially be infected with *R. parkeri* (Sumner et al., 2007).

A more recent study, occurring in 2016, collected ticks from three separate locations in southern Arizona (Allerdice et al., 2016). A total of 182 ticks belonging to *A. maculatum* group were collected from Pajarita Wilderness Area, San Pedro Riparian National Conservation Area, and Santa Rita Mountains in Tuscon, Arizona. These ticks were then tested using real-time PCR and semi-nested PCR methods for the detection of *R. parkeri*. In this study of 182 ticks collected, 44 tested positive for *R. parkeri.* This study provided evidence of established *A. maculatum* populations in southern Arizona that carry *R. parkeri*, and also proved there might be a great public health risk. The thought of a public health risk stems from the fact that most of the ticks collected in the year 2016 were collected from highly trafficked recreational areas (Allerdice et al., 2016).

Although Arizona has detected *A. maculatum* populations this is not the only tick species of significance that has been identified there. Eremeeva et al. (2006) collected

Rhipicephalus sanguineus ticks in eastern Arizona via flagging methods. They collected twenty ticks and used nested PCR methods to amplify the 17-kDA antigen gene of *Rickettsia.* A total of five ticks were positive for the *R. massiliae.* Before this study, it was thought that *R. massiliae* was not present in North America. This species of tick is known to bite humans and therefore this must be taken into consideration when humans present with tick bite related illness in eastern Arizona (Eremeeva et al., 2006).

Rhipicephalus sanguineus ticks are found across the world but reside predominantly in urban areas. Wikswo et al. (2007) examined ticks collected at a local residence in California. Researchers tested sixty-two ticks for *Rickettsia, Bartonella,* and *Erlichia* DNA. PCR methods revealed that one tick was positive for *R. rickettsii.* Some ticks tested positive for the presence of *Erlichia* and *Bartonella* DNA. However, the importance of this study is that this was the first time *R. rickettsii* was found present in *Rhipicephalus sanguineus* ticks.

The most common tick vector for *R. rickettsii* is thought to be *Dermacentor variabillis.* Hecht et al. (2019) conducted a multistate survey of *D. variabillis* for *Rickettsia* species. This study collected ticks for five years between 2012 and 2017 across twelve states. A total of 883 *D. variabillis* ticks were collected and their DNA extracted. Using real-time PCR methods, as well as nested PCR methods, the ticks were tested for *Rickettsia* species. The results showed that 203 ticks were positive for *Rickettsia* species bacteria, and of the 203 only one tick tested positive for *R. rickettsii.* This research

supports other studies that show the infrequent detection of *R. rickettsii* among *D. variabillis* ticks.

Another study conducted in North Carolina collected ticks from 32 counties over a five-year period. Kakumanu et al. (2018) collected a total of 532 ticks and extracted total genomic DNA from each individually. Using PCR methods, it was found that 291 ticks tested positive for *Rickettsia* species DNA and 52 ticks were co-infected with one or more *Rickettsia* spp. Of the infected ticks, 5 were positive for *R. rickettsii*, 7 were positive for *R. typhi*, and 156 were positive for *R. amblyommatis.* The results in this study showed greater diversity of *Rickettsia* sp. than previously conducted studies.

Figure III. - Female *Dermacentor andersoni* (Gibson, 2007)

Figure IV. - Female *Dermacentor variabillis* (Gibson, 2007)

Tick-borne *Rickettsia* in Eastern Texas

Mitchell et al. (2016) investigated the presence and distribution of ticks across the state of Texas. From 2006 to 2014 the Texas Department of State Health Services (DHSH) collected ticks removed from humans and sent them to the University of North Texas Health Science Center (UNTHSC-TBDL). Over this period, a total of 1,062 ticks were collected and screened for the presence of *Rickettsia, Borrelia,* and *Ehrlichia* spp. Thirteen tick species were identified, and each samples' DNA was tested in duplicate using PCR. Mitchell et al. (2016) reported that twenty-three percent of the collected ticks tested positive for at least one of these bacteria. However, most of the bacteria detected belonged to the spotted fever group rickettsiae. Although this study reports that the prevalence of these pathogens is low compared to other states there is still a need for more surveillance efforts and better characterization.

Mitchell et al. (2016) identified thirteen species of ticks that carry the *Rickettsia* pathogen, and one of these species is *Ixodes scapularis* better known as the black-legged tick. Although this species isn't as prominent in the transmission of *Rickettsia sp.* as *Dermacentor andersoni* and *D. variabilis* it is still an important public health risk. Billings et al. (1998) collected a singular tick in Anderson County, TX, and used various methods to test for *Rickettsia* sp. bacteria. They spread midgut tissue from the tick across a slide and stained it using the Gimenez staining technique. This slide was then analyzed by using antibody methods that cross react with many SFG rickettsiae. The DNA from the tick was also extracted and examined using PCR methods. A 17 kDa protein encoding

gene sequence was amplified and its sequence showed the highest similarity *R. australis* sequence. However, when examining the gltA nucleotide sequence the highest similarity was found with *R. hevetica.* Since these two comparisons led to different species similarities a final gene was examined. When looking at the rompA gene the greatest similarity was found with *R. montana.* The researchers created phylogenic trees which were similar with minor changes of the placement of *R. felis, R. akari,* and *R. australis.* According to these results, *Rickettsia* sp. carried by the *I. scapularis* tick is that of unknown origin. This is important information because this species could result in a rickettsiosis infection unrecognized by physicians (Billings et al., 1998).

