DETECTION OF TICK-BORNE PATHOGENS IN LAB REARED TICK COLONIES AND WILD POPULATIONS

By

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Abstract: Oklahoma State University has a Tick Rearing Facility that sells ticks worldwide. Ticks carry many pathogens that cause disease in animals. PCR protocols were developed in order to set up routine screening of the tick colonies for tick-borne pathogens commonly carried by ticks in Oklahoma. Of the six species reared, three were tested, *Amblyomma americanum, Ixodes scapularis,* and *Dermacentor variabilis.* A few wild populations were screened as well. They were screened for five pathogens, *Ehrlichia chaffeensis*, *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Rickettsia rickettsii* and *Francisella tularensis*. A method for DNA extraction was also developed using DNAzol® Direct (Molecular Research Center, Inc., Cincinnati, OH) and a beadbeater with zirconium/silica microbeads. Most screenings were negative with one positive for *Ehrlichia chaffeensis* in a wild tick. Positives for *Rickettsia amblyommii* and *Rickettsia montanensis* were found in *Amblyomma americanum* and *Dermacentor variabilis*. These initial results showed that the colony ticks are free of the five pathogens of interest, but contain related species which may have a symbiotic role with the ticks.

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CHAPTER I

INTRODUCTION

Oklahoma State University's Department of Entomology and Plant Pathology (Stillwater, OK) houses a tick rearing facility managed by Lisa Coburn. The facility currently rears and sells six hard tick species: *Ixodes scapularis*, *Amblyomma americanum*, *Dermacentor variabilis*, *Rhiphicephalus sanguineus*, *Amblyomma maculatum*, and *Dermacentor andersoni* following the procedures from Patrick and Hair (1975). The first three species listed are the most commonly sold by the facility and are the most important vectors of tick-borne pathogens. This facility was the first and is still the only place that can rear large quantities of *Ixodes scapularis*.

Ticks from this facility are sold worldwide to both industry and universities. Due to the nature of many experiments, dealing with live animals and pathogens, it is important to know that the ticks from this facility are free from pathogens. In the past, there has been no screening of the colonies and there are currently no screening procedures in place. Recently, purchasers of the ticks have asked for documentation showing that the ticks are free of certain pathogens, such as *Borrelia burgdorferi*. Occasionally, wild ticks are collected to add to the colonies to maintain colony stability. Oklahoma is a hotspot for tick-borne pathogens, so this might be a potential way of introducing pathogens to the colonies.

OVERALL GOAL AND OBJECTIVES

The overall goal was to develop assays and reagents to allow monitoring of lab reared tick colonies and research the emergence of tick-borne diseases in Oklahoma. There were three objectives to accomplish this:

- Develop PCR protocols for detection of tick-borne pathogens in lab reared and wild ticks
- Generate stock positive controls and storage system for PCR reactions
- Screen ticks from the Oklahoma State University Tick Rearing Facility and wild populations

The first objective allowed the tick rearing facility to monitor the colonies for tick-borne pathogens. This also allows future monitoring of emerging tick-borne pathogens in Oklahoma. It was important to develop easy to follow protocols so that anyone, even people without a molecular biology background, could perform the necessary steps. The second objective allows the tick rearing facility and Oklahoma State University researchers to have access to positive controls for future pathogen detection. This was very important because the positive controls for pathogens are difficult to obtain; and the ones that can be ordered are expensive. Only a small segment of DNA was stored to maintain a BSL-1 level lab. The ticks were screened for five pathogens, *Ehrlichia chaffeensis*, *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Rickettsia rickettsii*, and *Francisella tularensis*, in three tick species, *Amblyomma americanum*, *Ixodes scapularis*, and *Dermacentor variabilis*. The third objective was to determine if

the above pathogens are currently present in the Oklahoma State University Tick Rearing Facility colonies and a few wild populations.

CHAPTER II

REVIEW OF LITERATURE

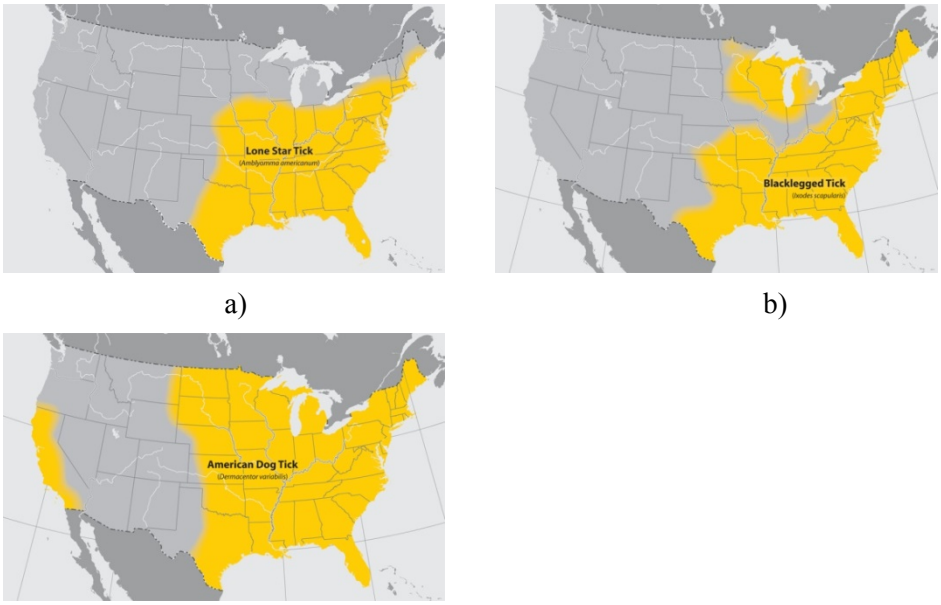
PREVALENT TICKS IN OKLAHOMA

Ticks are non-insect exoparasitic arthropods from the subphylum Chelicerata, class Arachnida, subclass Acari, and order Ixodida which currently has 907 identified species (Sonenshine and Roe, 2014). There are three families of ticks, Ixodidae, Argasidae, and Nutalliellidae (Bowman and Nutall, 2008). Ixodid ticks are the hard ticks and are well known vectors (an organism that transmits a disease or parasite from one animal or plant to another) of pathogens that affect both non-human animals and humans. In the United States, tick-borne diseases are the most common vector transmitted diseases (Donovan et al., 2002). Oklahoma is in what is known as the "tick belt" of the United States meaning there are large populations of ticks present. Many tick-borne pathogens are also present in Oklahoma. Tick populations can become extremely large very quickly as one female tick can lay up to three thousand eggs.

Oklahoma State University's tick rearing facility was established in the mid-1980s as a result of high amounts of tick research being carried out at Oklahoma State

University. Before the establishment of this facility, ticks were raised by individual researchers in their own lab. When the facility was first started, it was strictly for Oklahoma State University researchers. It wasn't until the mid-1990s that they started to sell the ticks to other universities and industry. There is currently no other facility like this in the world.

The three species of ticks that were tested, *Amblyomma americanum*, *Ixodes scapularis*, and *Dermacentor variabilis* are all found in Oklahoma (Figure 1) and are known to carry and transmit one or more of the pathogens that were focused on (see sections of Prevalent Tick-borne Pathogens in Oklahoma). They are also the three species frequently sold by the tick rearing facility. Other tick species are present in Oklahoma, but these three are the most common that transmit pathogens. All three species are members of the Ixodidae family (Mullen and Durden, 2009). They have four life states: egg, larvae, nymph, and adult. The three mobile life stages take one blood meal in order to molt or reproduce (Mullen and Durden, 2009). Due to this, tick-borne pathogens must be transmitted either transstatdially or transovarially within the tick populations (Bowman and Nuttall, 2008). Transstadial transmission takes place when a tick remains infected after a molt whereas transovarial transmission happens when a female passes the pathogen to her offspring. If the pathogen could not accomplish one of these types of transmissions, the tick would be able to pick up the pathogen, but not transmit it to another organism. This happens with some tick-borne pathogens if they are not in their primary vector.



a) b)

c)

Figure 1: Distribution maps of tick vectors common in Oklahoma, a) *Amblyomma americanum*, b) *Ixodes scapularis*, and c) *Dermacentor variabilis* (obtained from the CDC)

Amblyomma americanum

Amblyomma americanum, the Lone Star Tick, is one of the most notorious tick pest species in the United States (Mullen and Durden, 2009). Larvae are active during late summer, while nymphs and adults are active during late spring and early summer (Mullen and Durden, 2009). All mobile life stages (larvae, nymph, and adult) are very active and often parasitize humans (Mullen and Durden, 2009; Varela et al., 2004). They also parasitize almost any animal they can find (Mullen and Durden, 2009; Varela et al., 2004). However, white-tailed deer (*Odocoileus virginianus*) are the primary host for all three mobile life stages (Varela et al., 2004; Varela-Stokes, 2007). White-tailed deer are prevalent through much of the United States (Figure 2). In field studies of areas containing *A. americanum*, 80-100% of white-tailed deer sampled had all three mobile

life stages of *A. americanum* on them (Childs and Paddock, 2003). In Oklahoma, coyotes also serve as a host for all three mobile life stages (Childs and Paddock, 2003).

Figure 2: Distribution map of white-tailed deer (*Odocoileus virginianus*) in North America (obtained from Discoverlife.org)

Amblyomma americanum is known to transmit *Ehrlichia chaffeensis* and *Francisella tularensis*. It has been found to contain other pathogens, such as *Rickettsia rickettsii*, but not transmit them (Mullen and Durden, 2009). Though all three mobile life stages are able to transmit diseases, diseases are most commonly transmitted by the nymph and adult stages (Bowman and Nuttall, 2008). Components of *A. americanum* saliva have been shown to be bactericidal to some pathogens, such as *Borrelia burgdorferi* (Ledin et al., 2005).

Ixodes scapularis

Ixodes scapularis, the Blacklegged Tick, is found throughout the eastern, south-central, and Midwestern United States (Mullen and Durden, 2009). Larvae and nymphs

are found on small mammals while the adults are found on white-tailed deer (Mullen and Durden, 2009). The larvae are active during the summer, nymphs during late spring and early summer, and adults during fall and early spring (Mullen and Durden, 2009). Currently, *I. scapularis* is moving northward and farther inward in the United States (Hamer et al., 2012). The nymphal stage is the most likely to transmit disease (Mullen and Durden, 2009). It is the primary vector of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* (Mullen and Durden, 2009). Components of its saliva enhance the dissemination in vivo of *B. burgdorferi* (Ledin et al., 2005).

Dermacentor variabilis

Dermacentor variabilis, the American Dog Tick, is found throughout the eastern and south-central United States (Mullen and Durden, 2009). The larvae and nymphs are active during the late winter and spring while adults are active during late spring and early summer (Mullen and Durden, 2009). In Oklahoma, its main hosts are small rodents and dogs (Mani et al., 2012). The larval and nymphal stages have been found to survive without a blood meal (Wright and Barker, 2004). It is the major vector of *Rickettsia rickettsii* and *Francisella tularensis* (Mullen and Durden, 2009).

PREVALENT TICK-BORNE PATHOGENS IN OKLAHOMA

Due to Oklahoma's high abundance of ticks, there is also a high prevalence of tick-borne pathogens. The major ones of concern are *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, *Rickettsia rickettsii*, and *Francisella tularensis*. *Borrelia burgdorferi* is also a concern, but it is not as common in Oklahoma as the others. All five of these

pathogens are carried and transmitted by one of the ticks discussed in the previous section (section of Prevalent Ticks in Oklahoma).

Ehrlichia chaffeensis

Ehrlichia chaffeensis is an obligate, intracellular, gram-negative bacterium (Long et al., 2003; Mullen and Durden, 2009). It is the causative agent for Human Ehrlichiosis and is found in the southeastern and south-central United States (Mullen and Durden, 2009) (Figure 3). *Amblyomma americanum* is its primary vector (Long et al., 2003) and within tick populations, it is transmitted transstadially (Childs and Paddock, 2003; Cunha, 2000). White-tailed deer are not only the primary host for *A. americanum*, but they are also the primary reservoir for *E. chaffeensis* (Loftis et al., 2004; Long et al., 2003; Mullen and Durden, 2009; Verala-Stokes, 2007). They are able to maintain the infection with no clinical symptoms (Varela-Stokes, 2007). In Oklahoma, a large number of coyotes have been found to be infected with *E. chaffeensis* which suggests that they also may be reservoirs of the pathogen (Childs and Paddock, 2003). The number of cases of Ehrlichiosis is currently on the rise, most likely due to increases in population sizes of both *A. americanum* and White-tailed deer (Dumler et al., 2007).

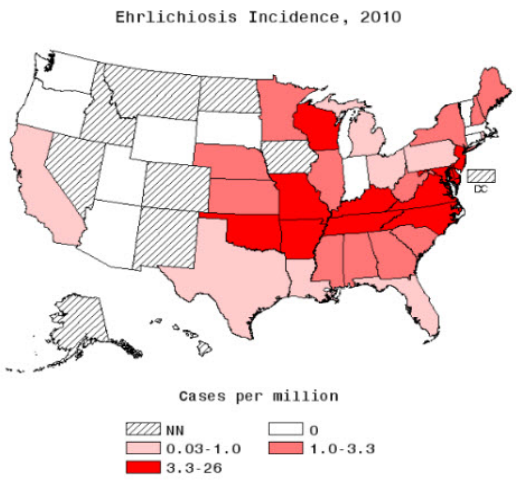


Figure 3: Distribution map of human cases of Ehrlichiosis in the United States (obtained from the CDC)

Borrelia burgdorferi

Borrelia burgdorferi is a spirochete, helically shaped, motile bacterium (Cunha, 2000). It is about 20-30µm in length and has flagella encased in a periplasmic space (Cunha, 2000). *Borrelia burgdorferi* has a unique characteristic; it contains linear plasmids that encode outer surface proteins (Cunha, 2000). It is the causative agent of Lyme disease which is currently the most commonly reported tick-borne disease in the United States (Childs and Paddock, 2003; Hamer et al., 2012; Mullen and Durden, 2009) (Figure 4). It is mostly found in the northeastern United States, but can be anywhere there is *Ixodes scapularis* and a suitable host. Within tick populations, it is transmitted transstadially (Mullen and Durden, 2009; Sonenshine and Mather, 1994). The main reservoirs are the white-footed mouse and the eastern chipmunk (Mullen and Durden, 2009, Hamer et al., 2012). *Borrelia burgdorferi* is currently moving northward and farther inward in the United States (Hamer et al., 2012).

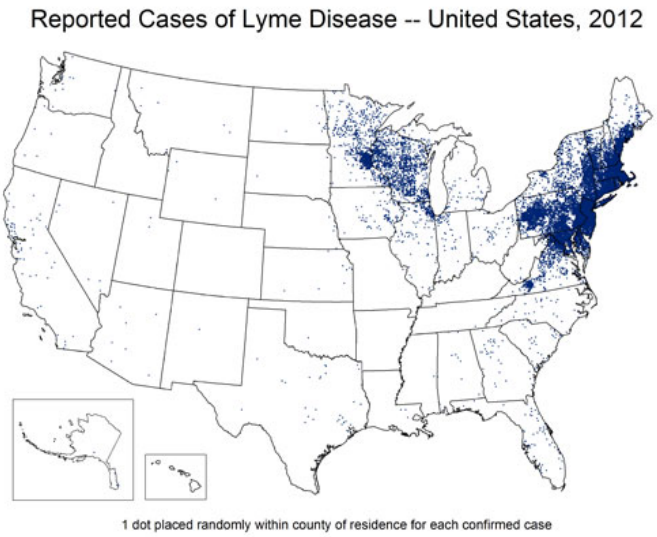


Figure 4: Distribution map of cases Lyme disease in the United States (obtained from the CDC)

Anaplasma phagocytophilum

Anaplasma phagocytophilum is an obligate, intracellular bacterium and is the causative agent of Human Granulocytic Anaplasmosis (Mullen and Durden, 2009). It was previously known as *Ehrlichia phagocytophilum*, but DNA studies have caused a change in the classification (Mullen and Durden, 2009). It is found in the northeastern, upper Midwestern and far-western United States (Mullen and Durden, 2009) (Figure 5). *Ixodes scapularis* is its primary vector (Mullen and Durden, 2009). Within tick populations, it is transmitted transstadially (Yoshimoto et al., 2012) and many animals serve as reservoirs (Maurin et al., 2003).

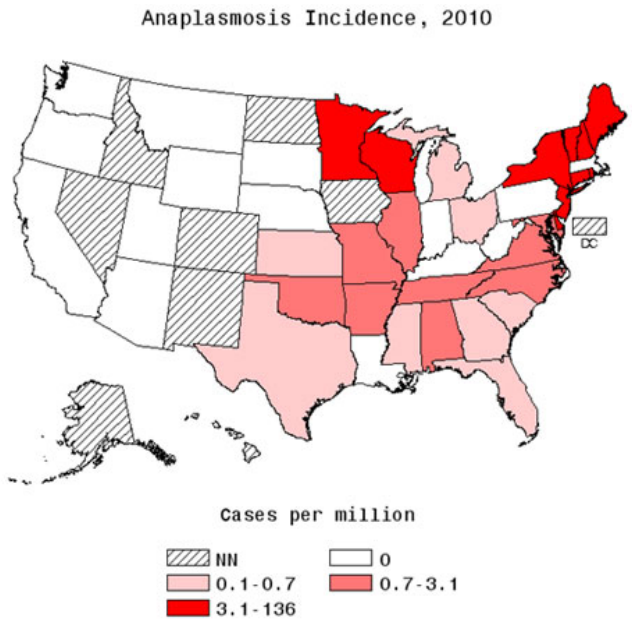


Figure 5: Distribution map of cases of human Anaplasmosis in the United States (obtained from the CDC)

Rickettsia rickettsii

Rickettsia rickettsii is an intracellular bacterium that multiplies freely in cytoplasm (Mullen and Durden, 2009). It is the causative agent of Rocky Mountain Spotted Fever and is found throughout the United States (Mullen and Durden, 2009) (Figure 6). *Dermacentor variabilis* is the primary vector in Oklahoma. In other parts of the United States, *Dermacentor andersoni* is the primary vector (CDC.gov). Within tick populations, it is transmitted transstadially and transovarially (Eremeeva et al., 2003; Mullen and Durden, 2009; Sonenshine and Mather, 1994). The larval and nymphal stages of *D. variabilis* are what maintain the infection from year-to-year (Mullen and Durden, 2009). *Rickettsia rickettsii* requires the ticks to feed for at least 10 hours in order for the tick to be able to transmit the pathogen (Mullen and Durden, 2009; Sonenshine and

Mather, 1994). This is because it is not virulent within the tick and only becomes virulent after feeding has started (Mullen and Durden, 2009).

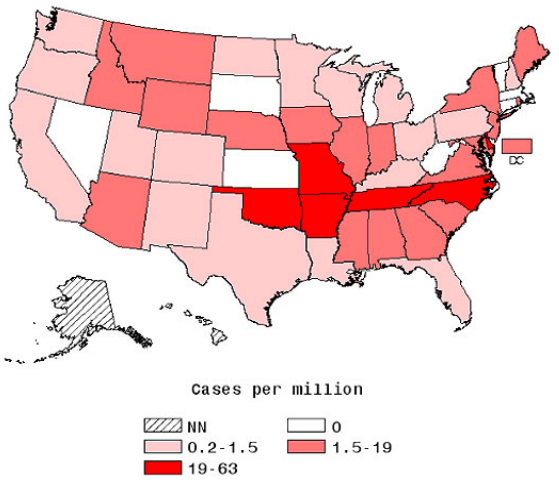


Figure 6: Distribution map of cases of Rocky Mountain Spotted Fever in the United States in 2010 (obtained from the CDC)

Francisella tularensis

Francisella tularensis is a pleomorphic, gram-negative, aerobic (Mullen and Durden, 2009) which is highly infectious (Mani et al., 2012). It is the causative agent of Tularemia (Mullen and Durden, 2009). It can be transmitted by many arthropods as well as by contact with infected animals (Mullen and Durden, 2009). It is found throughout the United States, but is concentrated mostly in the Midwest (Figure 7). The tick vector is mainly *Dermacentor variabilis*, but can also be transmitted by *Amblyomma americanum*. It is unclear if within tick populations the pathogen transmits transstadially or transovarially, but it seems to be more likely transtadially (Mullen and Durden, 2009; Mani et al., 2012). Peak human infections coincide with seasonal questing of adult *D.*

variabilis and *A. americanum* (Mani et al., 2012). Overwintering ticks have been shown to be reservoirs of the pathogen (Mani et al., 2012).