One way to combat TBDs is to survey ticks in the area, at the county level, and screen them for causative agents such as *Rickettsia* sp. bacteria. Mendell et al. (2019) collected and screened ticks in Walker county, Texas. They collected ticks at Huntsville State Park on three separate ocasions, June 2017, October 2017, and June 2018. Collection methods such as dragging were used to collect these ticks, and then they were returned to the lab and the nucleic acids were extracted. Most of the ticks collected were identified as *A. americanum,* but *A. maculatum, I. scapularis,* and *Dermacentor variabilis* were also found. They were then screened for various tick-borne pathogens through PCR techniques, and amplified DNA fragments were submitted for sequencing (Mendell et al., 2019). The sequencing revealed that all 68 *A. americanum* ticks collected tested positive for *R. amblyommatis.* In two of the *Ixodes scapularis* ticks, an unknown *Rickettsia* sp. was detected. The data collected was not only used to compare the frequency of ticks and

tick-borne diseases during different months, but also to provide data in hopes to target vector and disease control.

The proposed study is a continuation of efforts to study *Rickettsia* distribution in Nacogdoches, Texas. Early studies in this area were related to the detection of *R. felis* antibodies in cats in this region using specific recombinant antigen for *R. felis*. Wiggers et al. (2005) used 138 collected cat sera samples along with 10 samples provided by the Texas Department of Health (TDH). The samples provided by TDH were from confirmed cases of murine typhus. The purpose of the study was to determine whether the sera samples were infected with *R. typhi* or *R. felis.* The study revealed that three out of the 138 samples tested positive for *R. felis*, and 32 tested positive for *R. typhi* (this included the 10 controls from TDH). This study concluded that even though *R. felis* is more common than *R. typhi* the latter may be more pathogenic in humans.

Canterberry et al. (2015) investigated the frequency of ticks carrying *Rickettsia* in Nacogdoches County, TX. The study found that up to 70% of ticks may be carrying one or more species of *Rickettsia* with the potential to spread them to humans (Canterberry et al., 2015). In this study, thirty-five ticks were collected and their DNA was isolated. Using primers for the 17 kDa antigen for *Rickettsia* sp. PCR amplifications were performed on all collected ticks. Twenty-one ticks were determined to be positive for carrying *Rickettsia.* One tick determined to be *A. maculatum* was positive for *Rickettsia* presence and further PCR analysis using species specific primers showed that it was carrying *R. parkeri* (Canterberry et al., 2015). This study indicated that in Nacogdoches

County, when a patient presents with a certain clinical picture and has possible tick exposure then, a rickettsial infection should be considered by physicians.

Another study conducted in Nacogdoches, TX looked at *A. maculatum* populations and tested them for the presence of *R. parkeri* (Nathaniel, 2018). This study was conducted over a two-year period and a total of 49 ticks were collected. Using molecular techniques such as DNA extraction and PCR amplification, the ticks were identified as either positive or negative for carrying *Rickettsia* genus bacteria (Nathaniel,2018). The positive ticks were subjected to further PCR testing, and it was found that 26% of the original 49 collected ticks were positive for rickettsia bacteria, but only 6% were positive for *R. parkeri* (Nathaniel, 2018).

The proposed study had two main goals. The first goal was to detect *Rickettsia* in hard ticks collected in Nacogdoches, Texas. Once *Rickettsia* was detected the second goal was to characterize the species using molecular methods such as PCR. In this study we expected to collect various species of ticks such as *A. maculatum* and *I. scapularis* with more than half testing positive for carrying *Rickettsia* sp. Although, we expected to see various species of ticks we predicted *A. maculatum* would make up the majority of th collectd ticks. Also, we expected to detect rickettsial species *R. parkeri* based on the findings from previous studies in the area.

MATERIALS AND METHODS

Tick Collection

Ticks were collected in different localities in Nacogdoches, Texas. These localitites included places such as the SFA Experimental Forest (29’51”N, 94°38’42”W), Tucker Woods (37’47”N, 94°44’30”W), and Old Tyler Road (37’26”N, 94°39’56”W) (Figure V). Some ticks were collected in efforts by our lab and others were provided by Dr. Radulović. Collection dates ranged over two years, 2020-2021, and ticks were primarily collected in summer months ranging from April-June with a few collected in November. The flagging method was used for all collections. The flags used were 1 m^2 in size and were dragged over vegetation and checked every 5-10m for ticks. When the ticks were found on the flags they were placed inside a collection tube with a wet piece of paper or a blade of grass. This was done to prevent the ticks from desiccating before returning to the lab. Ticks were identified and sorted according to their species, sex, and developmental stage upon returning to the lab.

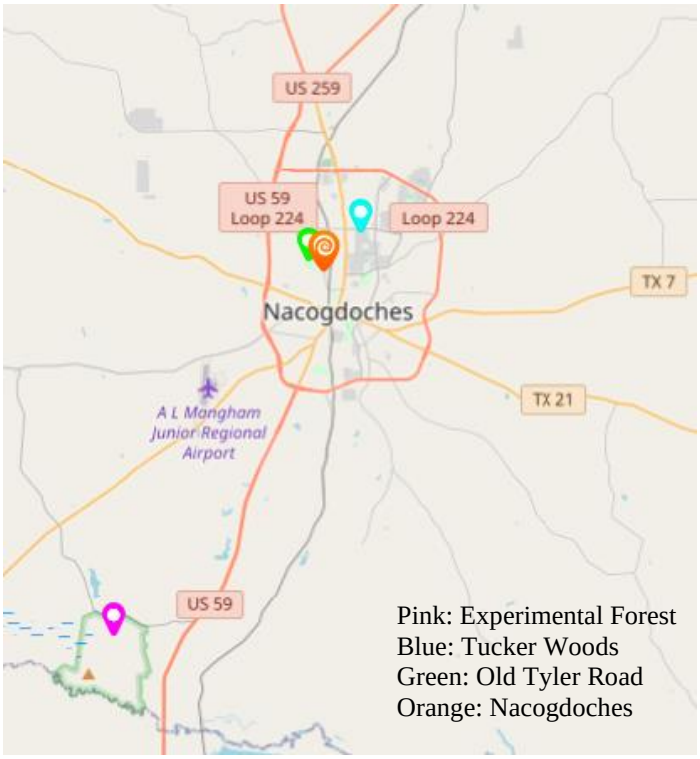


Figure V- Localitites for Tick Collection

DNA Extraction from Individual Ticks

After the ticks were identified and sorted DNA was extracted from each of them individually. This was done using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's recommended protocol with modifications for DNA extraction from individual ticks. The ticks were washed in 70% ethanol for two minutes to remove any external contaminants. Following this wash, another wash was performed using sterile nuclease-free water for 30 seconds. This step was repeated two more times to ensure all

the ethanol was washed from the ticks. The ticks were allowed to dry on sterile filter paper before placing them in 180 µL ATL buffer. Once in the buffer, the ticks were cut into small pieces using sterilized scissors. Following this step, 20 µL of proteinase K was added and the samples were vortexed for 15 seconds. After vortexing, the samples were incubated at 56°C for 3 hours and vortexed every 10-15 minutes. During this incubation proteins should be digested, and DNA should be released in the cell lysate. Once the incubation period finished, 200 µL of AL buffer was added and the samples were vortexed, followed by incubation of the mixture at 70°C for 10 minutes and the addition of 1 µL of RNA carrier. We used a 10 mg/mL solution of polyadenylic acid as an RNA carrier, which should enhance the recovery of low abundant DNA molecules. After vortexing the mixture with the RNA carrier, 230 µL of 96-100% ethanol was added, and the sample was vortexed. This sample mixture was then pipetted into the DNeasy Mini Spin Column provided in the kit, which is positioned on a 2 mL collection tube. The mixture causes a generation of positive charge in the membrane at the bottom of the spin column, which attracts negatively charged DNA molecules. In the next step, the column setup was centrifuged at 6,000xg for 1 minute, forcing pass of the mixture through the spin column membrane and the collection of the flow-through in a collection tube. DNA should stay bound on the spin column membrane, while the flow-through will be discarded. The column was then placed in a new collection tube and two steps of washing was performed using 500 µL of AW1 and AW2 buffers, respectively. The buffer was added to the column; centrifuged at 6,000xg for 1 min, and the flow-through was

discarded. After the washing steps, the empty column setup was centrifuged for three minutes at 20,000xg, to remove any residual ethanol that can interfere with downstream use of extracted DNA. Once finished, the collection tube was discarded, and the column was placed in a sterile 1.5 mL tube. A total of 200 µL of AE buffer was added directly on the membrane of the column. This was incubated for one minute at room temperature, to allow AE buffer to release DNA from the spin column membrane, followed by centrifugation for one minute at 6,000xg. Once finished the spin column was discarded and DNA was collected in the flow-through in the 1.5 mL tube. The concentration and quality of the extracted DNA was checked using NanoValue Spectrophotometer (GE). Finally, the DNA samples were stored at -20°C until further use.

PCR Based Detection of *Rickettsia* in Ticks

To determine if ticks were carrying *Rickettsia* sp. bacteria, PCR and semi-nested PCR methods were performed using eight sets of primers (Table II). The first set of primers targets the portion of the citrate synthase gene (gltA) that is highly conserved among *Rickettsia* sp. Successful amplification of this fragment indicated that the tick carried some species of the family *Rickettsiaceae* (Regnery et al., 1991). The second set of primers includes one forward and two reverse primers to perform a semi-nested PCR. These primers amplify a portion of the gene for the outer membrane protein A (ompA) which is present on the surface of the spotted fever group of *Rickettsia* and is commonly used for discrimination of this group from the other members of the family (Regnery et al., 1991). The third set of primers targets the portion of the 17kDa gene that is also

highly conserved among *Rickettsia* sp. Like the first set of primers successful amplification of this gene indicated that the tick carried some species of the family *Rickettsiaceae* (Chitanga et al., 2021; Shao et al., 2020).

A 25 µL PCR was prepared for each sample. The reactions consisted of 12.5 µL of 2XGoTaq (Promega), 1.25 µL of each forward and reverse primer (10 µM), 8 µL of nuclease-free water, and 2 µL of template DNA. For the second step of semi-nested PCR, we used 2 µL of 1:100 dilution of the first step PCR product as a template. The PCR program had a template as it is shown in Table III, with different annealing temperatures and extension times for different sets of primers.

The PCR products were stored at -20°C.

Table II. -Primer sequences used to amplify *Rickettsia* sp. bacteria.

Name	Sequence 5' →3'	Sense
RpCS.877p	GGGACCTGCTCACGGCGG	Forward
RpCs1258n	ATTGCAAAAAGTACAGTGAACA	Reverse
Rr190.70p	ATGGCGAATATTTCTCCAAAA	Forward
Rr190.701n	GTTCCGTTAATGGCAGCATCT	Reverse
Rr190.70p	ATGGCGAATATTTCTCCAAAA	Forward
Rr190.602n	AGTGCAGCATTCGCTCCCCCT	Reverse
Rr17k.1p	TTTACAAAATTCTAAAAACCAT	Forward
Rr17k.539n	TCAATTCACAACTTGCCATT	Reverse
Rr17k.90p	GCTCTTGCAACTTCTATGTT	Forward
Rr17k.539n	TCAATTCACAACTTGCCATT	Reverse

Table III. - PCR conditions for amplification of targeted *Rickettsia* gene fragments