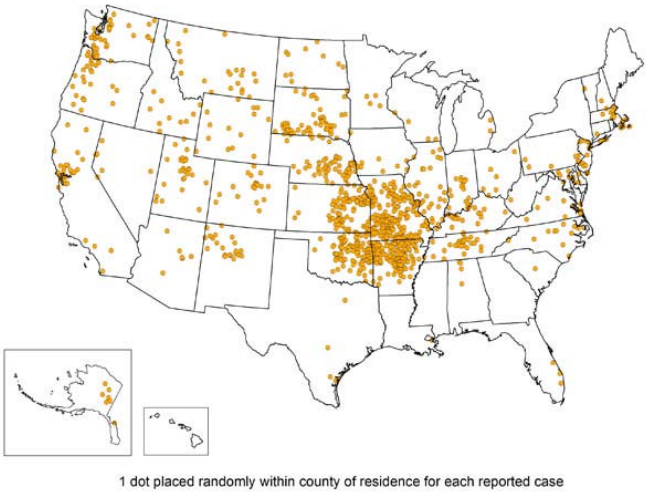


Figure 7: Distribution map of cases of Tularemia in the United States from 2004-2013 (obtained from the CDC)

Pathogens Found in Sheep and Rabbits

Three out of the five pathogens above are found in sheep, *Anaplasma phagocytophilum*, *Borrelia burgdorferi,* and *Francisella tularensis*. However, *B. burgdorferi* is not found in sheep in the United States, but is found in sheep in the United Kingdom, Denmark, and Sweden (Friðriksdóttir et al., 1992). Sheep serve as a reservoir for *A. phagocytophilum* in the U.S. (Ogden et al., 2003). In domestic animals, sheep are the primary host of *Francisella tularensis* (Kahn and Line, 2005). A review of the literature did not find any incidence of *Ehrlichia chaffeensis* or *Rickettsia rickettsii* in sheep.

Four out of the five pathogens above are found in rabbits, *Anaplasma phagocytophilum*, *Borrelia burgdorferi* (Yabsley et al., 2006), *Rickettsia rickettsii* (Joshi

and Kovács, 2007), and *Francisella tularensis* (Kahn and Line, 2005). *Ehrlichia chaffeensis* antibodies have been found in rabbits (Spickler, 2013). All are found in the U.S. (Yabsley et al., 2006.; Joshi and Kovács, 2007; Kahn and Line, 2005; Spickler, 2013).

PATHOGEN DETECTION AND DNA EXTRACTION METHODS

PCR is the process of DNA amplification and is the suggested method for detection of all five pathogens (Cunha, 2010). It requires Mg^{++}, dNTPs, taq polymerase (often combined in a kit), primers, and a DNA template. Primers are sequences of nucleic acids that can be specific to a particular organism or non-specific to detect multiple organisms. The specificity of the primers depends on the intended use of PCR, with higher specificity being desired most often. A thermal cycler is required to perform the necessary temperature changes to complete the reaction. There are essentially three steps to a PCR reaction, denaturation (unzipping of the double strand), annealing (attachment of primers), and extension (adding dNTPs to form the copies strand). There is an initial denaturation followed by a series of cycles of denaturation, annealing, and extension which is followed by a final extension to ensure all copies finish.

PCR has been used to detect *E. chaffeensis* in ticks in many states (Varela et al., 2004). The most common primer sets for running PCR have been ECB/ECC and HE1/HE3 in a nested PCR. The use of HE1/HE3 in a nested PCR helps increase the specificity (Long et al., 2003; Varela et al., 2004). Varela-Stokes (2007) dissected ticks and used only certain tissues, midgut and salivary glands, to obtain DNA for PCR to

detect *E. chaffeensis*. She used only the ECB/ECC primer set. Loftis et al. (2004) used whole ticks that were frozen with liquid nitrogen and crushed with a pestle to screen for *E. chaffeensis*. They used the IsoQuick nucleic Acid Extraction Kit (Orca Research, INC., Bothell, WA) to extract the DNA from the tick samples. Long et al. (2003) used a bead mill homogenizer to obtain tick tissues and DNA was extracted using the QIAmp DNA MiniKit (Qiagen, Valencia, CA).

PCR has also been used for detection of *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Rickettsia rickettsii*. However, a review of the literature found that primer sets vary greatly. Also, many primer sets for *B. burgdorferi* and *R. rickettsii* are not species specific and depend on sequencing if a positive result is found. While PCR can be used for *F. tularensis* detection, the presence of *Francisella*-like organisms in ticks may make PCR challenging (Kugeler et al., 2005). The 16S rRNA gene is highly conserved among *Francisella* species and should not be used to detect *F. tularensis* (Kugeler et al., 2005). Kugeler et al. (2005) used real-time PCR with a multi-target Taqman to detect three proteins, ISF*tu*2, *tul*4, and *igl*C. They state that in order to verify that *F. tularensis* is the organism present, all three proteins must be found. There are no commonly used primers to detect *F. tularensis* with PCR.

In order to run PCR to detect pathogens in ticks, DNA must first be extracted from the ticks. There are many ways of performing DNA extractions, but ticks' exoskeleton can make it difficult. Crowder et al. (2010) states that the best way to obtain total nucleic acids from ticks is with a combination of different sized, high density yttria stabilized zirconium oxide beads in a lysis buffer, sonicated poly A, and proteinase K solution. This allows the homogenization of the ticks as well as the lysis of the bacteria

and protozoa that may be present in the ticks. The homogenized/lysed solution can then be used in an extraction kit (DNA, RNA, viral, etc.). This method is less time consuming than the use of a mortar and pestle or even an enzymatic lysis method. They found that with the use of yttria stabilized zirconium oxide beads instead of steel or zirconium-silica beads, there was no PCR inhibition in downstream applications. They also found that Qiagen DNeasy columns (Qiagen, Valencia, CA) work just as well as other columns, but cost less.

Halos et al. (2004) stated that DNA extraction from ticks is highly susceptible to degradation. Taq-polymerase inhibitors may also be present which could affect PCR amplification downstream. Crowder et al. (2010) used a silica-gel column to remove any PCR inhibitors and cellular debris from the nucleic acids. Halos et al. (2004) compared three methods of tick DNA extraction: first, ATL buffer with proteinase K and Qiamp DNA extraction kit (tissue protocol) (Qiagen, Valencia, CA), second, Dounce mortar with proteinase L and phenol-chloroform/absolute ethanol/TE buffer, and third, sterile microbeads (glass and steel) with ATL buffer and Qiamp DNA extraction kit (Qiagen, Valencia, CA). They found that the third method had the highest DNA extraction efficiency (100%) followed by the second method (97%) then the first method (77%). They also found that the third method worked well on small samples, such as using nymphs or even larvae. They recommend that when detecting pathogens, a positive control of the 16S rNA gene from the ticks be used to show the quality of the DNA being amplified. The use of the mortar was found to be efficient, but very time consuming.

CHAPTER III

METHODOLOGY

DNA EXTRACTION

DNA extractions were performed using the whole tick. All lab reared ticks were obtained from the Oklahoma State University Tick Rearing Facility. Three species of ticks were examined, *Ixodes scapularis*, *Amblyomma americanum*, and *Dermacentor variabilis*. The ticks were frozen at -80°C until dead, usually at least 24 hours before use. Wild ticks from two of Oklahoma's State Parks, Greenleaf (Muskogee, OK) and Sequoyah (Hulbert, OK), were collected by Jessica Mitcham (Master's student, Oklahoma State University, Dr. Bruce Noden Lab). They were collected by flag dragging. In the field they were placed in 70% ethanol and were stored in 70% ethanol in the lab.

Total genomic DNA from the ticks was extracted using DNAzol® Direct (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's protocol. DNAzol® Direct is a one-step DNA extraction method that allows the DNA product to go straight into a PCR reaction. The DNA extractions were performed using a single tick, no pools were performed. The tick was added to a pre-weighed and labeled 2ml sterile

polypropylene microvial (Biospec Products, Bartlesville, OK) designed specifically for use with the Mini-Beadbeater-1™ and Mini-Beadbeater-16™ (Biospec Products, Bartlesville, OK). DNAzol® Direct was added to each tick at 100µl per 0-10mg of tick tissue. The ticks were then incubated at 80-90°C for 15min. Zicronium/silica (z/s) beads (Biospec Products, Bartlesville, OK), 1.0mm and 2.5mm, were then added to each tick. For nymphs, ten 1.0mm z/s beads and two 2.5mm z/s bead were added to the tick. For adults, twenty 1.0mm z/s beads and four 2.5mm z/s beads were added to the tick. The tubes were then shaken in either the Mini-Beadbeater-1™ at 2-3 cycles at 4,200oscillations/min for 1.5min or the Mini-Beadbeater-16™ for 2-3 cycles at 3,450oscillations/min for 1.5min. The adults usually needed 3 cycles to completely homogenize, but this varied by individual tick. The tubes were then centrifuged at 13,000xg for 1min to separate the body parts from the DNA product. As much of the DNA product as possible was then transferred to a 1.5ml tube, trying to leave behind as many tick parts as possible. The DNA extractions were then stored at -20°C and used for PCR.

PCR AMPLIFICATION OF DNA

PCR amplification was used to screen for five species of pathogens, *Ehrlichia chaffeensis*, *Borrelia burgdorferi*, *Rickettsia rickettsii*, *Anaplasma phagocytophilum*, and *Francisella tularensis*, in three species of ticks, *Amblyomma americanum*, *Ixodes scapularis*, and *Dermacentor variabilis*. Each tick species was only tested for the pathogens that are most commonly found in them. *Amblyomma americanum* was

screened for *Ehrlichia chaffeensis*, *Francisella tularensis*, and *Rickettsia rickettsii*. Although *Amblyomma americanum* is not known to transmit *Rickettsia rickettsii*, it has been found to carry it. *Ixodes scapularis* was screened for *Borrelia burgdorferi* and *Anaplasma phagocytophilum*. *Dermacentor variabilis* was screened for *Rickettsia rickettsii* and *Francisella tularensis*.

All PCR reactions were run using one of two PCR mixes, Hotstart PCR-To-Gel Master Mix, 2x (Amresco, LLC., Solon, OH) (contains Mg^{++}, dNTPs, Taq DNA polymerase, and loading dye) or Hotstart Taq Master Mix, 2x (Amresco, LLC., Solon, OH) (contains Mg^{++}, dNTPs, and Taq DNA polymerase). All PCR reactions were carried out in 25µl reactions comprised of 12.5µl PCR master mix, 0.5µl of each primer (starting concentration 100pmole/µl), 11µl of sterile water, and 0.5µl of template. Hotstart Taq Master Mix, 2X was used when the PCR product was going to be used for sequencing or cloning. To use the Hotstart Taq Master Mix, 2X for sequencing, the product needed to have excess primers and nucleotides removed using USB® ExoSAP-IT® PCR Product Cleanup (Affymetrix, Inc., Santa Clara, CA) following the manufacturer's protocol. All PCR products were stored at 4°C for up to 3 weeks.

All PCR products were visualized using an agarose gel with electrophoresis. Gels were made with 1X TBE buffer and 2% Agarose (Low-EEO/Multi-Purpose/Molecular Biology Grade, Fisher Bioreagents) (Fisher Scientific, Waltham, MA). Each gel was stained with ethidium bromide at 0.5µl/ml (placed in each individual gel when making, not stained after running). Each gel was run at 100V for 60-70min. All gels were run with the BenchTop 100bp DNA Ladder (Promega, Madison, WI) to indicate size of product. All gels were photographed using a White/Dual UV Transilluminator (VWR

International, Radnor, PA) at UV 302nm wave length with a triangular hood with an attached Canon Power Shot A3300IS camera and a Canon Selphy CP800 compact photo printer (Canon, Ōta, Tokyo, Japan).

Ehrlichia chaffeensis

Ehrlichia chaffeensis MO strain DNA was provided by Susan Little, Oklahoma State University (Stillwater, OK) in 2010. It was stored at -20°C. PCR was performed following a modified program from Jaworski et al. (2013). A nested set of primers was used, ECB/ECB followed by HE1/HE3 (Jaworski et al., 2013) (Table 1). The primer sets targeted the 16S rRNA gene. Primer set ECB/ECC will amplify many *Ehrlichia* and *Anaplasma* species. The nested primer set HE1/HE3 will amplify *Ehrlichia chaffeensis*. Both primer sets were tested at different temperatures to determine the best temperature used for annealing. The primer set ECB/ECC used the following program, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 65°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The primer set HE1/HE3 used the following program, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 58°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The products from ECB/ECC were diluted 1/2000 before running with HE1/HE3. The DNA fragment length for ECB/ECC is 479bp long and 390bp long for HE1/HE3. These primers sets were always run using Hotstart Taq Master Mix, 2X. They were found to react (produce bands) with Hotstart PCR-To-Gel Master Mix, 2X. No other primer sets were found to react with the PCR mix.

Borrelia burgdorferi

Borrelia burgdorferi strain B31 (ATCC® 35210™) (genomic DNA) was purchased from American Type Culture Collection (Manassas, VA) and stored at -20°C. A primer set targeting the *osp*A gene was used, ospA2/ospA4 (Scott et al., 2012) (Table 1). This primer set is not specific to ***B. burgdorferi***, but will detect all *Borrelia* species. Any positive from this primer set was sent for sequencing. The following program was used, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 55°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The DNA fragment length is 156bp long.

Anaplasma phagocytophilum

Anaplasma phagocytophilum DNA was obtained from Dr. Nicholson, Ricketsial Zoonoses Branch, Centers for Disease Control and Prevention. A nested set of primers were used, GE3a/GE10R followed by GE9F/GE2 (CDC, 2010), to target the 16S rRNA gene (Table 1). The primer set GE3a/GE10R used the following program, initial denaturation at 95°C for 5min continued with 40 cycles of 95°C (denaturing) for 30sec, 55°C (annealing) for 30sec, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The primer set GE9F/GE2 used the following program, initial denaturation at 95°C for 5min continued with 30 cycles of 95°C (denaturing) for 30sec, 55°C (annealing) for 30sec, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The

products from GE3a/GE10R were diluted 1/2000 before running with GE9F/GE2. The DNA fragment length for GE3a/GE10R is 932bp long and 546bp long for GE9F/GE2.

Rickettsia rickettsii

Rickettsia rickettsii DNA was obtained from Dr. Nicholson, Ricketsial Zoonoses Branch, Centers for Disease Control and Prevention. A primer set targeting the 17-kDA antigen gene was used, TZ15/TZ16 (CDC, 2010) (Table 1). This primer set is not specific to *Rickettsia rickettsii*, but is instead specific to *Rickettsia* spotted fever group. Any positive result from this primer set was gel extracted and sent for sequencing. The following program was used, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 55°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The DNA fragment length is 247bp long.

Francisella tularensis

Francisella tularensis DNA was obtained from Dr. Ramachandran, Oklahoma Animal Disease Diagnostic Laboratory, Oklahoma State University (Stillwater, OK). A primer set targeting the IS*Ftu*2 gene was used, ISFtu2F/ISFtu2R (Versage et al., 2003) (Table 1). The following program was used, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 55°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The DNA fragment length is 97bp long.

Tick DNA

The quality of the DNA extractions was checked by amplifying the tick gene 16S rRNA using the primer set TQ16S+1F/TQ16S-2R (Halos et al., 2004; Crowder et al., 2010). The following program was used, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 55°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The DNA fragment length is 320bp long.

Primers

All primers were obtained from the literature or the CDC. They were all ordered from Integrated DNA Technologies (IDT) (Coralville, IA) and restored to 100pmoles/µl. The detection limits of the primer sets were tested through a series of ten-fold dilutions, 200000copies/µl to 2copies/µl, of gel extracted plasmid DNA for each pathogen. PCR reactions were done at 0.5µl, resulting in 100000copies/0.5µl to 1copy/0.5µl. A NanoDrop UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA) was used to determine DNA concentration of plasmid DNA. Thermo Scientific's DNA copy number calculator webtool (http://www.thermoscientificbio.com/webtools/copynumber/) was used to determine the copy number of the desired sequence in the plasmid DNA. Specificity for each primer set was tested against *Amblyomma americanum*, *Ixodes scapularis*, *Dermacentor variabilis*, *Anaplasma phagocytophilum*, *Anaplasma odocoilei*, *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, *Francisella tularensis*, and *Rickettsia rickettsii*. The primer set TZ15/TZ16 was also tested with *Rickettsia parkeri*. The primer

set TQ16S+1F/TQ16S-2R was also tested with *Amblyomma maculatum*, *Rhipicephalus sanguineus*, and *Dermacentor andersoni*.

Table 1: Target gene, name, sequence, Tm value, fragment size, and reference for all primer sets used

Targeted Gene	Primer Name	Sequence (5' → 3')	Tm (°C)	Fragment Length (bp)		Reference
Ehrlichia chaffeensis 16S rRNA	ECC	AGAACGAACGCTGGCGGCAAGCC	66.8	479	390	Jaworski et al., 2013 Heise, 2007
	ECB	CGTATTACCGCGGCTGCTGGCA	64.6			
	HE1	CAATTGCTTATAACCTTTTGGTTATAAAT	51.6	390		
	HE3	TATAGGTACCGTCATTATCTTCCCTAT	54.3			
Borrelia burgdorferi ospA	ospA2	GTTTTGTAATTTCAACTGCTGACC	53.2	156		Scott et al., 2012
	ospA4	CTGCAGCTTGGAATTCAGGCACTTC	60.5			
Anaplasma phagocytophilum 16S rRNA	GE3a	CACATGCAAGTCGAACGGATTATTC	56.5	932	546	CDC, 2010
	GE10R	TTCCGTTAAGAAGGATCTAATCTCC	53.9			
	GE9F	AACGGATTATTCTTTATAGCTTGCT	52.7	546		
	GE2	GGCAGTATTAAAAGCAGCTCCAGG	58.1			
Rickettsia rickettsii 17-kDA antigen	TZ15	TTCTCAATTCGGTAAGGGC	52.3	247		CDC, 2010
	TZ16	ATATTGACCAGTGCTATTTC	42.4			
Francisella tularensis ISFtu2	ISFtu2F	TTGGTAGATCAGTTGGTGGGATAAC	56.4	97		Versage et al., 2003
	ISFtu2R	TGAGTTTTACCTTCTGACAACAATATTTC	54.1			
Tick DNA 16S rRNA	TQ16S +1F	CTGCTCAATGATTTTTTAAATTGCTGTGG	56.1	320		Crowder et al., 2010 Halos et al., 2004
	TQ16S -2R	ACGCTGTTATCCCTAGAG	50.6			

GEL EXTRACTIONS

Gel extractions were performed after some gel runs of PCR products. This enabled pure DNA to be obtained and sequenced. This was not always necessary if a valid sequence was obtained from a PCR cleanup. On gels done for screening, extractions were done on any positive result to verify the organism.

Empty 1.5ml tubes were labeled and weighed. Once a gel had been run and a band was seen, the band was cut out using a gel cutting tip (Axygen, Union city, CA). The tansilluminator (described in the section PCR Amplification of DNA) was kept turned on during the cutting of the band. Long sleeves and a UV face shield were worn during the process. The gel slice (cut band) was placed in a pre-weighed 1.5ml tube and reweighed (in some cases, multiple bands from the same PCR product were combined in one tube). Once the gel slice weight was determined, the extraction was performed using Purelink® Quick Gel Extraction Kit (Invitrogen™, Grand Island NY) following the manufacturer's protocol. Before the extraction was sent for sequencing, a NanoDrop (Thermo Scientific, Waltham, MA) was used to determine the concentration of DNA in the gel extraction.

SEQUENCING

Purified PCR products (gel extracted or cleaned up) and plasmid DNA were sent to the Oklahoma State University Core Facility (Stillwater, OK) for sequencing. PCR products were sent in a volume of 10µl [one per primer (20µl per primer set)] at 5ng/µl. Plasmid DNA was sent in a volume of 10µl [one per primer (20µl per primer set)] at

50ng/µl. Primers were sent in a volume of 10µl at 5pmoles/µl. The recovered sequences were visualized with the use of the computer program FinchTV (Geospiza, Seattle, WA). This allowed for the determination of the quality of the sequence. The online tool BLAST (NCBI, Bethesda, MD) was used to verify the identity of the sequences. BLAST was not used for all sequences, plasmid DNA sequences were aligned with verified sequences with the program ClustalW/X (Science Foundation Ireland, Dublin 2, Ireland). This showed the similarity of the sequences.