Stage	Temperature	Duration	Number of Cycles
Initial denaturation	95°C	5 minutes	1x
Denaturation	95°C	30 seconds	35x
Annealing	**45°C (OmpA)** **47°C (GltA)** **57°C (17-kDa)**	30 seconds (OmpA, GltA) 1 minute (17-kDa)	
Extension	72°C	1 minute (OmpA, 17-kDa) 30 seconds (GltA)	
Final extension	72°C	7 minutes	1x

Purification of DNA from Agarose Gel and Sequencing

The PCR products were resolved on a 1.5% agarose gel for 30 minutes at 100V. The appropriate band was excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The excised gel was weighed, and the appropriate amount of membrane binding buffer was added to the tube with gel slices. The tube was then incubated at 60°C with mixing until the gel was completely dissolved. This solution

was then pipetted into a provided spin column and the column was placed inside a collection tube. The column setup was centrifuged at 16,000xg for one minute, and the flow through was discarded, while DNA fragments should stay bound to the membrane at the bottom of the spin column. A total of 500 µL of membrane wash solution was added to the spin column and the column setup was centrifuged at 16,000 x g for one minute. After the flow-through is removed another wash step with 500 µL of membrane wash solution was performed, but centrifugation was performed for three minutes at maximal speed to remove all residual ethanol. The spin column was transferred to a sterile 1.5 mL tube and 50 µL of nuclease-free water was applied directly to the membrane at the bottom of the column. This was incubated at room temperature for one minute to allow the release of DNA molecules from the membrane, and then centrifuged at 16,000xg for one minute. Following this step, the column was discarded, and the DNA collected in the 1.5mL tube was stored at -20°C until further use.

The concentration of the purified DNA was checked using the NanoValue Spectrophotometer (GE). If the concentration was too low, then the samples were dried using the SpeedVac vacuum concentrator and then reconstituted in 20 µL of nuclease-free water. Once the concentration of DNA fragments was sufficient, sequencing reactions were prepared. These reactions were prepared by mixing recommended amounts of purified PCR product and the forward primer. These reactions were sent to Eurofins Genomics in Louisville, Kentucky sequencing service for sequencing.

Bioinformatic Analysis

Obtained sequences were processed using BioEdit software to obtain the maximal order of nucleotides with high confidence. These processed sequences were used in BLAST search in GenBank database to check the similarity with previously deposited sequences. Results of BLAST search were used for confirmation of amplification of a *Rickettsia* DNA sequence, as well as an indication of *Rickettsia* species that is present in examined tick sample.

To further characterize detected *Rickettsia* species, a phylogenetic tree using targeted sequences was constructed. Appropriated sequences of GltA, OmpA, and 17kDa genes that originate from different *Rickettsia* species were downloaded from GenBank. Alignment and trimming of downloaded sequences, as well as sequences obtained from our samples, was performed using BioEdit. Processed sequences were used for phylogenetic tree construction with a neighbor-joining method, by using BioEdit software and phylogeny.fr online tool. The position of sequences obtained from our samples in phylogenetic trees give a close insight into molecular characteristics of *Rickettsia* species we detected using molecular methods.

RESULTS AND DISCUSSION

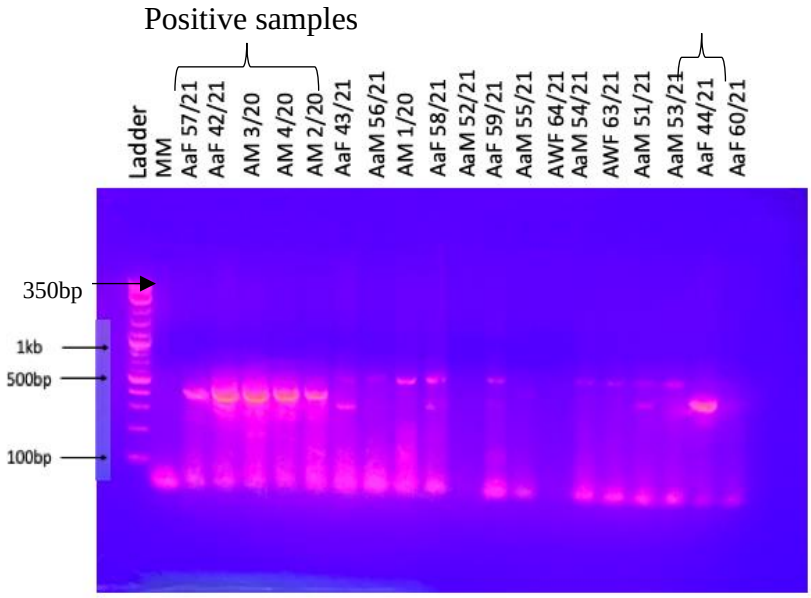
Molecular detection of *Rickettsia* in collected ticks

A total of 47 ticks were collected from locations around Nacogdoches, Tx. Of these collected ticks 13 were identified as *Amblyomma americanum,* 9 as *Dermacentor variabillis,* 6 as *Amblyomma maculatum,* and 19 as *Ixodes scapularis.* Of the *A. americanum* ticks there was 1 positive male and 3 positive females, *D. variabillis* had only 1 positive male, *A. maculatum* had 1 positive female and 3 positive males, *I. scapularis* had 12 positive females (Table IV). All 47 of the tick samples were analyzed via PCR methods using two of the target genes (GltA and 17kDa). After this was done only the positive samples were amplified using the third genetic marker (OmpA). This was done because the first two target genes are strictly used to determine if any *Rickettsia* is present however, the third target gene is used to distinguish the spotted fever group. Figures below represent PCR products resolved on agarose gel electrophoresis (Figures VI, VII, VIII). The first lane in the images is the DNA ladder which allows us to compare our band size to known sizes. The second lane "MM" is the master mix that was used in each sample. This was ran on the gel to ensure no contaminates were present. Upon doing this analysis we found that 21 of the 47 ticks (44%) were positive with *Rickettsia* sp. bacteria.