CLONING

Cloning of purified PCR products (gel extracted or cleaned up) was performed using TOPO® TA Cloning Kit One Shot Top10 with chemically competent cells and vector pCR® 2.1-TOPO® TA (Invitrogen™, Grand Island, NY) following the manufacturer's protocol. This kit has a fast set-up time, 5min, allowing for many clones to be performed in a short amount of time. This kit also has an efficiency of 95%, allowing for a very high yield of the sequence of interest. Media filled petri-dishes (see Media Preparation below) were used after transformation to grow the cloned bacteria. The inoculated plates were incubated at 37°C overnight. The next day, two isolated, white colonies from each plate were selected by drawing a circle around them and numbering them 1 or 2. A mini-culture was made by placing 75µl LB/Kan liquid (see Media Preparation below) in a PCR tube (one tube per colony). The LB/Kan liquid was then warmed for 10min at 37°C. Sterile inoculation needles (VWR International, Radnor, PA) (one needle per plate, one end per colony) were used to collect the isolated, white

colonies by scraping. The needle was then swished around in the warm LB/Kan liquid. The tubes with colonies were incubated for 15min at 37°C. Next, 20µl of nuclease free water was placed in a PCR tube (one per mini-culture) and 2µl of mini-culture were added. This was then incubated at 95°C for 5min. This was then used for PCR (program varied by organism) followed by electrophoresis to verify the quality of the cloned colony. Based on the quality of the bands seen, only two colonies per cloning were grown into larger cultures. During the PCR and gel running, the mini-culture was stored at 4°C, but was used before the end of the day to grow larger cultures. This prevented cell death. The scraped plates were stored at 4°C for up to one month.

The larger cultures were made by adding 7ml of LB/Kan liquid to a sterile, flat bottom culture flask (Falcon®, Corning Inc., New York, NY) (one per mini-culture). The entire mini-culture was added to the flask. The flasks were incubated overnight at 37°C with shaking. The following day, 5ml of the culture was used for plasmid preps and the leftover was used for one glycerol stock (see Plasmid Preparation, Glycerol Stock Preparation, and Storage below).

Media Preparation

The media for cloning was made using ImMedia™ Growth Medium, agar, kanamycin X-gal/IPTG (Invitrogen™, Grand Island, NY) and ImMedia™ Growth Medium, liquid, kanamycin (Invitrogen™, Grand Island, NY) following the manufacturer's protocols. These kits allowed for a fast set-up of media, about 5min. Sterile petri dishes, 100x115mm (Fisher Scientific, Waltham, MA) were used for casting the agar plates. All plates were poured on a sterilized lab bench (using 10% bleach

followed by 70% ethanol). The amount of media poured was just enough to cover the bottom of the petri dish. The plates were left open at room temperature for 35min to cool and set. After 35min, the lids were placed back on the plates and taped on so they would not accidentally come off (only two small pieces of tape were used to prevent condensation from forming on the plates). The plates were then placed in their original plastic sleeve and stored at 4°C. The plates were poured no more than one week in advance. The liquid media was stored in a glass bottle with a lid at 4°C.

PLASMID PREPARATION, GLYCEROL STOCK PREPARARTION, AND STORAGE

To isolate the cloned DNA from the cultures, PureLink® Quick Plasmid Miniprep Kit (Invitrogen™, Grand Island, NY) was used following the manufacturer's centrifuge protocol. The maximum of 5ml of culture was used for each plasmid preparation. The resulting plasmid DNA concentration was determined using a NanoDrop (Thermo Scientific, Waltham, MA), then sent for sequencing. If the sequence came back as the desired sequence, the plasmid DNA was diluted to 1pg/µl using 0.1X TE buffer and aliquoted into samples of 50µl. The aliquots were frozen at -80°C with 81 tubes per box. In the future, when screenings need to be performed, one aliquot can be thawed at a time and will be PCR ready.

One or two glycerol stocks were made from the remaining culture not used for plasmid preps. Aliquots of 1ml at 15% glycerol were prepared in sterile 1.2ml cryogenic vials (Nalgene, Thermo Scientific, Waltham, MA). The glycerol stock(s) was stored at -

20°C for short term storage. After the sequence for the plasmid prep was received and showed the sequence of interest (matched verified sequence used for cloning), large quantities of glycerol stocks were made to fill one freezer box, 72 vials per box. The glycerol stocks that were made from the left over culture were used to grow large cultures. LB/Kan liquid was placed in four sterile flat bottom culture flasks with 20ml in each flask. A sterile inoculation needle was used to stab/scrape the glycerol stock to be used without the need to thaw. The needle tip was then dipped and swished around in the LB/Kan liquid (one needle was used for all four scrapings). The flasks were incubated overnight at 37°C with shaking. Sterile glycerol was then added to each flask so that the total volume was 15% glycerol. The flasks were shaken well to mix. The culture was then poured into a sterile 10ml flask. This allowed the use of a repeat pipette to be used since it couldn't reach the bottom of the flask. The culture with glycerol was aliquoted into 1ml in each 1.2ml cryogenic vial. These glycerol stocks were stored at -80°C for long term storage. In the future, the glycerol stocks can be used to grow more culture which can be used to make more plasmid DNA or more glycerol stocks.

CHAPTER IV

RESULTS

TICK DNA

PCR Assay Development

The primer set TQ16S+1F/TQ16S-2R was tested against six tick species to ensure that it worked across multiple tick species. This primer set was used to ensure that the DNA extraction was successful. A specific primer for each species of tick was not necessary. The primer set was found to work for all six species (Figure 8). The thermal cycler program that worked was, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 55°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The DNA fragment length was about 320bp long.

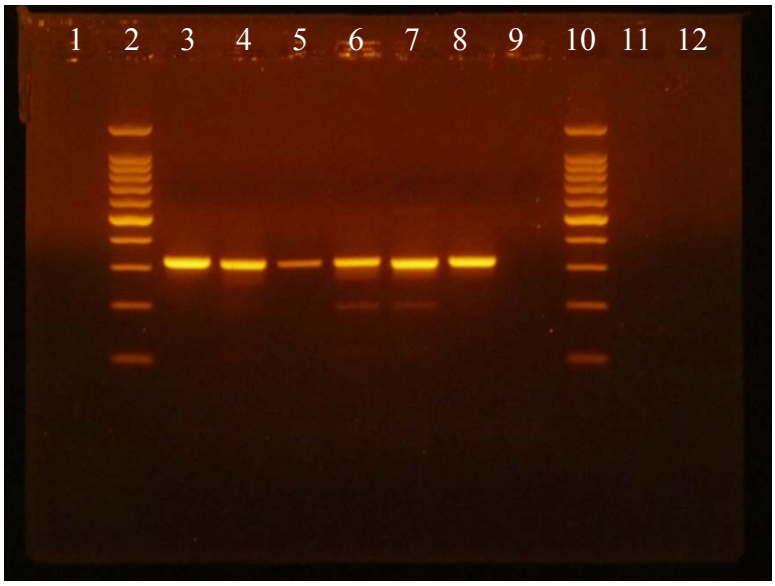


Figure 8: Tick 16S rRNA gene, 2% agarose gel run for 1hour 10min at 100V. DNA extractions from six different tick species, nymphal stage. Lanes: 1) blank, 2) 100bp DNA ladder, 3) *Amblyomma americanum*, 4) *Amblyomma maculatum*, 5) *Rhipicephalus sanguineous*, 6) *Dermacentor variabilis*, 7) *Dermacentor andersoni*, 8) *Ixodes scapularis*, 9) negative 10) 100bp DNA ladder 11-12) blank.

Sequencing

Sequences from only one tick species, *Ixodes scapularis*, were obtained. This was to ensure that the primers were amplifying the correct gene. A sequence for both the forward primer, TQ16S+1F, and the reverse primer, TQ16S-2R, were obtained. After receiving them, the online tool BLAST for nucleotides (NCBI, Bethesda, MD) was used to verify them. Both the forward and reverse sequences matched *Ixodes scapularis* isolate IS988 16S ribosomal RNA gene, partial sequence; mitochondrial (Accession number: KF146639.1) with E-value $1e^{-136}$ (Table 2). Many other matches were found, but all were for *Ixodes* 16S rRNA.

Table 2: Forward and reverse sequences for *Ixodes scapularis* cleaned PCR product using the primer set TQ16S+1F/TQ16S-1R. Both sequences matched *Ixodes scapularis* isolate IS988 16S ribosomal RNA gene, partial sequence; mitochondrial (Accession number: KF146639.1) with E-value $1e^{-136}$ using BLAST for nucleotides (NCBI).

Primer	Sequence (5' → 3')	BLAST Match and E-value
TQ16S+1F	GATTTTGCCCTTACCAAAGGTTTTGAATAAGATTTTAATGAGTGCTAAGAGAATGATTAAACAATTAAAAGCTTTCTTTAATTAAAAAATTAAATTTAATTTTTTTAGTGCAAAAGCAAAAATAAAATTTAGGGACAAGAAGACCCTATGAATTTTTATTGTTAATAATTAATATTTTAGTTATTAATAATTTAATTGGGGCGATAAAAAAAGAATAAGTATCTTTTAGTTATAAAATAAGTTCCGTTTTTAGCGATTAAATGAAATAAATACTCTAGGGATAACAGCGTAA	*Ixodes scapularis* isolate IS988 16S ribosomal RNA gene, partial sequence; mitochondrial (Accession number: KF146639.1) E-value: $1e^{-136}$
TQ16S-2R	CCAAATTCCCCCAAAAAAGGGGAATTTATTTTTATAACTAAAAAGATACTTATTCTTTTTTTATCGCCCCAATTAAATTATTAATAACTAAAATATTAATTATTAACAATAAAAATTCATAGGGTCTTCTTGTCCCTAAATTTTATTTTTGCTTTTGCACTAAAAAAATTAAATTTAATTTTTTAATTAAAGAAAGCTTTTAATTGTTTAATCATTCTCTTAGCACTCATTTAAAATCTTATTTCAATACCTTTGTATAGTCAAAATACCACAGCAATTTAAAAAATCATTGAGCAGA	

Specificity

The specificity for the primer set TQ16S+1F/TQ16S-2R was tested with *Amblyomma americanum* nymph and adult, *Dermacentor variabilis* nymph and adult, *Ixodes scapularis* nymph and adult, *Anaplasma phagocytophilum*, *Anaplasma odocoilei*, *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, *Francisella tularensis*, and *Rickettsia rickettsii*. TQ16S+1F/TQ16S-2R was found to be specific for tick DNA (Figure 9).

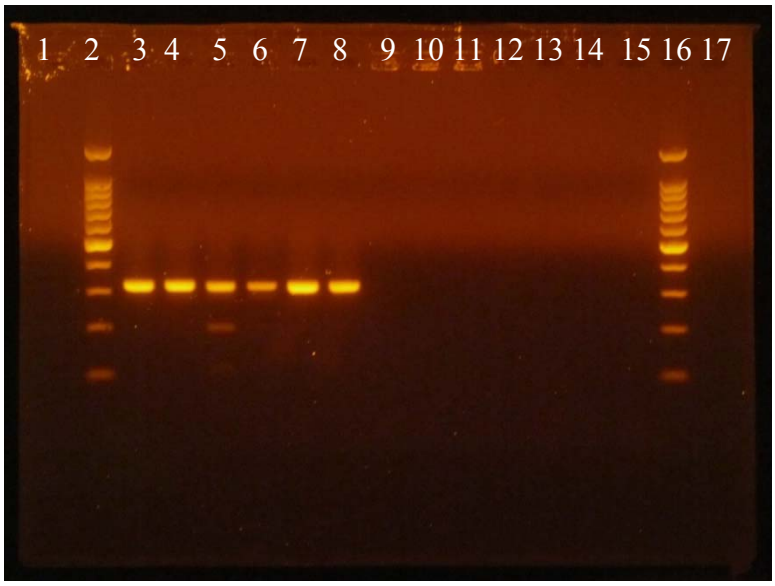


Figure 9: Specificity of primer set TQ16S+1F/TQ16S-2R. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) *A. americanum* adult, 4) *A. americanum* nymph, 5) *D. variabilis* adult, 6) *D. variabilis* nymph, 7) *I. scapularis* adult, 8) *I. scapularis* nymph, 9) *A. phagocytophilum*, 10) *A. odocoilei*, 11) *B. burgdorferi*, 12) *E. chaffeensis*, 13) *F. tularensis*, 14) *R. rickettsii*, 15) negative, 16) 100bp DNA ladder, 17) blank.

EHRLICHIA CHAFFEENSIS

PCR Assay Development

The nested primer sets, ECB/ECC followed by HE1/HE3, were found to be successful in amplifying *Ehrlichia chaffeensis*. Although the literature has both primer sets running with an annealing temperature of 55°C, it was found that this temperature could cause some non-specific banding. Temperature gradients were performed on both primer sets to determine a new annealing temperature, for ECB/ECC, 50-65°C and 60-65°C and for HE1/HE3, 50-65°C and 50-60°C (Figures 10, 11, 12, and 13). For ECB/ECC, 65°C was found to work well, and for HE1/HE3, 58°C was found to work the best (Figure 10). The following thermal cycler program was used for ECB/ECC, initial

denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 65°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The following thermal cycler program was used for HE1/HE3, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 65°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The final DNA fragment length was 390bp long.

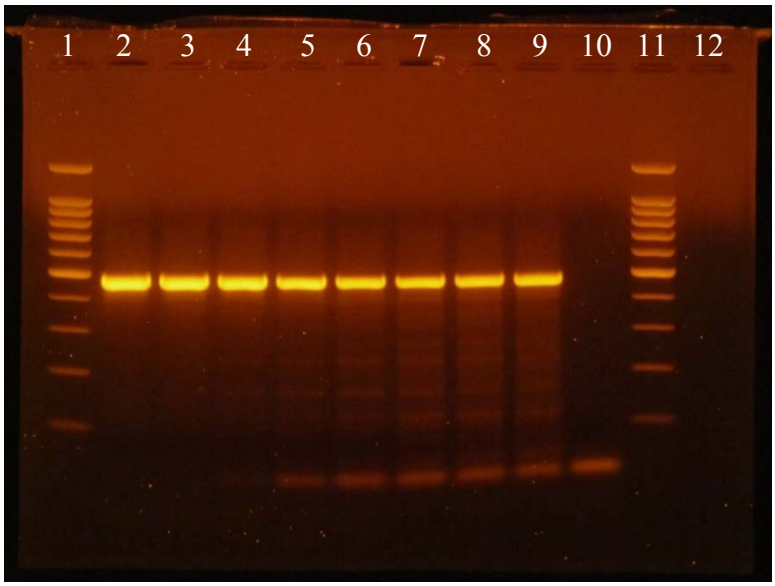


Figure 10: *Ehrlichia chaffeensis* primer set ECC/ECB temperature gradient from 50-65°C. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) 100bp DNA ladder, 2) 65°C, 3) 63.9°C, 4) 62.1°C, 5) 59.4°C, 6) 55.9°C, 7) 53.4°C, 8) 51.4°C, 9) 50°C, 10) negative at 55.9°C, 11) 100bp DNA ladder, 12) blank.

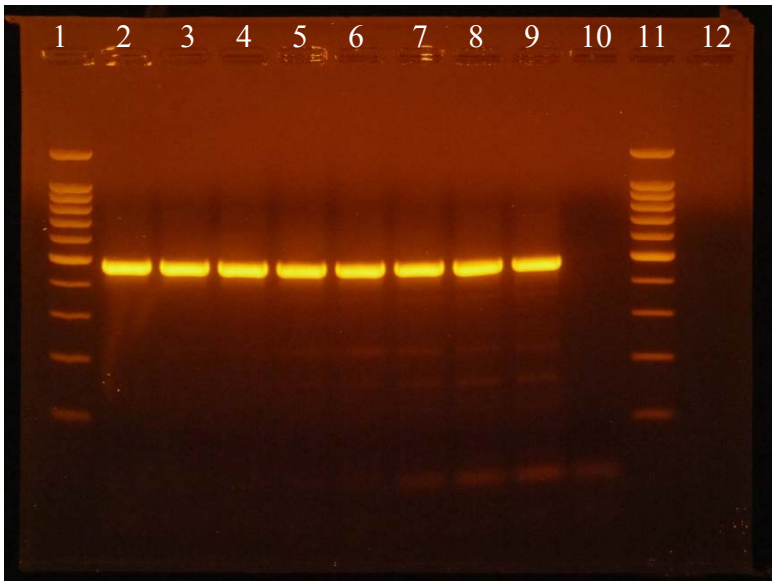


Figure 11: *Ehrlichia chaffeensis* primer set ECB/ECC temperature gradient 60-65°C. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) 100bp DNA ladder, 2) 65°C, 3) 64.7°C, 4) 64.1°C, 5) 63.1°C, 6) 62°C, 7) 61.2°C, 8) 60.5°C, 9) 60°C, 10) negative at 62°C, 11) 100bp DNA ladder, 12) blank.

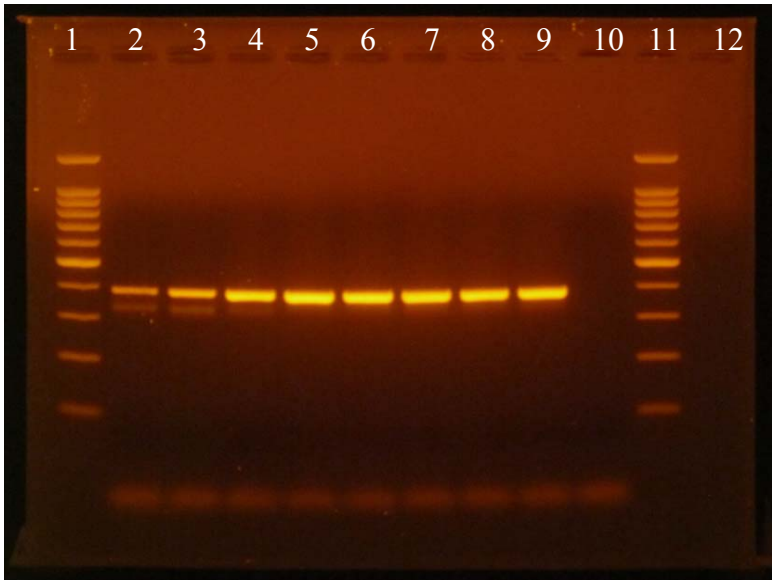


Figure 12: *Ehrlichia chaffeensis* primer set HE1/HE3 temperature gradient 50-65°C. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) 100bp DNA ladder, 2) 65°C, 3) 63.9°C, 4) 62.1°C, 5) 59.4°C, 6) 55.9°C, 7) 53.4°C, 8) 51.4°C, 9) 50°C, 10) negative at 55.9°C, 11) 100bp DNA ladder, 12) blank.

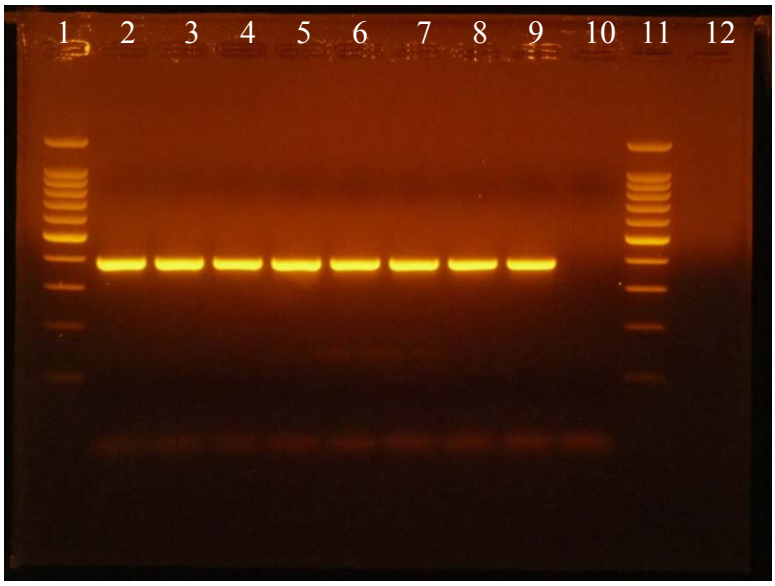


Figure 13: *Ehrlichia chaffeensis* primer set HE1/HE3 temperature gradient 50-60°C. 2% agarose gel run for 1hour 10min at 100V.Lanes: 1) 100bp DNA ladder, 2) 60°C, 3) 59.3°C, 4) 58.1°C, 5) 56.3°C, 6) 54°C, 7) 52.3°C, 8) 50.9°C, 9) 50°C, 10) negative at 54°C, 11) 100bp DNA ladder, 12) blank.