Table IV. - Tick samples collected for analysis

	Amblyomma americanum		*Dermacentor variabillis*		*Amblyomma maculatum*		*Ixodes scapularis*	
	Postive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Male	1	5	1	7	3	0	0	3
Female	3	4	0	1	1	1	12	4
Nymph	0	0	0	0	0	1	0	0
Total Positive	4		1		4		12	

Figure VI - Gel electrophoresis of PCR targeting the GltA gene



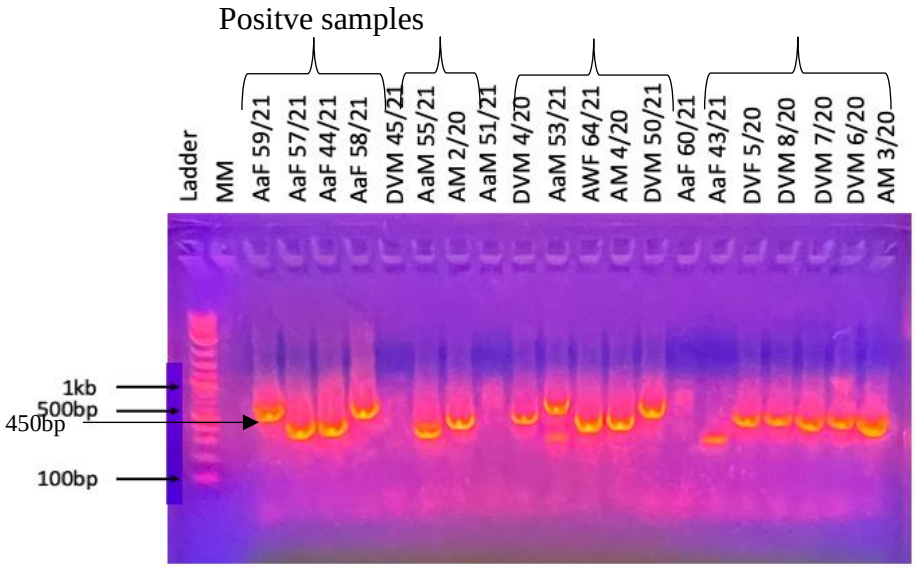


Figure VII- Gel electrophoresis of PCR targeting the 17kDa gene

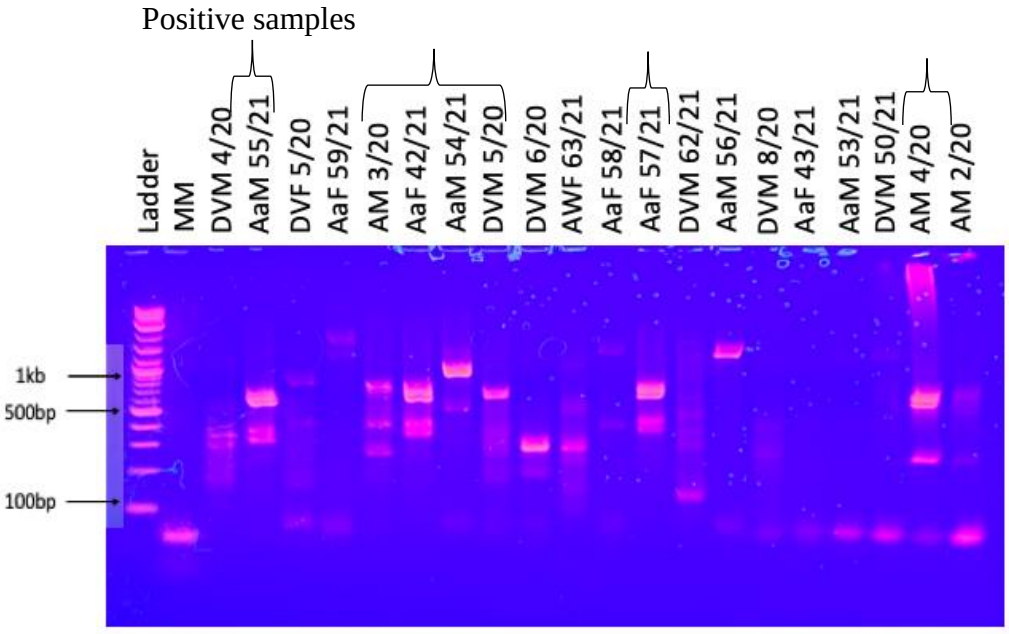


Figure VIII- Gel electrophoresis of PCR targeting the OmpA gene

Sequencing analysis of detected *Rickettsia*

Sequencing results from the 21 PCR positive samples were obtained and analyzed. After trimming these sequences according to the primers used we ended up with 306bp for GltA, 377bp for 17kDa, and 444bp for OmpA. This was very close to the expected sizes for each gene which was 350bp, 450bp, and 500bp respectively. Once the sequences were trimmed they were run in blast to find sequences with the most similarity. Sequences with high similarity were downloaded and trimmed to match the amount of base pairs our obtained sequences had. The most similar GenBank hits for each sequence for each genetic marker can be seen in Tables V, VI, and VII. The goal was to download two sequences for each *Rickettsia* sp. from GenBbank, this was done for almost all available species. When sequences only had one entry that matched the obtained sequences then the one was downloaded. After all the sequences from GenBank were downloaded they were entered into Bioedit along with our trimmed sequences. This was done separately for all three genetic markers. Once all the sequences were aligned and trimmed they were submitted to phylogeny.fr an online tool, which resulted in three separate phylogentic trees (Figures IX, X, XI)

Table V- GenBank blast results for GltA genetic marker

Genetic Marker	**Sample**	**Most Similar Hit**	**Query Cover**	**% Identity**
GltA	AM 2/20, AM4/20, AM3/20	Candidatus *Rickettsia andeanae*	100%	100%
	AaF 57/21, AaF 42/21, AaF 44/21	*Rickettsia amblyommatis*	100%	100%
	48F20, 40F20, 43F20, 39F20, ISF40/21	*Rickettsia tamurae*	100%	99.02%
	41F20, 57F20	*Rickettsia tamurae*	100%	98.69%
	42F20	*Rickettsia lusitaniae*	100%	97.06%
	23F20	*Rickettsia lusitaniae*	100%	97.39%

Table VI- GenBank blast results for 17kDa genetic marker

Genetic Marker	**Sample**	**Most Similar Hit**	**Query Cover**	**% Identity**
17kDa	AaM 55/21	*Rickettsia amblyommii*	100%	99.47%
	AmF 64/21	*Candidatus Rickettsia andeanae*	100%	99.47%
	41F20, 48F20, 40F20, 44F20	*Rickettsia monacensis*	100%	98.41%
	43F20, 39F20	*Rickettsia monacensis*	100%	98.67%
	38F20	*Rickettsia asembonensis*	100%	97.88%

Table VII- GenBank blast results for OmpA genetic marker

Genetic Marker	Sample	Most Similar Hit	Query Cover	% Identity
OmpA	AaF 42/21, AaM 55/21, AaF 44/21, AaF 57/21	*Rickettsia amblyommatis*	100%	100%
	AM3/20, AM4/20	*Candidatus Rickettsia andeanae*	100%	99.55%
	AmF 64/21	*Candidatus Rickettsia andeanae*	100%	99.32
	DVM 5/20	*Rickettsia amblyommatis*	100%	99.77%
	38F20	*Rickettsia tamurae*	100%	93.95%
	49F20, 41F20	*Rickettsia monacensis*	100%	93.92%
	ISF 40/21	*Rickettsia monacensis*	100%	90.85%
	42F20	*Rickettsia monacensis*	100%	91.91%