Sequencing and Cloning

Sequences from *Ehrlichia chaffeensis* with both primer sets, ECB/ECC and HE1/HE3, were obtained to verify that the positive control was *E. chaffeensis* and that the primer set was amplifying the correct gene. All sequences were verified with the online tool BLAST for nucleotides (NCBI, Bethesda, MD). Both the forward and reverse sequences of both ECB/ECC and HE1/HE3 matched *Ehrlichia chaffeensis* strain Arkansas 16S ribosomal RNA gene, complete sequence (Accession number: NR_074500.1) with an E-value of 0.0 (Table 3). Many other *Ehrlichia* species were matched as well, but all were for the 16S rRNA gene or complete genome.

The PCR products that provided the ECB/ECC sequences in Table 3 were used for cloning. After colonies were picked and mini cultures grown, 2µl of each mini culture

was added to 20µl nuclease free water. These were then incubated at 95°C for 5 min. The resulting products were used directly in PCR reactions. The gel run of the PCR products showed the quality of the colony (Figure 14). Although the two colonies appeared very differently on the gel, both were chosen to grow into cultures for plasmid preps. They were both sequenced and based on the sequences, lane 3 (colony 223) was chosen for use as the positive control (Figure 14). The plasmid DNA sequences obtained for that colony were matched to the ECB/ECC sequences using the program ClustalW/X (Science Foundation Ireland, Dublin 2, Ireland). They aligned with the ECB/ECC sequences below. Only the PCR products from ECB/ECC were used for the positive control storage. The primer set HE1/HE3 can be used with the plasmid DNA obtained from the ECB/ECC PCR product.

Table 3: *Ehrlichia chaffeensis* forward and reverse sequences of DNA from primer sets ECC/ECB and HE1/HE3. Forward and reverse sequences from both ECC/ECB and HE1/HE3 matched *Ehrlichia chaffeensis* strain Arkansas 16S ribosomal RNA gene, complete sequence (Accession number: NR_074500.1) with E-value of 0.0 using BLAST for nucleotides (NCBI).

Primer	Sequence (5' → 3')	BLAST Match and E-value
ECC	CTGAGTTTATTGCTAAGAACCTTATCGCTAGTGAC GGAAATTGCTTATACCTTTTGGTTAAAATAATTGTT AGTGGCAGACGGGTGAGTAATGCGTAGGAATCTAC CTAGTAGTATGGAATAGCCATTAGAAATGATGGGT AATACTGTATAATCCCTGCGGGGGAAAGATTTATC GCTATTAGATGAGCCTACGTTAGATTAGCTAGTTG GTAAGGTAATGGCTTACCAAGGCTATGATCTATAG CTGGTCTGAGAGGACGATCAGCCACACTGGAACTG AGATACGGTCCAGACTCCTACGGGAGGCAGCAGTG GGGAATATTGGACAATGGGCGAAAGCCTGATCCA GCTATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTG TAAAACTCTTTCAATAGGGAAGATAATGACGGTAC CTATAGAAGAAGTCCCGGCAAACTCTGTGCCAGCA GCCGCGGTAATACGA	*Ehrlichia chaffeensis* strain Arkansas 16S ribosomal RNA gene, complete sequence (Accession number: NR_074500.1) E-value: 0.0

ECB	ATTGGGAAGATTTCGGGAATCTTCTTTAGGTACCG TCATTATCTTCCCTATTGAAAGAGTTTTACAACCCG AAGGCCTTCTTCACTCACGCGGCATAGCTGGATCA GGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGC CTCCCGTAGGAGTCTGGACCGTATCTCAGTTCCAG TGTGGCTGATCGTCCTCTCAGACCAGCTATAGATC ATAGCCTTGGTAAGCCATTACCTTACCAACTAGCT AATCTAACGTAGGCTCATCTAATAGCGATAAATCT TTCCCCCGCAGGGATTATACAGTATTACCCATCATT TCTAATGGCTATTCCATACTACTAGGTAGATTCCTA CGCATTACTCACCCGTCTGCCACTAACAATTATTTA TAACCAAAAGGTTATAAGCAATTGTCCGTTCGACT TGCATGTGTTAGGCTTGCCGCCA	*Ehrlichia chaffeensis* strain Arkansas 16S ribosomal RNA gene, complete sequence (Accession number: NR_074500.1) E-value: 0.0
HE1	AGCCCGTTATTTGTTGGCCGACGGGTGAGTATGCG TAGGATCTACCTAGTAGTATGGAATAGCCATTAGA AATGATGGGTAATACTGTATAATCCCTGCGGGGGA AAGATTTATCGCTATTAGATGAGCCTACGTTAGAT TAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCTA TGATCTATAGCTGGTCTGAGAGGACGATCAGCCAC ACTGGAACTGAGATACGGTCCAGACTCCTACGGGA GGCAGCAGTGGGGAATATTGGACAATGGGCGAAA GCCTGATCCAGCTATGCCGCGTGAGTGAAGAAGGC CTTCGGGTTGTAAAACTCTTTCAATAGGGAAGATA ATGACGGTACCTATAGAAGAAGTCCCGGCAAACTC TGTGCCAGCAGCCGCGGTAATACGA	*Ehrlichia chaffeensis* strain Arkansas 16S ribosomal RNA gene, complete sequence (Accession number: NR_074500.1) E-value: 0.0
HE3	CTTGTGGGTTTTTCACCCGAGGCCTTCTTCACTCAC GCGGCATAGCTGGATCAGGCTTTCGCCCATTGTCC AATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGG ACCGTATCTCAGTTCCAGTGTGGCTGATCGTCCTCT CAGACCAGCTATAGATCATAGCCTTGGTAAGCCAT TACCTTACCAACTAGCTAATCTAACGTAGGCTCAT CTAATAGCGATAAATCTTTCCCCCGCAGGGATTAT ACAGTATTACCCATCATTTCTAATGGCTATTCCATA CTACTAGGTAGATTCCTACGCATTACTCACCCGTCT GCCACTAACAATTATTTATAACCAAAAGGTTATAA GCAATTGTCCGTTCGACTTGCATGTGTTAGGCTTGC CGCCAGCGTTCGTTCAAA	

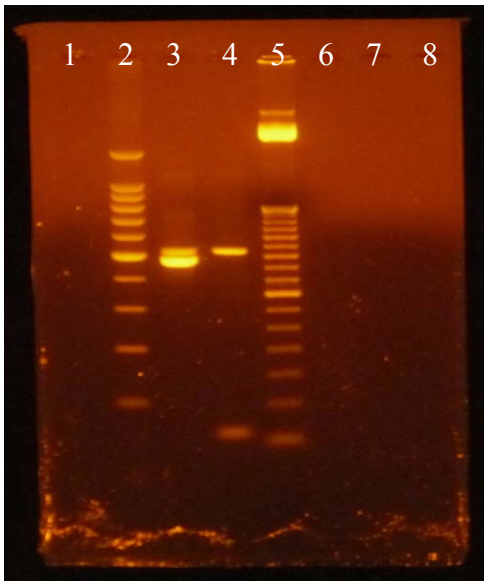


Figure 14: *Ehrlichia chaffeensis* clone quality check. 2% agarose gel run for 1hour at 100V. Both lanes chosen for culture growth and plasmid prep, but lane 3 was chosen based on sequence data. Lanes: 1) blank, 2) 100bp DNA ladder, 3) colony 223, 4) colony 224, 5) 50bp DNA ladder, 6-8) blank.

Storage

Cultures were grown from the *E. chaffeensis* colony 223 for glycerol stocks. The glycerol stocks were 15% glycerol and 85% culture. Each individual cryovial was labeled: *E. chaffeensis* 15% glycerol cell culture 01/23/2014. Seventy-two cyrovials were placed in a freezer box labeled: *Ehrlichia chaffeensis* 15% glycerol cell culture 01/23/2014. The plasmid DNA was diluted to 1pg/µl and aliquoted into 81 tubes, each labeled: *E. chaffeensis* Plasmid DNA 1pg/µl 0.1X TE buffer. They were placed in a freezer box labeled: *Ehrlichia chaffeensis* Plasmid DNA 1pg/µl at 50pg per tube 0.1X TE buffer 01/08/2014. Both the glycerol stocks and plasmid DNA were stored at -80°C for long term storage. They will remain with the freezer owned by the Tick Rearing Facility (Oklahoma State University).

Sensitivity and Specificity

The sensitivity of both primer sets, ECB/ECC and HE1/HE3, were tested with ten-fold dilutions using plasmid DNA. The plasmid DNA was gel purified before use for sensitivity. An initial dilution to 200,000copies/µl was performed. This was then diluted in a ten-fold dilution down to 2copies/µl. Only 0.5µl was used in PCR reactions to have a scale of 100,000copies/0.5µl down to 1copy/0.5µl. The primer set ECB/ECC was found to detect 100copies/0.5µl well, but also had a very faint band for 10copies/0.5µl (Figure 15). The primer set HE1/HE3 was tested from the PCR products obtained from testing ECB/ECC. It was found to detect down to 1copy/0.5µl (Figure 16). Without nesting, HE1/HE3 was only able to detect down to 100copies/0.5µl (Figure 17).

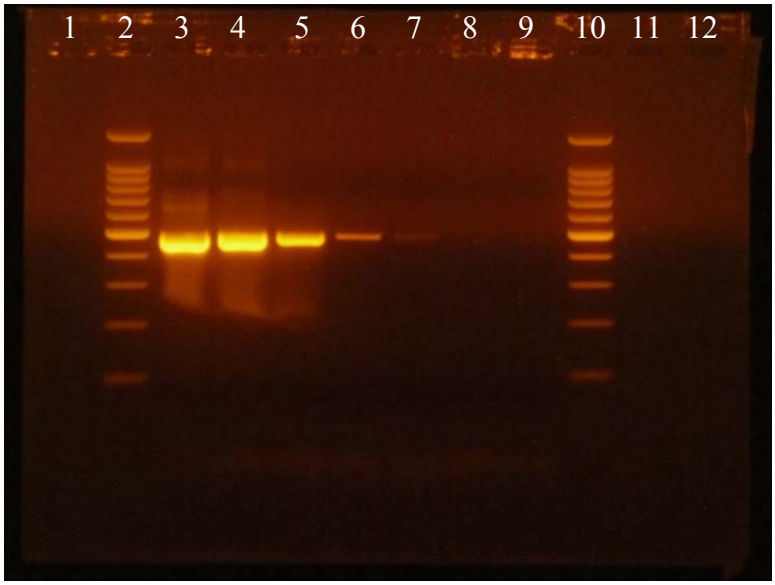


Figure 15: *Ehrlichia chaffeensis* plasmid DNA sensitivity test 100,000copies/0.5µl to 1copy/0.5µl with ECB/ECC. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) 100,000copies/0.5µl, 4) 10,000copies/0.5µl, 5) 1,000copies/0.5µl, 6) 100copies/0.5µl, 7) 10copies/0.5µl, 8) 1 copy/0.5µl, 9) negative, 10) 100bp DNA ladder, 11 and 12) blank.

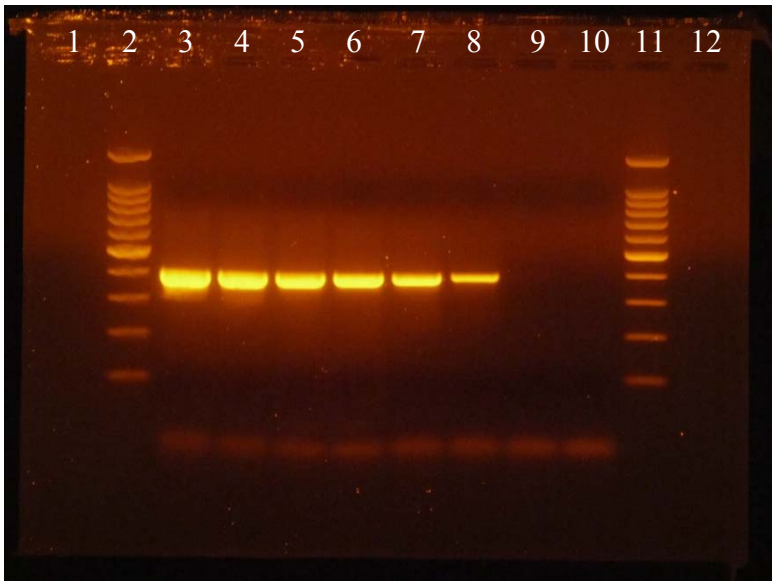


Figure 16: *Ehrlichia chaffeensis* plasmid DNA sensitivity testing 100,000copies/0.5µl to 1copy/0.5µl with HE1/HE3 nested. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) 100,000copies/0.5µl, 4) 10,000copies/0.5µl, 5) 1,000copies/0.5µl, 6) 100copies/0.5µl, 7) 10copies/0.5µl, 8) 1 copy/0.5µl, 9) nested negative, 10) negative, 11) 100bp DNA ladder, 12) blank.

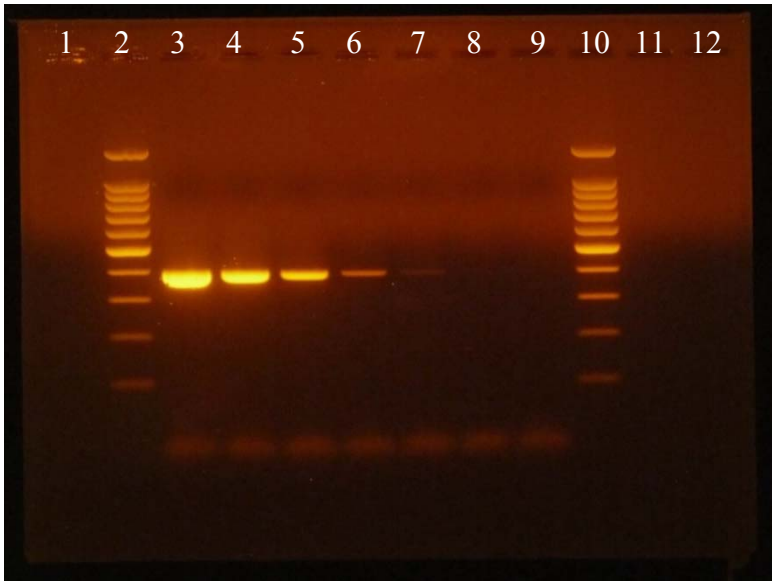


Figure 17: *Ehrlichia chaffeensis* plasmid DNA sensitivity testing 100,000copies/0.5µl to 1copy/0.5µl with HE1/HE3 not nested. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) 100,000 copies/0.5µl, 4) 10,000copies/0.5µl, 5) 1,000copies/0.5µl, 6) 100copies/0.5µl, 7) 10copies/0.5µl, 8) 1 copy/0.5µl, 9) negative, 10) 100bp DNA ladder, 11 and 12) blank.

To test specificity, both primer sets were tested with *Amblyomma americanum* nymph and adult, *Dermacentor variabilis* nymph and adult, *Ixodes scapularis* nymph and adult, *Anaplasma phagocytophilum*, *Anaplasma odocoilei*, *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, *Francisella tularensis*, and *Rickettsia rickettsii*. The primer set ECB/ECC was found to detect both *Anaplasma* species and *Ehrlichia chaffeensis* and had some non-specific bands with the ticks (Figure 18). The primer set HE1/HE3 was found to only detect *E. chaffeensis* (Figure 19).

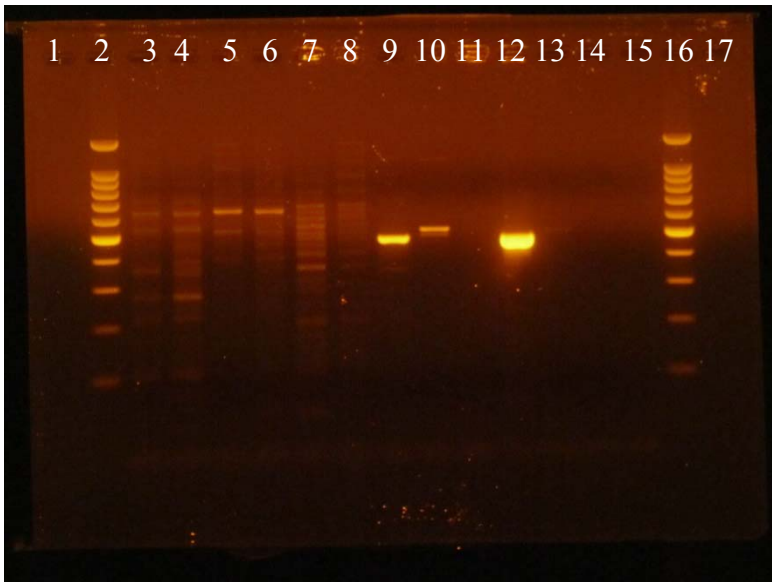


Figure 18: Specificity of primer set ECB/ECC. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) *A. americanum* adult, 4) *A. americanum* nymph, 5) *D. variabilis* adult, 6) *D. variabilis* nymph, 7) *I. scapularis* adult, 8) *I. scapularis* nymph, 9) *A. phagocytophilum*, 10) *A. odocoilei*, 11) *B. burgdorferi*, 12) *E. chaffeensis*, 13) *F. tularensis*, 14) *R. rickettsii*, 15) negative, 16) 100bp DNA ladder, 17) blank.

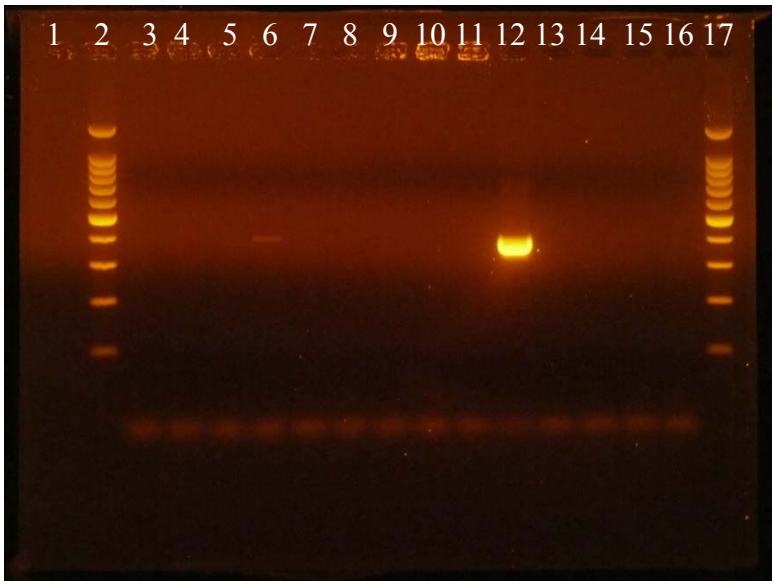


Figure 19: Specificity of primer set HE1/HE3 nested. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) *A. americanum* adult, 4) *A. americanum* nymph, 5) *D. variabilis* adult, 6) *D. variabilis* nymph, 7) *I. scapularis* adult, 8) *I. scapularis* nymph, 9) *A. phagocytophilum*, 10) *A. odocoilei*, 11) *B. burgdorferi*, 12) *E. chaffeensis*, 13) *F. tularensis*, 14) *R. rickettsii*, 15) negative, 16) 100bp DNA ladder, 17) blank.

BORRELIA BURGDORFERI

PCR Assay Development

The primer set, ospA2/ospA4, was found to be successful in amplifying *Borrelia burgdorferi*. It was not tested with other *Borrelia* species. Three different annealing temperatures, 45°C, 50°C, and 55°C, were tested for this primer set (Figure 20). The following thermal cycler program was found to be successful, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 55°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The resulting DNA fragment size was 156bp long.

Figure 20: *Borrelia burgdorferi* with primer set ospA2/ospA4. 2% agarose gel run for 1hour at 100V. Different annealing temperatures. Lanes: 1) blank, 2) 100bp DNA ladder, 3) 45°C, 4) negative 45°C, 5) 50°C, 6) negative 50°C, 7) 55°C, 8) negative 55°C, 9-15) other primer set testing not in association with ospA2/ospA4. 16 and 17) blank.

Sequencing and Cloning

Sequences from *Borrelia burgdorferi* from primer set, ospA2/ospA4, were obtained to verify that the positive control was *B. burgdorferi* and that the primer set was amplifying the correct gene. The online tool BLAST for nucleotides (NCBI, Bethesda, MD) was used to verify all sequences. Both the forward and reverse sequences of ospA2/ospA4 matched *Borrelia burgdorferi* isolate BbC65 outer surface protein A gene, complete cds (Accession number: KJ830728.1) with E-values of $1e^{-53}$ (forward) and $1e^{-48}$ (reverse) (Table 4). Other matches were found, but all were for *B. burgdorferi* outer surface protein or complete genome.