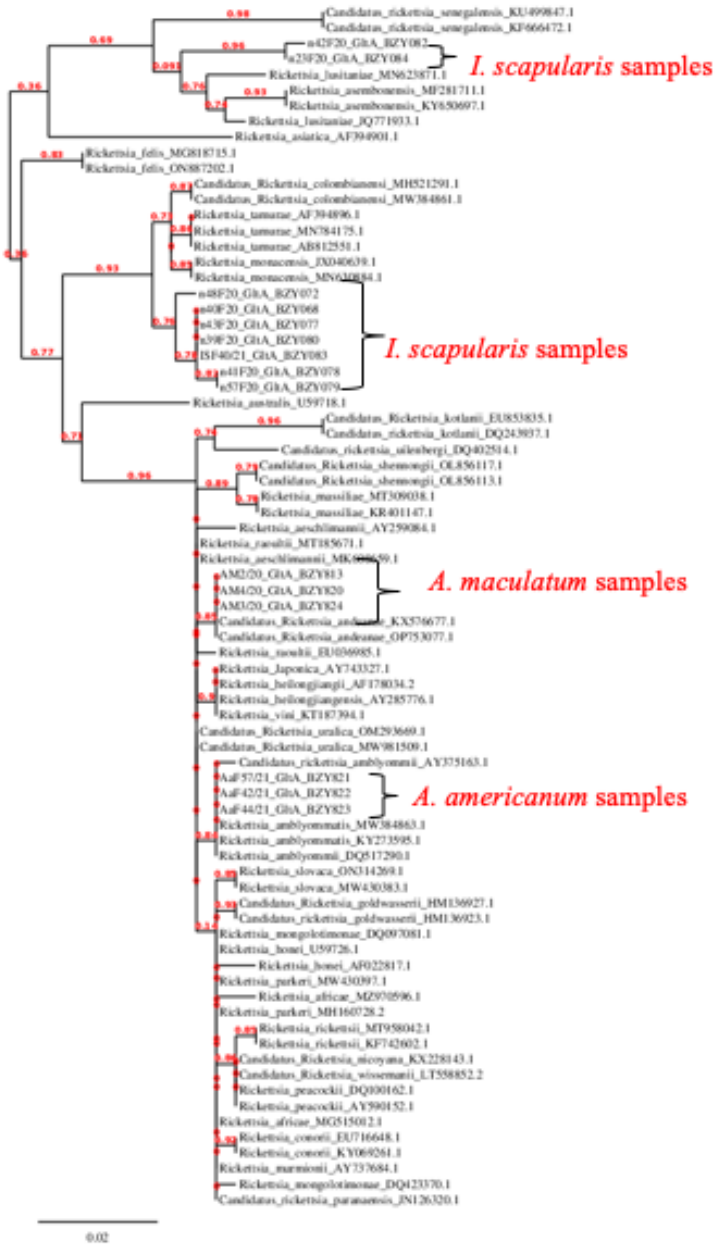


Figure IX- Phylogenetic tree created using GltA genetic marker

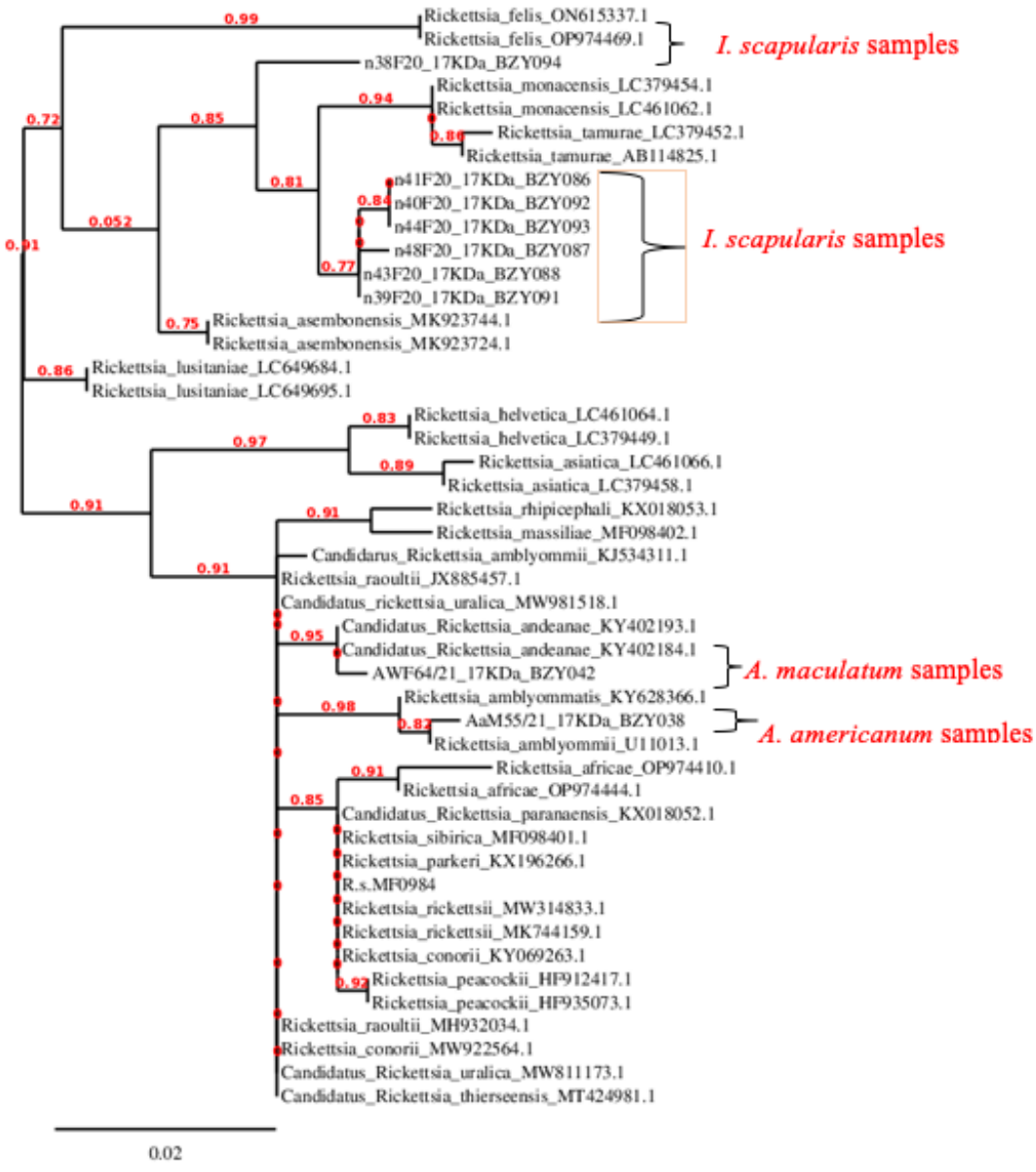


Figure X- Phylogenetic tree created using 17kDa genetic marker

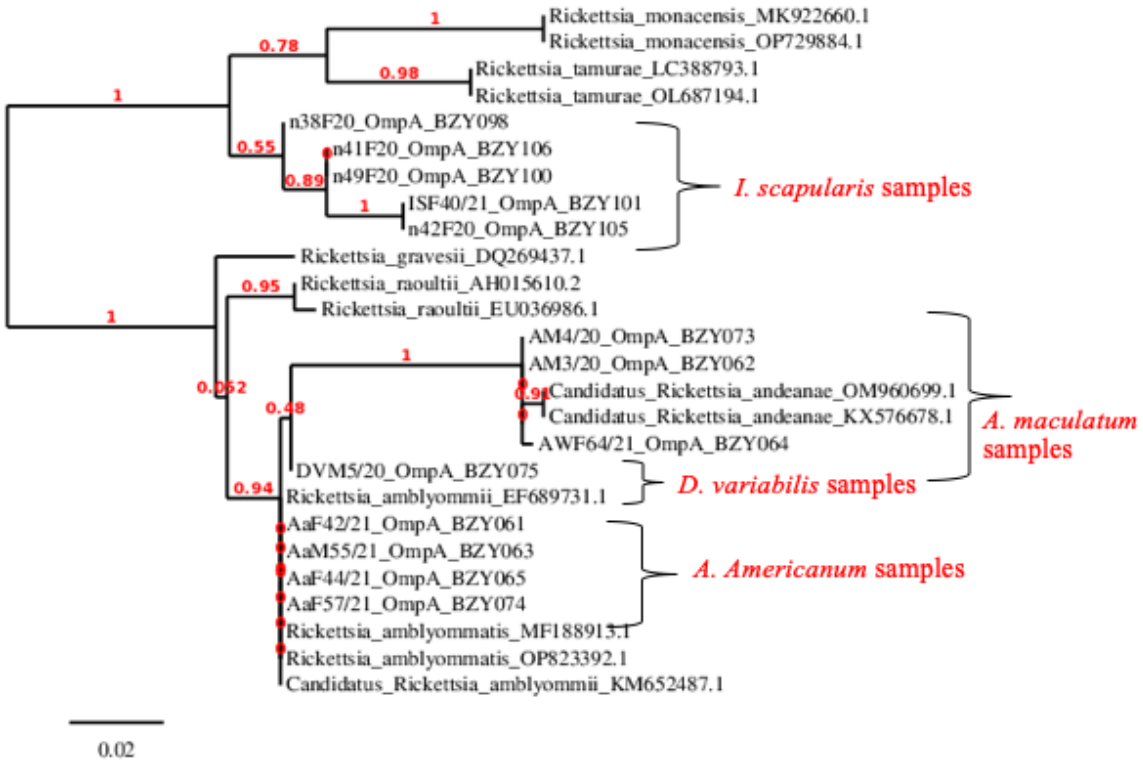


Figure XI- Phylogenetic tree created using OmpA genetic marker

Phylogenetic analysis of detected Rickettsia

Through PCR amplification and sequencing we were able to retrieve 15 rickettsial GltA partial sequences from analyzed ticks. The most of these sequences originated from *I. scapularis* ticks (9 sequences), while 3 sequences originated from each, *A. americanum* and *A. maculatum*. Phylogenetic tree revealed that sequences originating from *A. americanum* ticks cluster together with GenBank sequences that belong to *R. amblyommatis*, and obviously belong to this rickettsial species. In similar manner we can conclude that sequences originating from *A. maculatum* ticks belong to *R. andeanae.* On the other hand, sequences originating from *I. scapularis* ticks form two separate clusters. One cluster containing 5 sequences is related to *R. monacensis, R. tamurae,* and *R. colombianensis* sequences retrieved from GenBank, while another cluster containing 2 sequences is related to *R. lusitaniae, R. asembonensis,* and *R. senegalensis*. However, percentage of identity of sequences from these clusters with related sequences from GenBank was below 98.37%-98.69%, indicating that these sequences belong to *Rickettsia* species that do not have deposited sequences of GltA in GenBank, or that are not described yet.

We were able to retrieve 9 rickettsial 17kDa partial sequences from analyzed ticks. The majority of these sequences originated from *I. scapularis* ticks (7 sequences), while 1 sequence originated from each, *A. americanum* and *A. maculatum.* When looking at the second tree we can see a separate cluster just as we saw in the phylogenetic tree using the GltA gene. This cluster is made up of the same samples as the first cluster with

the addition of two *I. scapularis* sequences. This supports the theory that these sequences belong to *Rickettsia* species that are not described yet. This tree also has an *A. americanum* sample which is the most closely related to *R. amblyommii*, and an *A. maculatum* sample which is most closely related to *R. andeanae.*

Our final tree was created using 13 rickettsial OmpA partial sequences from analyzed ticks. Unlike the first two trees the majority of the sequences did not originate from *I. scapularis* ticks (5 sequences), however *A. maculatum* and *A. americanum* where similar to the first tree with 3 sequences and 4 sequences respectively. This tree helps confirm what was found in the first two trees. We can see that the 3 *A. maculatum* sequences are most closely related to *R. andeanae* while the 4 *A. americanum* sequences are closest to *R. amblyommii and R. amblyommatis.* The unique thing about this tree is we had 1 *D. variabillis* sequence which was the closest related to *R. amblyommii* and *R. andeanae.* However, like the first tree the *I. scapularis* sequences formed their own cluster within the tree. This shows signs that this species has not yet been described. The results from all three trees coincide with eachother which gives confidence that the data collected is correct.

The data from this study shows that there are at least four species of ticks present in Nacogdoches, Texas and all four of these species can carry *Rickettsia* species bacteria. The bacteria detected from these analyses is diverse and showed at least six species of *Rickettsia* detected as well as the potential of a new *Rickettsia* species detected

CONCLUSION

This study was a continuation of efforts to understand the distribution of *Rickettsia* in Nacogdoches, Texas. We predicted the majority of ticks collected would be *A. maculatum* based on previous studies that focused soley on *A. maculatum* populations in the area. However, in this study there were only 6 *A. maculatum* ticks collected, and the majority of the ticks were determined to be *I. scapularis.* A total of 47 ticks were collected and 21 were found positive to be carrying various types of *Rickettsia* sp. This means 44% of collected ticks were positive for *Rickettsia,* although this is less than our prediction of 50% positive it is still a significant amount of ticks carrying *Rickettsia.* Canterbery et al. (2015) had similar results finding 21 out of 35 ticks positive (60%) for carrying *Rickettsia* in Nacogdoches. However, the 2015 study only characterized one *Rickettsia* species, *R. parkeri.* Our study detected and characterized six species of *Rickettsia,* but we did not detect *R. parkeri* in any of the positive ticks. This was surprising because Blakely (2018) collected 49 *A. maculatum* ticks in Nacogdoches, Texas and determined 6% were positive for carrying *R. parkeri.* However, our study did detected a potential new species. This isn't the only study in east Texas to detect an uncharacterized species of *Rickettsia* in ticks. Mendell et al. (2019) screened ticks in Walker county, Texas and they also detected an unknown *Rickettsia* sp. Like our study

this unknown *Rickettsia* sp. was detected in *I. scapularis* ticks. Mendell et al. (2019) only detected the species in two ticks while our study detected the unknown species in 11 ticks. The findings from our study support the conclusions drawn from the Walker county study that this detected species is a new *Rickettsia* sp.

This study was successful in detecting and characterizing *Rickettsia* sp. carried by ticks in Nacogdoches, Texas. The ticks in this study were collected while walking on trails in wooded areas. They were collected from the brush surrounding these trails in the summer months. Also, out of the 4 tick species collected there was not one species more likely to be carrying *Rickettsia.* This means if you come in contact with a tick in Nacogdoches, Texas, no matter the species, you have a chance of coming in contact with *Rickettsia.* This information would be useful to citizens of Nacogdoches, Texas as well as physicians in the area. Knowledge of where ticks are collected and when allows citizens to be more cautious in those areas as well as areas with similar features. For physicians this information could be useful if a person presents with certain symptoms and has been in these areas or in contact with ticks in the area.

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