The PCR products that provided the ospA2/ospA4 sequences in Table 4 were used for cloning. After colonies were picked and mini cultures grown, 2µl of each mini

culture was added to 20µl nuclease free water. These were then incubated at 95°C for 5 min. The resulting products were used directly in PCR reactions. The gel run of the PCR products showed the quality of the colony (Figure 21). All the colonies appeared to be of similar quality. Lanes 3 (colony 121) and 8 (colony 142) were chosen to grow cultures for plasmid preps. Both were sent for sequences and lane 8 (colony 142) was chosen for positive control storage. The plasmid DNA sequence obtained for that colony was matched to the ospA2/ospA4 sequences using the program ClustalW/X (Science Foundation Ireland, Dublin 2, Ireland). They aligned with the ospA2/ospA4 sequences below.

Table 4: *Borrelia burgdorferi* forward and reverse sequences of DNA from primer set ospA2/ospA4. Both sequences matched *Borrelia burgdorferi* isolate BbC65 outer surface protein A gene, complete cds (Accession number: KJ830728.1) with E-values of $1e^{-53}$ (forward) and $1e^{-48}$ (reverse) using BLAST for nucleotides (NCBI).

Primer	Sequence (5' → 3')	BLAST Match and E-value	
ospA2	ACAGATGGGTGCATTTGAGTCGTATT GTTGTACTGTAATTGTGTTTTCTTTTGT AAACACAAGGTCTTTAGTTTTTTTACT GTTTACAGTAATTGTTAAAGTTGAAGT GCCTGAATTCCAAGCTGCAGAGGAGC	E-value: $1e^{-53}$	*Borrelia burgdorferi* isolate BbC65 outer surface protein A gene, complete cds (Accession number: KJ830728.1)
ospA4	TCCTTTACTTACTGTAACAGTAAAAAC TAAAGACCTTGTGTTTACAAAAGAAA ACACAATTACAGTACAACAATACGAC TCAAATGGCACCAAATTAGAGGGGTC AGCAGTTGAAATTACAAAACAACCGT	E-Value: $1e^{-48}$	

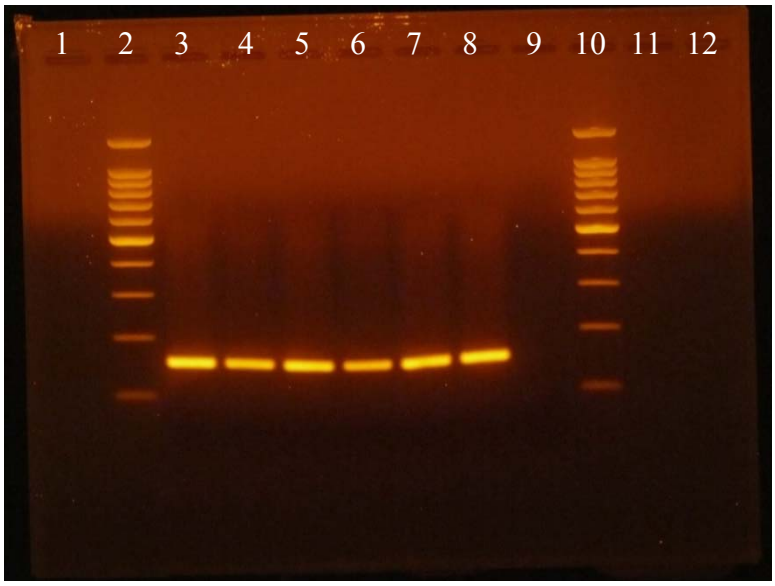


Figure 21: *Borrelia burgdorferi* clone quality check. 2% agarose gel run for 1hour at 100V. Lanes 3 and 8 chose for plasmid prep, lane 8 chosen for culture growth. Lanes: 1) blank, 2) 100bp DNA ladder, 3) colony 121, 4) colony 122, 5) colony 131, 6) colony 132, 7) colony 141, 8) colony 142, 9) negative, 10) 100bp DNA ladder, 11 and 12) blank.

Storage

Cultures were grown from the *B. burgdorferi* colony 142 for glycerol stocks. The glycerol stocks were 15% glycerol and 85% culture. Each individual cryovial was labeled: *B. burgdorferi* 15% glycerol cell culture 04/11/2015. Seventy-two cyrovials were placed in a freezer box labeled: *Borrelia burgdorferi* 15% glycerol cell culture 04/11/2015. The plasmid DNA was diluted to 1pg/µl and aliquoted into 81 tubes, each labeled: *B. burgdorferi* Plasmid DNA 1pg/µl ospA2/4 0.1X TE buffer. They were placed in a freezer box labeled: *Borrelia burgdorferi* Plasmid DNA 1pg/µl at 50pg per tube 09/03/2014 ospA2/4. Both the glycerol stocks and plasmid DNA were stored at -80°C for long term storage. They will remain with the freezer owned by the Tick Rearing Facility (Oklahoma State University).

Sensitivity and Specificity

The sensitivity of the primer set, ospA2/ospA4, was tested with a ten-fold dilution using plasmid DNA. The plasmid DNA was gel purified before use for sensitivity. An initial dilution to 200,000 /µl was performed. This was then diluted in a ten-fold dilution down to 2copies/µl. Only 0.5µl was used in PCR reactions to have a scale of 100,000copies/0.5µl down to 1copy/0.5µl. The primer set ospA2/ospA4 was found to detect 100copies/0.5µl well (Figure 22).

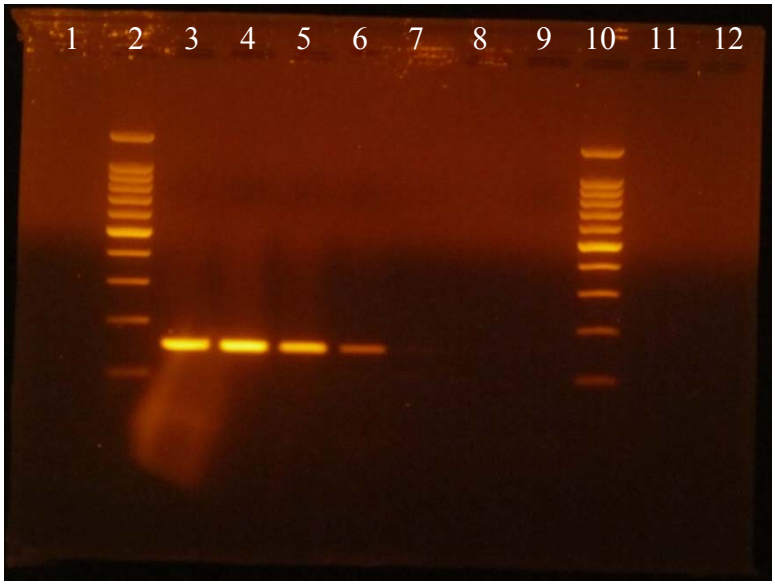


Figure 22: *Borrelia burgdorferi* plasmid DNA sensitivity testing 100,000copies/0.5µl to 1copy/0.5µl with ospA2/ospA4. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) 100,000 copies/0.5µl, 4) 10,000copies/0.5µl, 5) 1,000copies/0.5µl, 6) 100copies/0.5µl, 7) 10copies/0.5µl, 8) 1 copy/0.5µl, 9) negative, 10) 100bp DNA ladder, 11 and 12) blank.

To test specificity, the primer set was tested with *Amblyomma americanum* nymph and adult, *Dermacentor variabilis* nymph and adult, *Ixodes scapularis* nymph and adult, *Anaplasma phagocytophilum*, *Anaplasma odocoilei*, *Borrelia burgdorferi*,

Ehrlichia chaffeensis, *Francisella tularensis*, and *Rickettsia rickettsii*. It was found to detect only *Borrelia burgdorferi* with some slight non-specific banding in *D. variabilis* and *I. scapularis* (Figure 23).

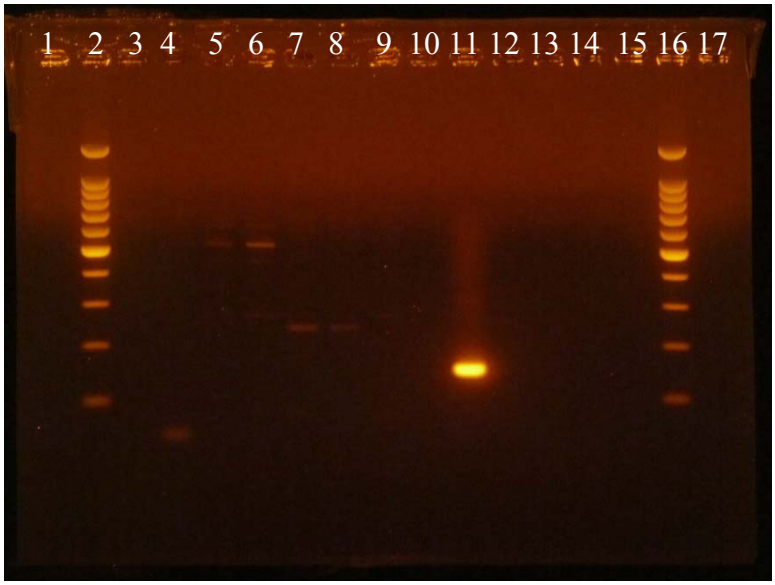


Figure 23: Specificity of primer set ospA2/ospA4. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) *A. americanum* adult, 4) *A. americanum* nymph, 5) *D. variabilis* adult, 6) *D. variabilis* nymph, 7) *I. scapularis* adult, 8) *I. scapularis* nymph, 9) *A. phagocytophilum*, 10) *A. odocoilei*, 11) *B. burgdorferi*, 12) *E. chaffeensis*, 13) *F. tularensis*, 14) *R. rickettsii*, 15) negative, 16) 100bp DNA ladder, 17) blank.

ANAPLASMA PHAGOCYTOPHILUM

PCR Assay Development

The nested primer sets, GE3a/GE10R followed by GE9F/GE2, were found to be successful in amplifying *Anaplasma phagocytophilum* (Figure 24). The following thermal cycler program was used for GE3a/GE10R, initial denaturation at 95°C for 5min continued with 40 cycles of 95°C (denaturing) for 30sec, 55°C (annealing) for 30sec, and

72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The following thermal cycler program was used for GE9F/2, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 65°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The final DNA fragment length was 546bp long.

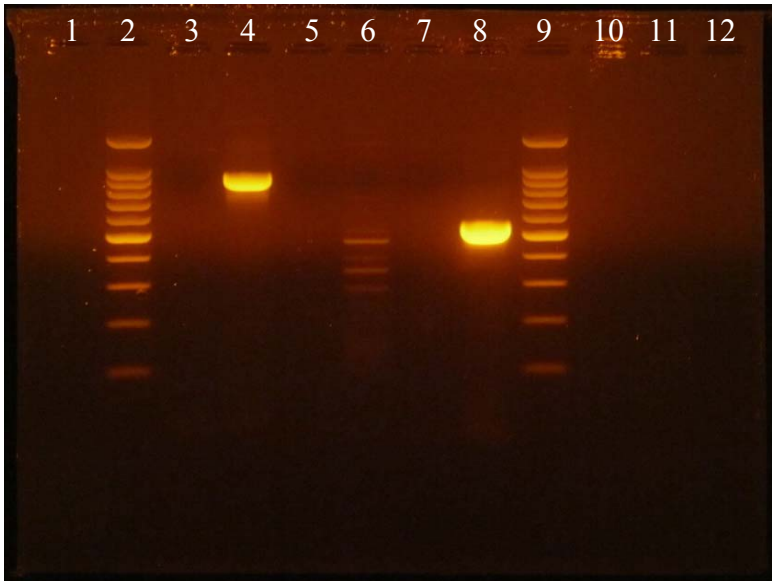


Figure 24: *Anaplasma phagocytophilum* with primer sets GE3a/GE10R and GE9F/GE2. 2% agarose gel run for 1hour 10min at 100V. Positive primer test with *A. phagocytophilum*. Lanes: 1) blank, 2) 100bp DNA ladder, 3) GE3a/GE10R negative, 4) GE3a/GE10R positive , 5) GE9F/GE2 not nested negative, 6) GE9F/GE2 not nested positive, 7) GE9F/GE2 nested negative, 8) GE9F/GE2 nested positive, 9)100bp DNA ladder, 10-12) blank.

Sequencing and Cloning

Sequences from *Anaplasma phagocytophilum* with both primer sets, GE3a/GE10R and GE9F/GE2, were obtained to verify that the positive control was *A. phagocytophilum* and that the primer set was amplifying the correct gene. All sequences

were verified with the online tool BLAST for nucleotides (NCBI, Bethesda, MD). Both the forward and reverse sequences of GE3a/GE10 matched *Anaplasma phagocytophilum* strain ApMuc02c 16S ribosomal RNA gene, partial sequence (Accession number: JX73652.1) with E-value of 0.0 (Table 5). Many other *Anaplasma phagocytophilum* and a few *Ehrlichia* species were matched as well, but all were for the 16S rRNA gene or complete genome. Both the forward and reverse sequences for GE9F/GE2 matched *Anaplasma phagocytophilum* strain ApMuc02c 16S ribosomal RNA gene, partial sequence (Accession number: JX73652.1) with E-value of 0.0 as well (Table 5).

The PCR products that provided both the GE3a/GE10R andGE9F/GE2 sequences in Table 5 were used for cloning. After colonies were picked and mini cultures grown, 2µl of each mini culture was added to 20µl nuclease free water. These were then incubated at 95°C for 5 min. The resulting products were used directly in PCR reactions. The gel run of the PCR products showed the quality of the colonies (Figures 25 and 26). All the colonies appeared to be of similar quality. From Figure 25, lanes 3 (colony 121) and 8 (colony 142) and from Figure 26, lanes 3 (colony 121) and 5 (colony 131) were chosen to grow cultures for plasmid preps. All four plasmid preps were sent for sequencing. The plasmid DNA sequences obtained for colonies 142 (GE3a/GE10R) and 121 (GE9F/GE2) were matched to the GE3a/GE10R and GE9F/GE2 sequences using the program ClustalW/X (Science Foundation Ireland, Dublin 2, Ireland). Both sets of sequences aligned with the sequences below. Colonies 142 (GE3a/GE10R) and 121 (GE9F/GE2) were chosen for positive control storage.

Table 5: *Anaplasma phagocytophilum* forward and reverse sequences of DNA from primer sets GE3a/GE10R and GE9F/GE2. Forward and reverse sequences from both GE3a/GE10R and GE9F/GE2 matched *Anaplasma phagocytophilum* strain ApMuc02c 16S ribosomal RNA gene, partial sequence (Accession number: JX73652.1) with E-value of 0.0 using BLAST for nucleotides (NCBI).

Primer	Sequence (5' → 3')	BLAST Match and E-value
GE3a	TAAGGCCTTGCATAAGATAGTTAGTGGCAGACGGGTGAGTA ATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGA AATGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGATT TATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTTGGTA GGGTAAAGGCCTACCAAGGCGATGATCTATAGCTGGTCTGA GAGGATGATCAGCCACACTGGAACTGAGATACGGTCCAGAC TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCG CAAGCCTGATCCAGCTATGCCGCGTGAGTGAGGAAGGCCTT AGGGTTGTAAAACTCTTTCAGTAGGGAAGATAATGACGGTA CCTACAGAAGAAGTCCCGGCAAACTCCGTGCCAGCAGCCGC GGTAATACGGAGGGGGCAAGCGTTGTTCGGAATTATTGGGC GTAAAGGGCATGTAGGCGGTTCGGTAAGTTAAAGGTGAAAT GCCAGGGCTTAACCCTGGAGCTGCTTTTAATACTGCCAGACT AGAGTCCGGGAGAGGATAGCGGAATTCCTAGTGTAGAGGTG AAATTCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGG CTATCTGGTCCGGTACTGACGCTGAGGTGCGAAAGCGTGGG GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAA CGATGAGTGCTGAATGTGGGGATTTTTTATCTCTGTGTTGTA GCTAACGCGTTAAGCACTCCGCCTGGGGACTACGGTCGCAA GACTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGG TGGAGCATGTGGTTTAATTCGATGCAACGCGAAAAACCTTA CCACTCCTTGACATGGAGATTAGATCCTTCTTTACGAAAAAG GT	*Anaplasma phagocytophilum* strain ApMuc02c 16S ribosomal RNA gene, partial sequence (Accession number: JX73652.1) E-value: 0.0
GE10R	CAGTTCCGCAGTGCTAGGTTTTTCGCGTTGCATCGAATTAAA CCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTT GAGTTTTAGTCTTGCGACCGTAGTCCCCAGGCGGAGTGCTTA ACGCGTTAGCTACAACACAGAGATAAAAAATCCCCACATTC AGCACTCATCGTTTACAGCGTGGACTACCAGGGTATCTAATC CTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTACCGG ACCAGATAGCCGCCTTCGCCACTGGTGTTCCTCCTAATATCT ACGAATTTCACCTCTACACTAGGAATTCCGCTATCCTCTCCC GGACTCTAGTCTGGCAGTATTAAAAGCAGCTCCAGGGTTAA GCCCTGGCATTTCACCTTTAACTTACCGAACCGCCTACATGC CCTTTACGCCCAATAATTCCGAACAACGCTTGCCCCCTCCGT ATTACCGCGGCTGCTGGCACGGAGTTTGCCGGGACTTCTTCT GTAGGTACCGTCATTATCTTCCCTACTGAAAGAGTTTTACAA CCCTAAGGCCTTCCTCACTCACGCGGCATAGCTGGATCAGGC TTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAG GAGTCTGGACCGTATCTCAGTTCCAGTGTGGCTGATCATCCT CTCAGACCAGCTATAGATCATCGCCTTGGTAGGCCTTTACCC TACCAACTAGCTAATCTAACATAGGCTCATCTAATAGCGATA AATCTTTCCCCCGCAGGGATTATACAGTATTACCCACCATTT CTAGTGGCTATCCCATACTACTAGGTAGATTCCTATGCATTA CTCACCCGTCTGCCACTAACTATTCTTTATAGCAAGCTATAA AGAATAATCCGTTCGCTTTGGCATGGTGA	
GE9F	AACTGGATTGCTATATGACAGACGGGTGAGTATGCATAGGA	*Anaplasma*

	ATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGT AATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTA GATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAAAGGC CTACCAAGGCGATGATCTATAGCTGGTCTGAGAGGATGATC AGCCACACTGGAACTGAGATACGGTCCAGACTCCTACGGGA GGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT CCAGCTATGCCGCGTGAGTGAGGAAGGCCTTAGGGTTGTAA AACTCTTTCAGTAGGGAAGATAATGACGGTACCTACAGAAG AAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGG AGGGGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCA TGTAGGCGGTTCGGTAAGTTAAAGGTGAAATGCCAGGGCTT AACCCTGGAGCTGCTTTTAATACTGCCAAA	*phagocytophilum* strain ApMuc02c 16S ribosomal RNA gene, partial sequence (Accession number: JX73652.1) E-value: 0.0
GE2	GCGGTCCAGGCATTTCCCTTTACTTACCGAACCGCCTACATG CCCTTTACGCCCAATAATTCCGAACAACGCTTGCCCCCTCCG TATTACCGCGGCTGCTGGCACGGAGTTTGCCGGGACTTCTTC TGTAGGTACCGTCATTATCTTCCCTACTGAAAGAGTTTTACA ACCCTAAGGCCTTCCTCACTCACGCGGCATAGCTGGATCAGG CTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAG GAGTCTGGACCGTATCTCAGTTCCAGTGTGGCTGATCATCCT CTCAGACCAGCTATAGATCATCGCCTTGGTAGGCCTTTACCC TACCAACTAGCTAATCTAACATAGGCTCATCTAATAGCGATA AATCTTTCCCCCGCAGGGATTATACAGTATTACCCACCATTT CTAGTGGCTATCCCATACTACTAGGTAGATTCCTATGCATTA CTCACCCGTCTGCCACTAACTATTCTTTATAGCAAGCTATAA AGATAATCCGTAGAAGACGGGTGAGTAATGCATAGGAATCT ACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATA CTGTATAATCCCCTGCGGGGGAAAGATTTATCGCTATTAGAT GAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAAAGGCCTA CCAAGGCGATGATCTATAGCTGGTCTGAGAGGATGATCAGC CACACTGGAACTGAGATACGGTCCAGACTCCTACGGGAGGC AGCAGTGGGGAATATTGGACAATGGGCGCAAGCCCTGATCC AGCTATGCCGCGTGAGTGAGGAAGGCCCTTAGGGTTGTAAA ACTCTTTCAGTAGGGAAGATAATGACGGTACCTACAGAAGA AGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGGA GGGGGCAAAGCGTTGTCGGAAATATTGGGCGTAAAGGGCAT GTAGGCGGTTCGGTAAGTAAAGGTGAAATGCCAGGGCTTAA CCTGGAGCTGCTTTTTATACCTGCCA	

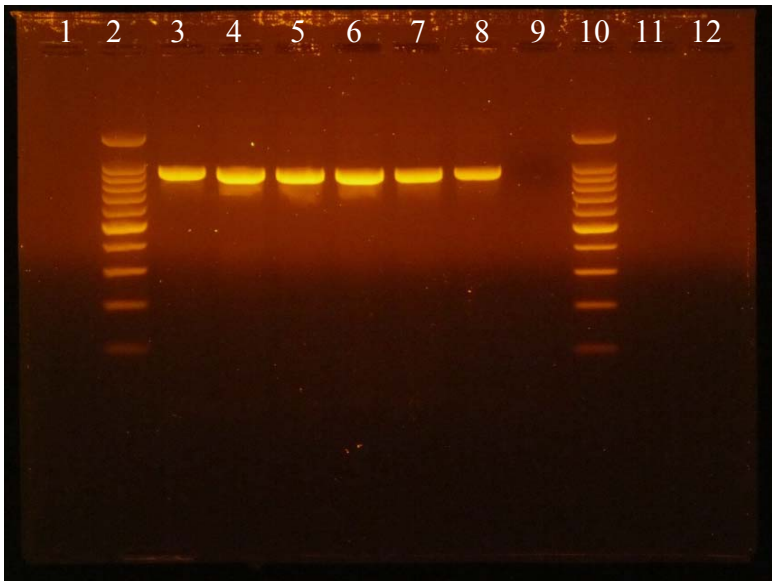


Figure 25: *Anaplasma phagocytophilum* clone quality check for primer set GE3a/GE10R. 2% agarose gel run for 1hour at 100V. Lanes 3 and 8 chosen for plasmid preps, lane 8 chosen for culture growth. Lanes: 1) blank, 2) 100bp DNA ladder, 3) colony 121, 4) colony 122, 5) colony 131, 6) colony 132, 7) colony 141, 8) colony 142, 9) negative, 10) 100bp DNA ladder, 11 and 12) blank.

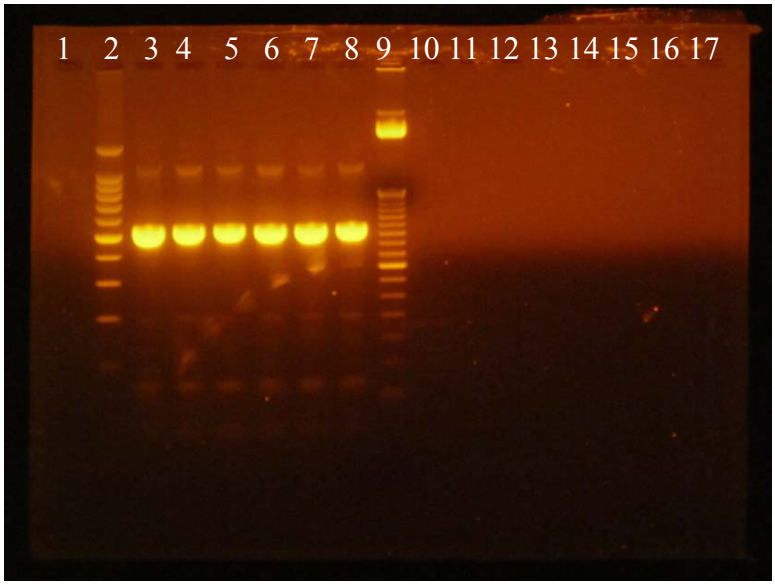


Figure 26: *Anaplasma phagocytophilum* clone quality check for primer set GE9F/GE2. 2% agarose gel run for 1hour at 100V. Lanes 3 and 5 chosen for plasmid prep, lane 3 chosen for culture growth. Lanes: 1) blank, 2) 100bp DNA ladder, 3) colony 121, 4) colony 122, 5) colony 131, 6) colony 132, 7) colony 141, 8) colony 142, 9) 50bp DNA ladder, 10 through 17) blank.

Storage

Cultures were grown from *A. phagocytophilum* colony 142 (GE3a/GE10) and 121 (GE9F/GE2) for glycerol stocks. The glycerol stocks were 15% glycerol and 85% culture. Each individual cryovial for colony 121(GE9F/GE2) was labeled: *A. phago.* 15% glycerol cell culture 05/13/2014. Seventy-two cyrovials were placed in a freezer box labeled: *Anaplasma phagocytophilum* 15% glycerol cell culture 05/13/2014. The plasmid DNA was diluted to 1pg/µl and aliquoted into 81 tubes, each labeled: *A. phago.* Plasmid DNA 1pg/µl 0.1X TE buffer. They were placed in a freezer box labeled: *Anaplasma phagocytophilum* plasmid DNA 1pg/µl at 50pg per tube 0.1X TE buffer 01/08/2014. For colony 142 (GE3a/GE10), each cryovial was labeled: *A. phago.* 15% glycerol cell culture 04/11/2015. Seventy-two cryovials were placed in a freezer box labeled: *Anaplasma phagocytophilum* 15% glycerol cell culture 04/11/2015. The plasmid DNA was diluted to 1pg/µl and aliquoted into 81 tubes, each labeled: *A. phago.* Plasmid DNA 1pg/µl GE3a/10 0.1X TE buffer. They were placed in a freezer box labeled: *Anaplasma phagocytophilum* Plasmid DNA 1pg/µl at 50pg per tube 09/12/2014 GE3a/10. Both the glycerol stocks and plasmid DNA were stored at -80°C for long term storage. They will remain with the freezer owned by the Tick Rearing Facility (Oklahoma State University).

Sensitivity and Specificity

The sensitivity of both primer sets, GE3a/GE10R and GE9F/GE2, were tested with ten-fold dilutions using plasmid DNA. The plasmid DNA was gel purified before use for sensitivity. An initial dilution to 200,000copies/µl was performed. This was then diluted in a ten-fold dilution down to 2copies/µl. Only 0.5µl was used in PCR reactions

to have a scale of 100,000copies/0.5µl down to 1copy/0.5µl. The primer set GE3a/GE10R was found to detect 10copies/0.5µl well (Figure 27). The primer set GE9F/GE2 was tested from the PCR products obtained from testing GE3a/GE10R. It was found to detect down to 10copies/0.5µl (Figure 28). Without nesting, GE9F/GE2 was only able to detect down to 10,000copies/0.5µl (Figure 29).

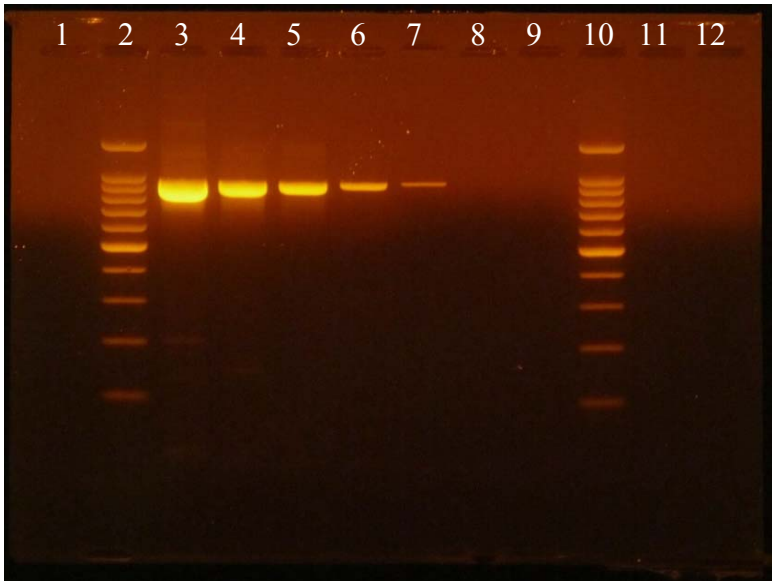


Figure 27: *Anaplasma phagocytophilum* plasmid DNA sensitivity testing 100,000copies/0.5µl to 1copy/0.5µl with GE3a/GE10R. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) 100,000 copies/0.5µl, 4) 10,000copies/0.5µl, 5) 1,000copies/0.5µl, 6) 100copies/0.5µl, 7) 10copies/0.5µl, 8) 1 copy/0.5µl, 9) negative, 10) 100bp DNA ladder, 11 and 12) blank.

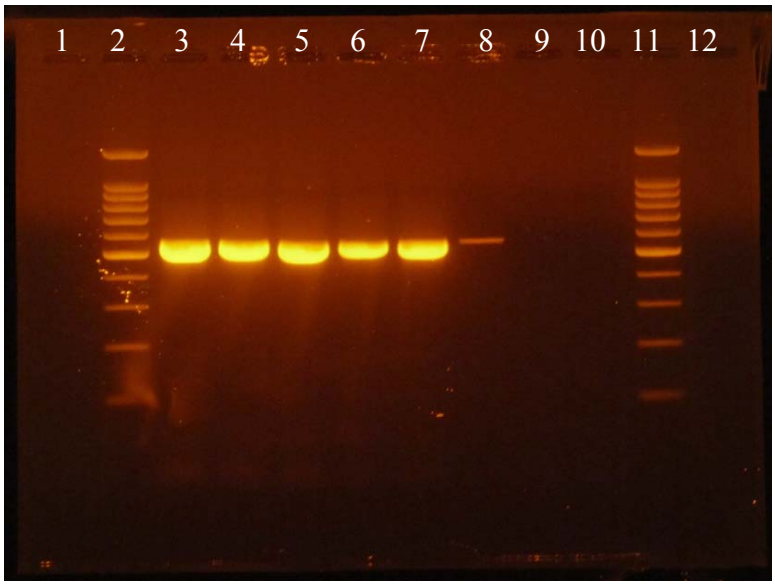


Figure 28: *Anaplasma phagocytophilum* plasmid DNA sensitivity testing 100,000copies/0.5µl to 1copy/0.5µl with GE9F/GE2 nested. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) 100,000 copies/0.5µl, 4) 10,000copies/0.5µl, 5) 1,000copies/0.5µl, 6) 100copies/0.5µl, 7) 10copies/0.5µl, 8) 1 copy/0.5µl, 9) nested negative, 10) negative, 11) 100bp DNA ladder, 12) blank.

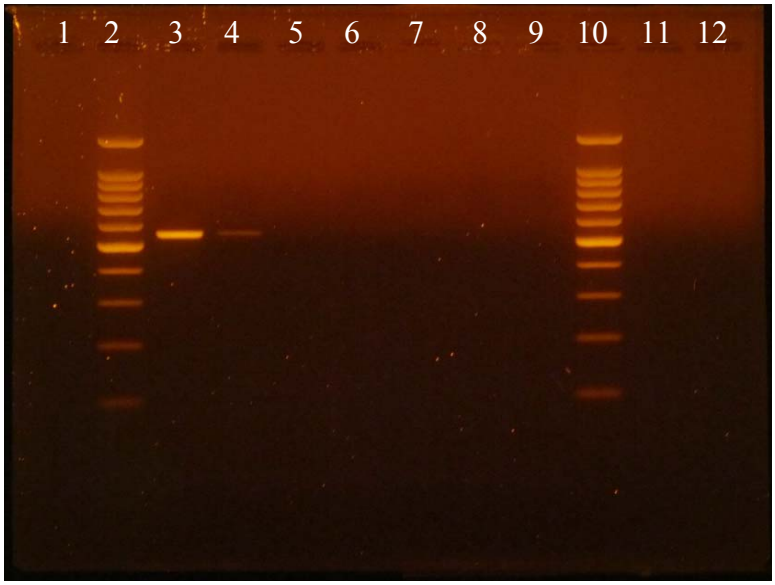


Figure 29: *Anaplasma phagocytophilum* plasmid DNA sensitivity testing 100,000copies/0.5µl to 1copy/0.5µl with GE9F/GE2 not nested. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) 100,000 copies/0.5µl, 4) 10,000copies/0.5µl, 5) 1,000copies/0.5µl, 6) 100copies/0.5µl, 7) 10copies/0.5µl, 8) 1 copy/0.5µl, 9) negative, 10) 100bp DNA ladder, 11 and 12) blank.

To test specificity, both primer sets were tested with *Amblyomma americanum* nymph and adult, *Dermacentor variabilis* nymph and adult, *Ixodes scapularis* nymph and adult, *Anaplasma phagocytophilum*, *Anaplasma odocoilei*, *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, *Francisella tularensis*, and *Rickettsia rickettsii*. The primer set GE3a/GE10R was found to detect *Anaplasma phagocytophilum* and had some non-specific bands with a few of the ticks (Figure 30). The primer set GE9F/GE2 was found to only detect *A. phagocytophilum* (Figure 31).

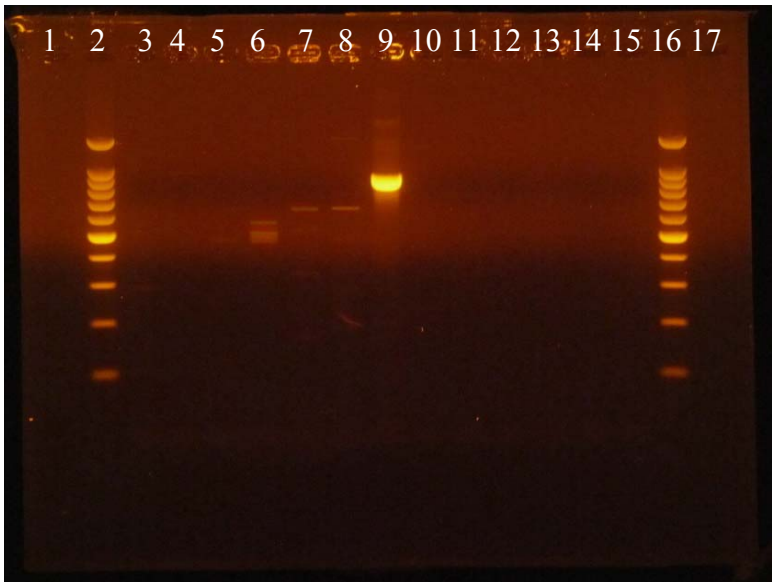


Figure 30: Specificity of primer set GE3a/GE10R. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) *A. americanum* adult, 4) *A. americanum* nymph, 5) *D. variabilis* adult, 6) *D. variabilis* nymph, 7) *I. scapularis* adult, 8) *I. scapularis* nymph, 9) *A. phagocytophilum*, 10) *A. odocoilei*, 11) *B. burgdorferi*, 12) *E. chaffeensis*, 13) *F. tularensis*, 14) *R. rickettsii*, 15) negative, 16) 100bp DNA ladder, 17) blank.

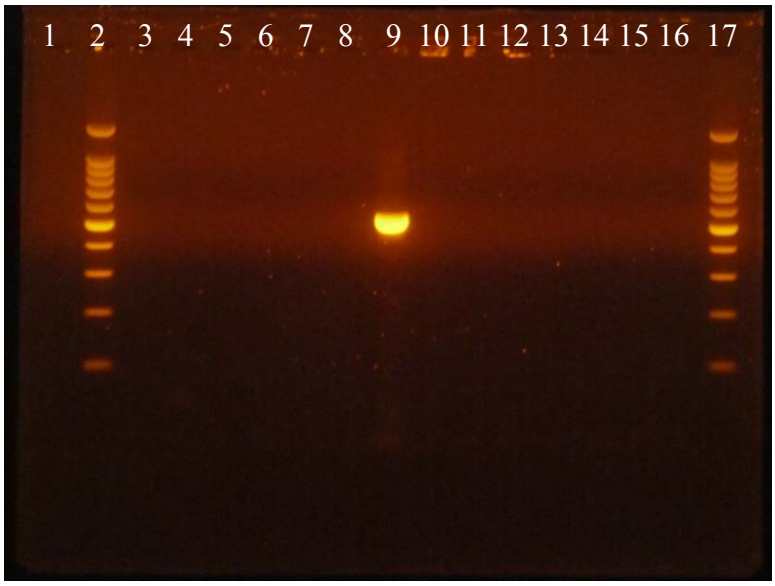


Figure 31: Specificity of primer set GE9F/GE2 nested. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) *A. americanum* adult, 4) *A. americanum* nymph, 5) *D. variabilis* adult, 6) *D. variabilis* nymph, 7) *I. scapularis* adult, 8) *I. scapularis* nymph, 9) *A. phagocytophilum*, 10) *A. odocoilei*, 11) *B. burgdorferi*, 12) *E. chaffeensis*, 13) *F. tularensis*, 14) *R. rickettsii*, 15) negative, 16) 100bp DNA ladder, 17) blank.

RICKETTSIA RICKETTSII

PCR Assay Development

The primer set, TZ15/TZ16, was found to be successful in amplifying *Rickettsia* species (spotted fever group). Two different annealing temperatures, 47°C and 55°C, were tested for this primer set (Figure 32). The following thermal cycler program was found to be successful, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C (denaturing) for 1min, 55°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The resulting DNA fragment size was 230bp long.

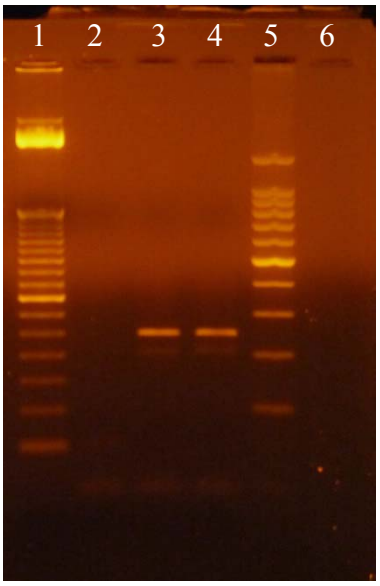


Figure 32: *Rickettsia rickettsii* with primer set TZ15/TZ16. 2% agarose gel run for 1hour at 100V. Different annealing temperatures. Lanes: 1) 50bp DNA ladder, 2) negative, 3) 55°C, 4) 47°C, 5) 100bp DNA ladder, 6) blank

Sequencing and Cloning

Sequences for *Rickettsia rickettsii* with primer set, TZ15/TZ16, were obtained to verify that the positive control was *Rickettsia rickettsii* and that the primer set was amplifying the correct gene. All sequences were verified with the online tool BLAST for nucleotides (NCBI, Bethesda, MD). Both the forward and reverse sequences of TZ15/TZ6 matched *Rickettsia rickettsii* isolate Colatina1 17kDa surface protein gene, partial cds (Accession number: KC845924.1) with E-values of $3e^{-102}$ (forward) and $4e^{-100}$ (reverse) (Table 6). Many other *Rickettsia* species were matched as well, but all were for the 17kDa gene or complete genome.

The PCR product that provided the sequences in Table 6 was used for cloning. After colonies were picked and mini cultures grown, 2µl of each mini culture was added to 20µl nuclease free water. These were then incubated at 95°C for 5 min. The resulting

products were used directly in PCR reactions. The gel run of the PCR products showed the quality of the colonies (Figures 33). All the colonies appeared to be of similar quality. From Figure 33, lanes 3 (colony 121), 6 (colony 131), and 9 (colony 141) were chosen to grow cultures for plasmid preps. All three plasmid preps were sent for sequencing. The plasmid DNA sequences obtained were matched to the TZ15/TZ16 sequences using the program ClustalW/X (Science Foundation Ireland, Dublin 2, Ireland). All sequences aligned with the sequences below. Colony 121 was chosen for positive control storage.

Table 6: *Rickettsia* species forward and reverse sequences of DNA from primer set TZ15/TZ16. Both sequences matched *Rickettsia rickettsii* isolate Colatina1 17kDa surface protein gene, partial cds (Accession number: KC845924.1) with E-values of $3e^{-102}$ (forward) and $4e^{-100}$ (reverse) using BLAST for nucleotides (NCBI).

Primer	Sequence (5' → 3')	BLAST Match and E-value	
TZ15	AACAGGATTTAAATGGCATAGGTGTA GGGTGCATTACTTGGAGCAGTTCTTG GTGGACAAATCGGTGCAGGTATGGAT GAACAGGATAGAAGACTTGCAGAGCT TACCTCACAGAGAGCTTTAGAAACAG CTCCTAGTGGTAGTAACGTAGAATGG CGTAATCCGGATAACGGCAATTACGG TTACGTAACACCTAATAAAACTTATA GAAATAGCACTGGTCAATATATA	E-value: $3e^{-102}$	*Rickettsia rickettsii* isolate Colatina1 17kDa surface protein gene, partial cds (Accession number: KC845924.1)
TZ16	AAGTTGGGTTAGATGGGGTTAAGCTA CCGTAATTGCCGTTATCCGGATTACGC CATTCTACGTTACTACCACTAGGAGCT GTTTCTAAAGCTCTCTGTGAGGTAAGC TCTGCAAGTCTTCTATCCTGTTCATCC ATACCTGCACCGATTTGTCCACCAAG AACTGCTCCAAGTAATGCACCTACAC CTACTCCAACAAGCTGTCCTTTGCCCT TACCGAATTGAGAAATA	E-value: $4e^{-100}$	

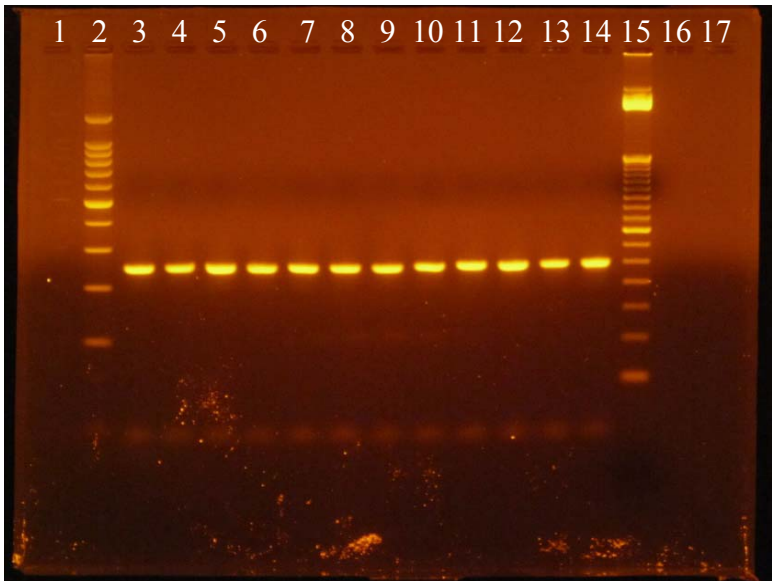


Figure 33: *Rickettsia rickettsii* clone quality check for primer set TZ15/TZ16. 2% agarose gel run for 1hour at 100V. Lanes 3, 6, and 9 chosen for plasmid preps, lane 8 chosen for culture growth. Lanes: 1) blank, 2) 100bp DNA ladder, 3) colony 121, 4) colony 122, 5) colony 123, 6) colony 131, 7) colony 132, 8) colony 133, 9) colony 141, 10) colony 142, 11) colony 143, 12) colony 151, 13) colony 152, 14) colony 153, 15) 50bp DNA ladder, 16 and 17) blank.

Storage

Cultures were grown from *Rickettsia rickettsii* colony 121 for glycerol stocks. The glycerol stocks were 15% glycerol and 85% culture. Each individual cryovial was labeled: *Rickettsia* sp. 15% glycerol cell culture 01/30/2014. Seventy-two cyrovials were placed in a freezer box labeled: *Rickettsia* sp. 15% glycerol cell culture 01/30/2014. The plasmid DNA was diluted to 1pg/µl and aliquoted into 81 tubes, each labeled: *Rickettsia* sp. Plasmid DNA 1pg/µl 0.1X TE buffer. They were placed in a freezer box labeled: *Rickettsia* sp. Plasmid DNA 1pg/µl at 50pg per tube 0.1X TE buffer 01/10/2014. Both the glycerol stocks and plasmid DNA were stored at -80°C for long term storage. They will remain with the freezer owned by the Tick Rearing Facility (Oklahoma State University). The positive controls were made from *Rickettsia rickettsii* but were labeled as *Rickettsia*

sp. due to the primer set being non-specific and any positive band found would be the same size as the positive control.

Sensitivity and Specificity

The sensitivity of the primer set, TZ15/TZ16, was tested with a ten-fold dilution using plasmid DNA. The plasmid DNA was gel purified before use for sensitivity. An initial dilution to 200,000copies/µl was performed. This was then diluted in a ten-fold dilution down to 2copies/µl. Only 0.5µl was used in PCR reactions to have a scale of 100,000copies/0.5µl down to 1copy/0.5µl. The primer set TZ15/TZ16 was found to detect 100copies/0.5µl well and showed light banding at 10copies/0.5µl (Figure 34).

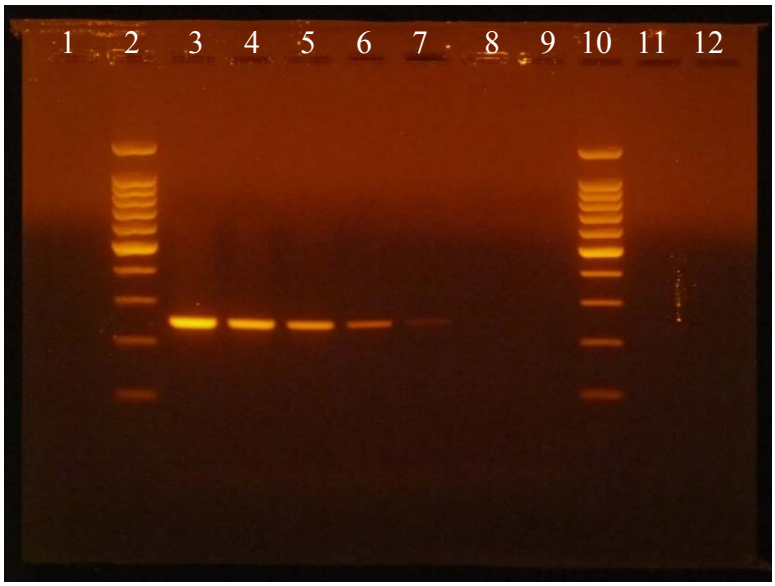


Figure 34: *Rickettsia rickettsii* plasmid DNA sensitivity testing 100,000copies/0.5µl to 1copy/0.5µl with ospA2/ospA4. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) 100,000 copies/0.5µl, 4) 10,000copies/0.5µl, 5) 1,000copies/0.5µl, 6) 100copies/0.5µl, 7) 10copies/0.5µl, 8) 1 copy/0.5µl, 9) negative, 10) 100bp DNA ladder, 11 and 12) blank.

To test specificity, the primer set was tested with *Amblyomma americanum* nymph and adult, *Dermacentor variabilis* nymph and adult, *Ixodes scapularis* nymph and adult, *Anaplasma phagocytophilum*, *Anaplasma odocoilei*, *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, *Francisella tularensis*, and *Rickettsia rickettsii*. It was found to detect only *Rickettsia rickettsii* (Figure 35).

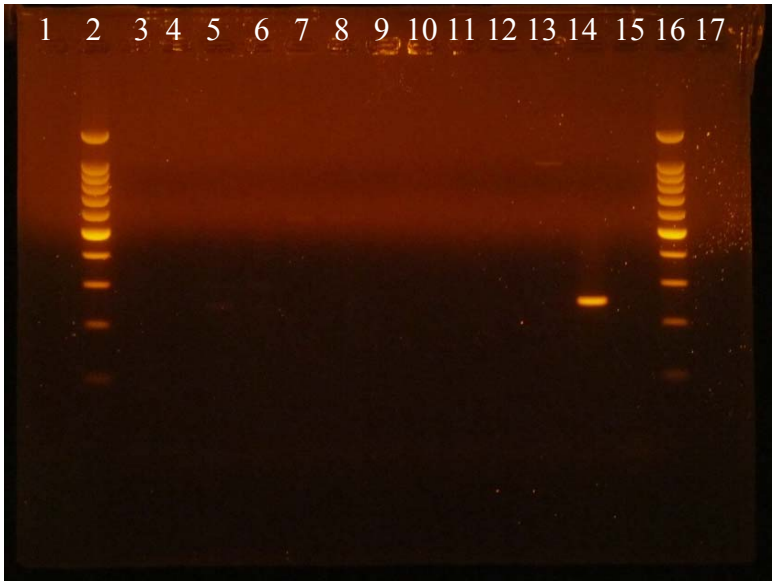


Figure 35: Specificity of primer set TZ15/TZ16. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) *A. americanum* adult, 4) *A. americanum* nymph, 5) *D. variabilis* adult, 6) *D. variabilis* nymph, 7) *I. scapularis* adult, 8) *I. scapularis* nymph, 9) *A. phagocytophilum*, 10) *A. odocoilei*, 11) *B. burgdorferi*, 12) *E. chaffeensis*, 13) *F. tularensis*, 14) *R. rickettsii*, 15) negative, 16) 100bp DNA ladder, 17) blank.

FRANCISELLA TULARENSIS

PCR Assay Development

The primer set, ISFtu2F/ISFtu2R, was found to be successful in amplifying *Francisella tularensis* (Figure 36). The following thermal cycler program was found to be successful, initial denaturation at 95°C for 5min continued with 30 cycles of 94°C

(denaturing) for 1min, 55°C (annealing) for 1min, and 72°C (extension) for 1min followed by a final extension at 72°C for 5min and ending with a hold at 4°C. The resulting DNA fragment size was 97bp long.

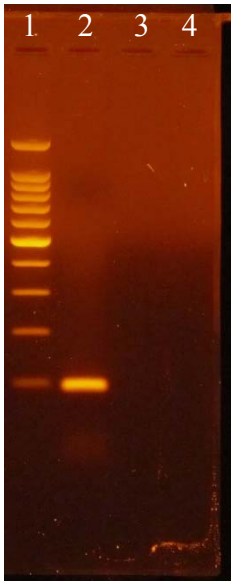


Figure 36: *Francisella tularensis* with primer set TZ15/TZ16. 2% agarose gel run for 1hour at 100V. Lanes: 1) 100bp DNA ladder, 2) *F. tularensis*, 3) negative, 4) blank.

Sequencing and Cloning

Sequences for *Francisella tularensis* with primer set, ISFtu2F/ISFtu2R, were obtained to verify that the positive control was *Francisella tularensis* and that the primer set was amplifying the correct gene. All sequences were verified with the online tool BLAST for nucleotides (NCBI, Bethesda, MD). Both the forward and reverse sequences of ISFtu2F/ISFtu2R matched *Francisella tularensis* subsp. novicida insertion sequence IstuF2 transposase gene, partial cds; O-antigen gene cluster, complete sequence; and transposase gene, complete cds (Accession number: EF059983.1) with E-values of $3e^{-21}$

(forward) and $3e^{-20}$ (reverse) (Table 7). Many other *Francisella tularensis* subspecies were matched as well, but all were for the ISFtu2 gene or complete genome.

The PCR product that provided the sequences in Table 7 was used for cloning. After colonies were picked and mini cultures grown, 2µl of each mini culture was added to 20µl nuclease free water. These were then incubated at 95°C for 5 min. The resulting products were used directly in PCR reactions. The gel run of the PCR products showed the quality of the colonies (Figures 37). All the colonies appeared to be of similar quality. From Figure 37, lanes 3 (colony 121) and 8 (colony 142) were chosen to grow cultures for plasmid preps. Both plasmid preps were sent for sequencing. The plasmid DNA sequences obtained were matched to the ISFtu2F/ISFtu2R sequences using the program ClustalW/X (Science Foundation Ireland, Dublin 2, Ireland). All sequences aligned with the sequences below. Colony 121 was chosen for positive control storage.

Table 7: *Francisella tularensis* forward and reverse sequences of DNA from primer set ISFtu2F/ISFtu2R. Both sequences matched *Francisella tularensis* subsp. novicida insertion sequence IstuF2 transposase gene, partial cds; O-antigen gene cluster, complete sequence; and transposase gene, complete cds (Accession number: EF059983.1) with E-values of $3e^{-21}$ (forward) and $3e^{-20}$ (reverse) using BLAST for nucleotides (NCBI).

Primer	Sequence (5'→ 3')	BLAST Match and E-value	
ISFtu2F	CCATTAATCATGCTTGACTGATGCT TTAGGTAATCCAATAGAAATATTG TTGTCAGAAGGTAAAACTCA	E-value: $3e^{-21}$	*Francisella tularensis* subsp. novicida insertion sequence IstuF2 transposase gene, partial cds; O-antigen gene cluster, complete sequence; and transposase gene, complete cds (Accession number: EF059983.1)
ISFtu2R	AATTGCGATACTAAAGCATCAGTC ATAGCATGGATTTTAGTGGTTATC CCACCAACTGATCTACCAAA	E-value: $3e^{-20}$	

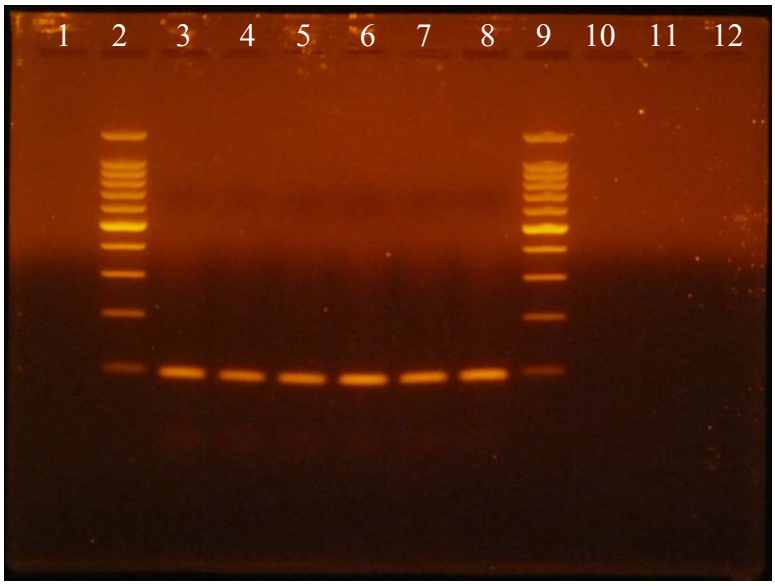


Figure 37: *Francisella tularensis* clone quality check for primer set ISFtu2F/ISFtu2R. 2% agarose gel run for 1hour at 100V. Lanes 3 and 8 chosen for plasmid preps, lane 8 chosen for culture growth. Lanes: 1) blank, 2) 100bp DNA ladder, 3) colony 121, 4) colony 122, 5) colony 131, 6) colony 132, 7) colony 141, 8) colony 142, 9) 100bp DNA ladder, 10-12) blank.

Storage

Cultures were grown from *Francisella tularensis* colony 121 for glycerol stocks. The glycerol stocks were 15% glycerol and 85% culture. Each individual cryovial was labeled: *F. tularensis* 15% glycerol cell culture 06/11/2014. Seventy-two cyrovials were placed in a freezer box labeled: *Francisella tularensis* 15% glycerol cell culture 06/11/2014. The plasmid DNA was diluted to 1pg/µl and aliquoted into 81 tubes, each labeled: *F. tularensis* Plasmid DNA 1pg/µl 0.1X TE buffer. They were placed in a freezer box labeled: *Francisella tularensis* Plasmid DNA 1pg/µl at 50pg per tube 0.1X TE buffer 06/04/2014. Both the glycerol stocks and plasmid DNA were stored at -80°C for long term storage. They will remain with the freezer owned by the Tick Rearing Facility (Oklahoma State University).

Sensitivity and Specificity

The sensitivity of the primer set ISFtu2F/ISFtu2R was tested with a ten-fold dilution using plasmid DNA. The plasmid DNA was gel purified before use for sensitivity. An initial dilution to 200,000copies/µl was performed. This was then diluted in a ten-fold dilution down to 2copies/µl. Only 0.5µl was used in PCR reactions to have a scale of 100,000copies/0.5µl down to 1copy/0.5µl. The primer set ISFtu2F/ISFtu2R was found to detect 1000copies/0.5µl well and showed light banding at 100copies/0.5µl (Figure 38).

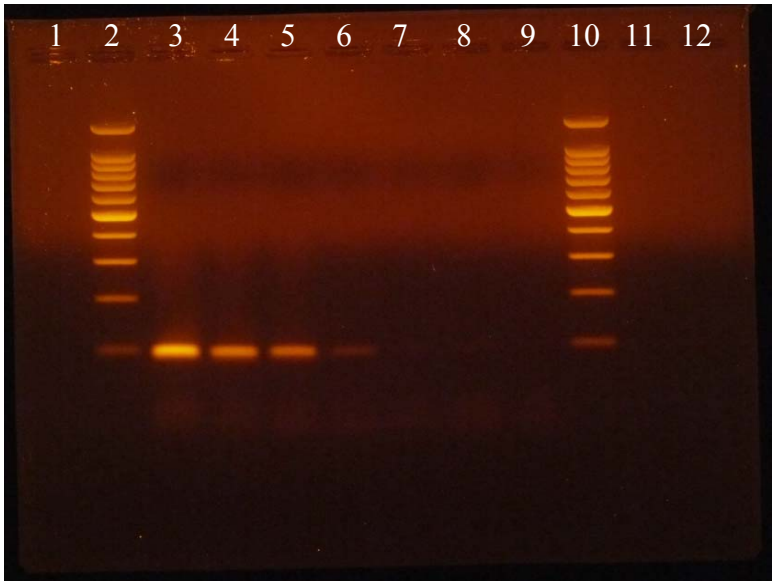


Figure 38: *Francisella tularensis* plasmid DNA sensitivity testing 100,000copies/0.5µl to 1copy/0.5µl with ISFtu2F/ISFtu2R. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) 100,000 copies/0.5µl, 4) 10,000copies/0.5µl, 5) 1,000copies/0.5µl, 6) 100copies/0.5µl, 7) 10copies/0.5µl, 8) 1 copy/0.5µl, 9) negative, 10) 100bp DNA ladder, 11 and 12) blank.

To test specificity, the primer set was tested with *Amblyomma americanum* nymph and adult, *Dermacentor variabilis* nymph and adult, *Ixodes scapularis* nymph and

adult, *Anaplasma phagocytophilum*, *Anaplasma odocoilei*, *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, *Francisella tularensis*, and *Rickettsia rickettsii*. It was found to detect only *Francisella tularensis* (Figure 39).

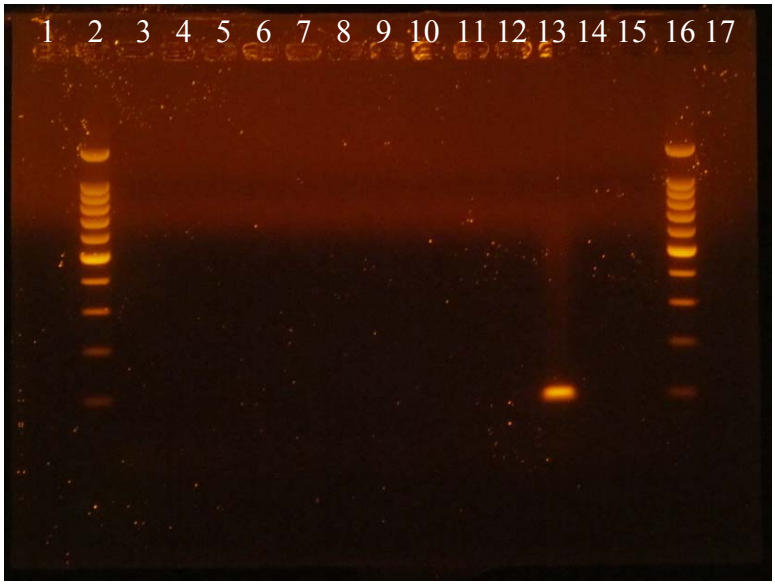


Figure 39: Specificity of primer set ISFtu2F/ISFtu2R. 2% agarose gel run for 1hour 10min at 100V. Lanes: 1) blank, 2) 100bp DNA ladder, 3) *A. americanum* adult, 4) *A. americanum* nymph, 5) *D. variabilis* adult, 6) *D. variabilis* nymph, 7) *I. scapularis* adult, 8) *I. scapularis* nymph, 9) *A. phagocytophilum*, 10) *A. odocoilei*, 11) *B. burgdorferi*, 12) *E. chaffeensis*, 13) *F. tularensis*, 14) *R. rickettsii*, 15) negative, 16) 100bp DNA ladder, 17) blank.

SCREENING

Tick DNA

Ticks from the Oklahoma State University Tick Rearing Facility and from a few Oklahoma State Parks were screened for pathogens. From the Tick Rearing Facility, adult and nymphs were tested from three species, *Amblyomma americanum*, *Ixodes scapularis*, and *Dermacentor variabilis* (Table 8). *Amblyomma americanum* (only *A. americanum* was collected) from three state parks, Greenleaf, Sequoyah, and Lake Thunderbird, were

screened (Table 9). Individual ticks were tested, no pooling was performed. All tick extractions were screened for the tick 16S rRNA gene with primer set TQ16S+1F/TQ16S-2R to insure that the extraction worked properly. All tick extractions performed tested positive. Figure 40 shows some of the tick extraction positive results. Each tick species was only screened for the most common pathogens found in that species. *Amblyomma americanum* was screened for *Ehrlichia chaffeensis*, *Francisella tularensis*, and *Rickettsia* species. *Ixodes scapularis* was screened for *Borrelia burgdorferi* and *Anaplasma phagocytophilum*. *Dermacentor variabilis* was screened for *Rickettsia* species and *Francisella tularensis*. See Tables in each pathogen section below for screening results.

Table 8: Tick groups from Oklahoma State University Tick Rearing Facility. Group numbers used for tables in pathogen screening sections below. A.a: *Amblyomma americanum*, D.v.: *Dermacentor variabilis*, I.s.: *Ixodes scapularis*, D.a.: *Dermacentor andersoni*, A.m.: *Amblyomma maculatum*, R.s.: *Rhiphicephalus sanguineus.*

Colony ticks			
Group number	**Species, life stage, & molt date**	**Group number**	**Species, life stage, & molt date**
1	A.a. nymphs 8/1/14	20	A.a. nymphs 9/21/14 wild
2	A.a. nymphs 8/15/14	21	A.a. adults 8/15/14
3	A.a. nymphs 8/29/14	22	A.a. adults 8/22/14
4	A.a. adults 7/25/14	23	A.a. adults 8/29/14
5	A.a. adults 8/1/14	24	D.v. adults 7/18/14
6	A.a. adults 8/8/14	25	D.v. adults 7/25/14
7	D.v. nymphs 7/11/14	26	D.v. adults 8/8/14
8	D.v. nymphs 8/8/14	27	I.s. nymphs 7/18/14
9	D.v. nymphs 9/5/14 wild mix	28	I.s. nymphs 7/25/14
10	D.v. adults 6/13/14	29	I.s. nymphs 8/1/14
11	D.v. adults 6/20/14	30	I.s. adults 6/20/14
12	D.v. adults 6/27/14	31	I.s. adults 6/27/14
13	I.s. nymphs 6/20/14	32	I.s. adults 7/4/14
14	I.s. nymphs 6/27/14	33	D.v. nymphs 4/18/14
15	I.s. nymphs 7/1/14	34	D.a. nymphs 1/28/14
16	I.s. adults 5/23/14	35	A.a. nymphs 6/6/14
17	I.s. adults 5/30/14	36	A.m. nymphs 4/26/14
18	I.s. adults 6/6/14	37	R.s. nymphs 4/29/14
19	A.a. nymphs 9/12/14 wild mix		

Table 9: Wild ticks collected from Oklahoma State Parks. Group numbers used for tables in pathogen screening sections below. All ticks collected were *Amblyomma americanum*.

Wild Ticks	
Group Number	**State Park & collection date**
W1	Greenleaf 7/3/14
W2	Greenleaf 6/3/14
W3	Greenleaf 5/19/14
W4	Sequoyah 7/3/14
W5	Sequoyah 6/4/14
W6	Sequoyah 5/19/14
W7	Lake Thunderbird 5/01/14
W8	Lake Thunderbird 6/30/14

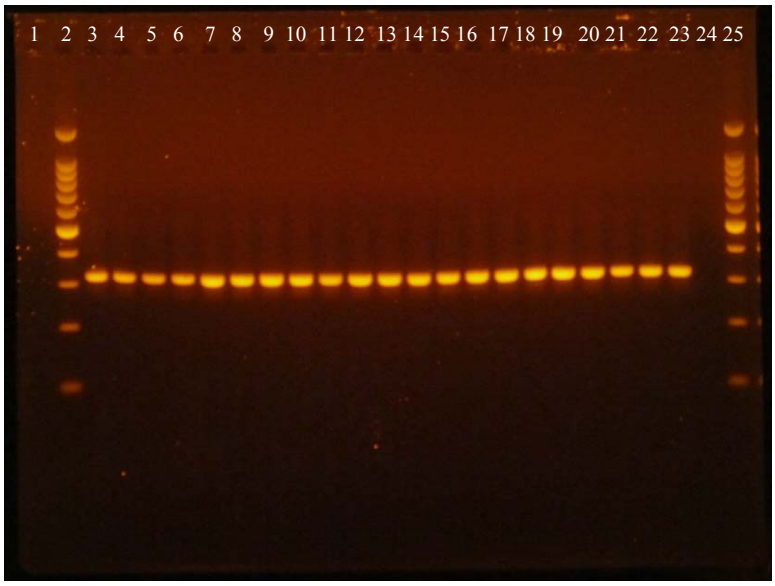


Figure 40: Tick 16S rRNA screening using primer set TQ16S+1F/TQ16S-2R. 2% agarose gel run at 1hour 10min at 100V. Each lane is an individual tick. Lanes: 1) blank, 2) 100bp DNA ladder, 3) group 2, 4) group 3, 5) group 19, 6) group 20, 7) group W2, 8-12) group W1, 13-17) group W3, 18-20) group W4, 21-23) group 35, 24) negative, 25) 100bp DNA ladder.

Ehrlichia chaffeensis

Amblyomma americanum was screened for *Ehrlichia chaffeensis* using nested primer sets ECB/ECC and HE1/HE3. Four ticks from each colony group and five from each wild group (Tables 8 and 9) were tested. Wild group 8 had only four ticks tested due to the small quantity collected. One wild tick was found positive for *E. chaffeensis* and all others were negative (Table 10). Figure 41 shows the one positive result and some of the negatives. The one positive sequence was verified with the online tool BLAST (NCBI) and matched *Ehrlichia chaffeensis* strain Arkansas 16S ribosomal RNA gene, complete sequence, Accession #: NR_074500.1.

Table 10: *Ehrlichia chaffeensis* screening of *A. americanum*. One positive found with BLAST match: *Ehrlichia chaffeensis* strain Arkansas 16S ribosomal RNA gene, complete sequence, Accession #: NR_074500.1. All others were negative.

Group Number	Number Positive	BLAST Match	Group Number	Number Positive	BLAST Match
1	0/4	n/a	23	0/4	n/a
2	0/4	n/a	35	0/4	n/a
3	0/4	n/a	W1	0/5	n/a
4	0/4	n/a	W2	1/5	*Ehrlichia chaffeensis* strain Arkansas 16S ribosomal RNA gene, complete sequence, Accession #: NR_074500.1
5	0/4	n/a	W3	0/5	n/a
6	0/4	n/a	W4	0/5	n/a
19	0/4	n/a	W5	0/5	n/a
20	0/4	n/a	W6	0/5	n/a
21	0/4	n/a	W7	0/5	n/a
22	0/4	n/a	W8	0/4	n/a

Figure 41: *Ehrlichia chaffeensis* screening of *A. americanum* using nested primer sets ECB/ECC and HE1/HE3. 2% agarose gel run for 1hour 10min at 100V. Each lane is an individual tick. Lanes: 1) blank, 2) 100bp DNA ladder, 3 and 4) group 22, 5-8) group 23, 9-12) group W2, 13) negative nested, 14) *E. chaffeensis* positive control nested, 15) negative not nested, 16) *E. chaffeensis* positive control not nested, 17) 100bp DNA ladder.

Borrelia burgdorferi

Ixodes scapularis was screened for *Borrelia burgdorferi* using primer set ospA2/ospA4. Four ticks from each group (Table 8 above) were tested. No positive results were found (Table 11). Figure 42 shows some of the negative results.

Table 11: *Borrelia burgdorferi* screening of *Ixodes scapularis*. No positives found.

Group Number	Number Positive	Group Number	Number Positive
13	0/4	27	0/4
14	0/4	28	0/4
15	0/4	29	0/4
16	0/4	30	0/4
17	0/4	31	0/4
18	0/4	32	0/4

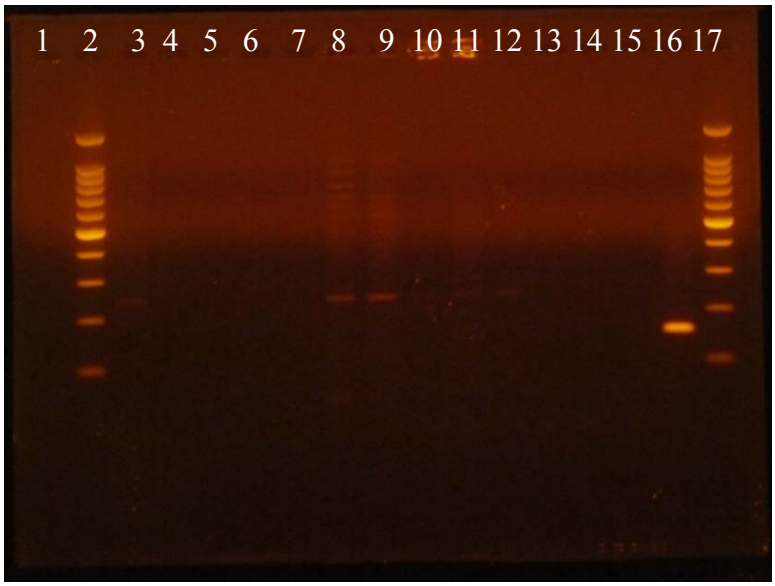


Figure 42: *Borrelia burgdorferi* screening of *I. scapularis* using primer set ospA2/ospA4. 2% agarose gel run for 1hour 10min at 100V. Each lane is an individual tick. Lanes: 1) blank, 2) 100bp DNA ladder, 3) group 13, 4-5) group 14, 6-7) group 15, 8) group 16, 9-10) group 17, 11-12) group 18, 13) group 27, 14) group 28, 15) negative, 16) *B. burgdorferi* positive control, 17) 100bp DNA ladder.

Anaplasma phagocytophilum

Ixodes scapularis was screened for *Anaplasma phagocytophilum* using nested primer sets GE3a/GE10R and GE9F/GE2. Four ticks from each group (Table 8 above) were tested. No positive results were found (Table 12). Figure 43 shows some of the negative results.

Table 12: *Anaplasma phagocytophilum* screening of *Ixodes scapularis*. No positives found.

Group Number	Number Positive	Group Number	Number Positive
13	0/4	27	0/4
14	0/4	28	0/4
15	0/4	29	0/4
16	0/4	30	0/4
17	0/4	31	0/4
18	0/4	32	0/4

Figure 43: *Anaplasma phagocytophilum* screening of *I. scapularis* using nested primer set GE9F/GE2. 2% agarose gel run for 1hour 10 min at 100V. Each lane is an individual tick. Lanes: 1) 100bp DNA ladder, 2-4) group 13, 5-8) group 14, 9-12) group 15, 13-14) group 16, 15) negative, 16) *A. phagocytophilum* positive control, 17) 100bp DNA ladder.

Rickettsia rickettsii

Amblyomma americanum and *Dermacentor variabilis* were screened for the presence of *Rickettsia rickettsii* using primer set TZ15/TZ16. Four ticks from each colony group and five from each wild group (Tables 8 and 9 above). Wild group 8 only had four tested due to the small quantity collected. A total of 37 out of 87 *A. americanum* had a positive result (Table 13). Figure 44 shows some of the positives and negatives. A total of 25 out of 40 *D. variabilis* had a positive result (Table 13). Figure 45 shows some of the positive and negatives. Using the online tool BLAST (NCBI) showed both *A. americanum* and *D. variabilis* were negative for *R. rickettsii*. However, *Amblyomma americanum* was positive for *R. amblyommii* (BLAST match: *Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1) and

Dermacentor variabilis was positive for *R. montanensis* (BLAST match: *Rickettsia montanensis* 17 kDa antigen gene, partial cds., Accession #: DQ402377.1). Although the latter sequences matched three other *Rickettsia* species equally, a search of the literature showed that only *R. montanensis* is found in the U.S. out of the four.

Table 13: *Rickettsia rickettsii* screening of *A. americanum* (groups 1-6, 19-23, 35, and W1-W8) and *D. variabilis* (groups 7-12, 24-26, and 33) using primer set TZ15/TZ16. All were negative for *R. rickettsii*. Thirty-seven *A. americanum* were positive for *Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1. Twenty-five *D. variabilis* were positive for *Rickettsia montanensis* 17 kDa antigen gene, partial cds., Accession #: DQ402377.1.

Group Number	Number Positive	BLAST match	Group Number	Number Positive	BLAST match
1	0/4	n/a	22	2/4	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1
2	0/4	n/a	23	3/4	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1
3	0/4	n/a	24	3/4	*Rickettsia montanensis* 17 kDa antigen gene, partial cds., Accession #: DQ402377.1
4	0/4	n/a	25	1/4	*Rickettsia montanensis* 17 kDa antigen gene, partial cds., Accession #: DQ402377.1
5	0/4	n/a	26	4/4	*Rickettsia montanensis* 17 kDa antigen gene, partial cds., Accession #: DQ402377.1
6	4/4	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1	33	4/4	*Rickettsia montanensis* 17 kDa antigen gene, partial cds., Accession #: DQ402377.1
7	4/4	*Rickettsia montanensis* 17 kDa antigen gene, partial cds., Accession #: DQ402377.1	35	4/4	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1

8	0/4	n/a	W1	2/5	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1
9	0/4	n/a	W2	4/5	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1
10	1/4	*Rickettsia montanensis* 17 kDa antigen gene, partial cds., Accession #: DQ402377.1	W3	4/5	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1
11	4/4	*Rickettsia montanensis* 17 kDa antigen gene, partial cds., Accession #: DQ402377.1	W4	3/5	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1
12	4/4	*Rickettsia montanensis* 17 kDa antigen gene, partial cds., Accession #: DQ402377.1	W5	3/5	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1
19	2/4	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1	W6	3/5	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1
20	0/4	n/a	W7	0/5	n/a
21	1/4	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1	W8	2/4	*Rickettsia amblyommii* isolate TX051 17 kDa antigen gene, partial cds., Accession #: EF689730.1

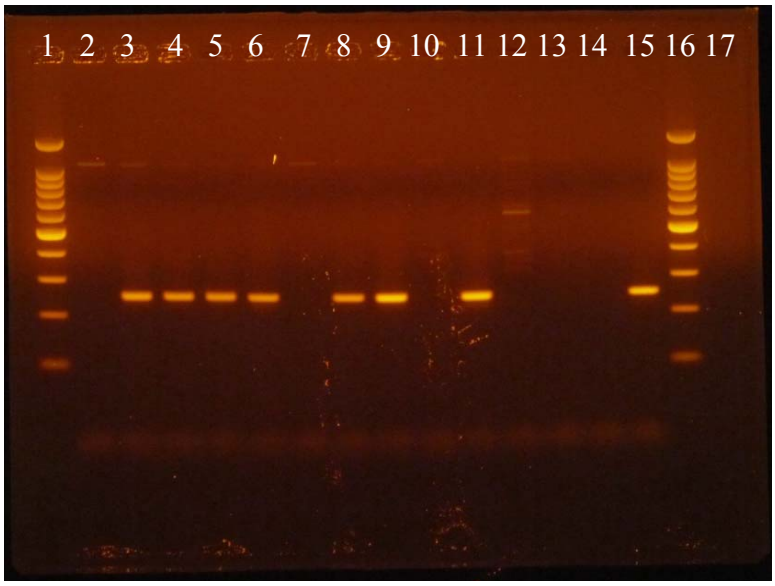


Figure 44: *Rickettsia rickettsii* screening of *A. americanum* using primer set TZ15/TZ16. 2% agarose gel run for 1hour 10min at 100V. Each lane is an individual tick. Lanes: 1) 100bp DNA ladder, 2) group W1, 3-7) group W3, 8-12) group W4, 13) group W6, 14) negative, 15) *R. rickettsii* positive control, 16) 100bp DNA ladder, 17) blank.

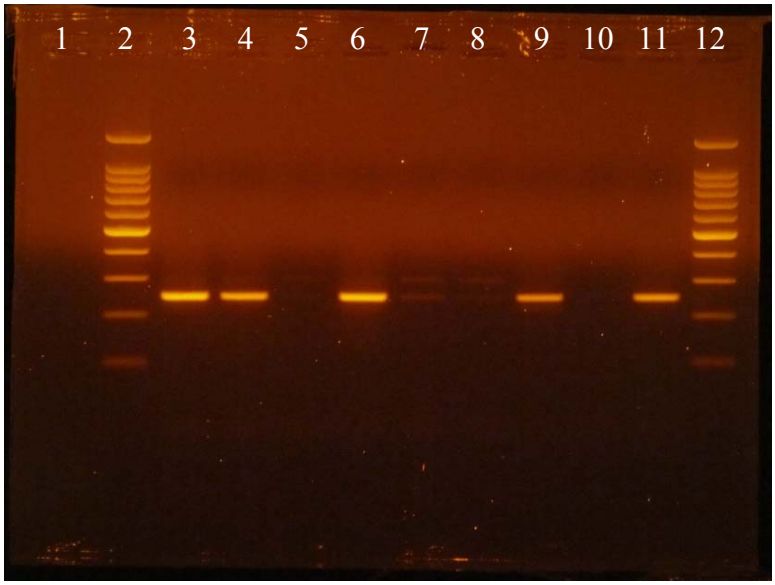


Figure 45: *Rickettsia rickettsii* screening of *D. variabilis* using primer set TZ15/TZ16. 2% agarose gel run for 1hour 10min at 100V. Each lane is an individual tick. Lanes: 1) blank, 2) 100bp DNA ladder, 3-6) group 24, 7-9) group 25, 10) negative, 11) *R. rickettsii* positive control, 12) 100bp DNA ladder.

Francisella tularensis

Amblyomma americanum and *Dermacentor variabilis* were screened for the presence of *Francisella tularensis* using primer set ISFtu2F/ISFtu2R. Four ticks from each group colony group and five from each wild group were tested (Tables 8 and 9 above). Wild group 8 only had four tested due to the small quantity collected. No positive results were found for either tick species (Table 14). Figures 46 and 47 show some of the negative results.

Table 14: *Francisella tularensis* screening of *A. americanum* (groups 1-6, 19-23, 35, and W1-W8) and *D. variabilis* (groups 7-12, 24-26, and 33) using primer set ISFtu2F/ISFtu2R. No positives found.

Group Number	Number Positive	Group Number	Number Positive
1	0/4	22	0/4
2	0/4	23	0/4
3	0/4	24	0/4
4	0/4	25	0/4
5	0/4	26	0/4
6	0/4	33	0/4
7	0/4	35	0/4
8	0/4	W1	0/4
9	0/4	W2	0/4
10	0/4	W3	0/4
11	0/4	W4	0/4
12	0/4	W5	0/4
19	0/4	W6	0/4
20	0/4	W7	0/4
21	0/4	W8	0/4

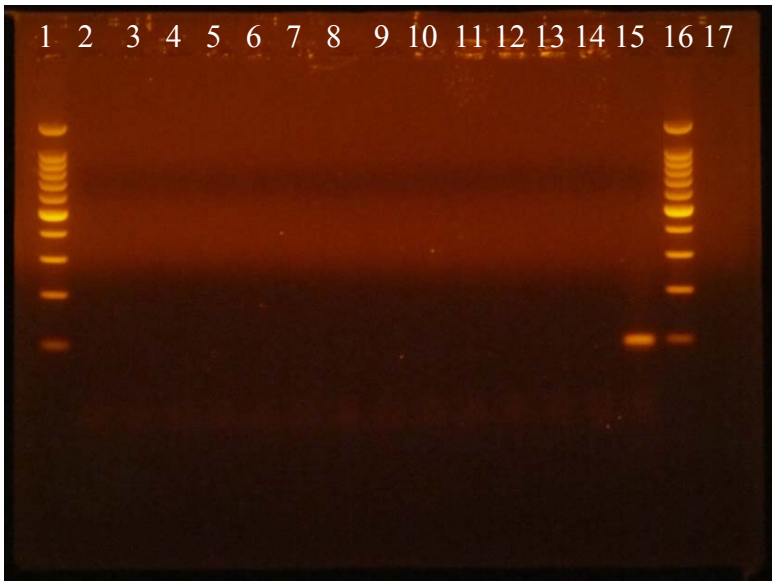


Figure 46: *Francisella tularensis* screening of *A. americanum* using primer set ISFtu2F/ISFtu2R. 2% agarose gel run for 1hour at 100V. Each lane is an individual tick. Lanes: 1) 100bp DNA ladder, 2) group 2, 3) group 3, 4) group 19, 5) group 30, 6) group W2, 7-11) group W1, 12-13) group W3, 14) negative, 15) *F. tularensis* positive control, 16) 100bp DNA ladder, 17) blank.

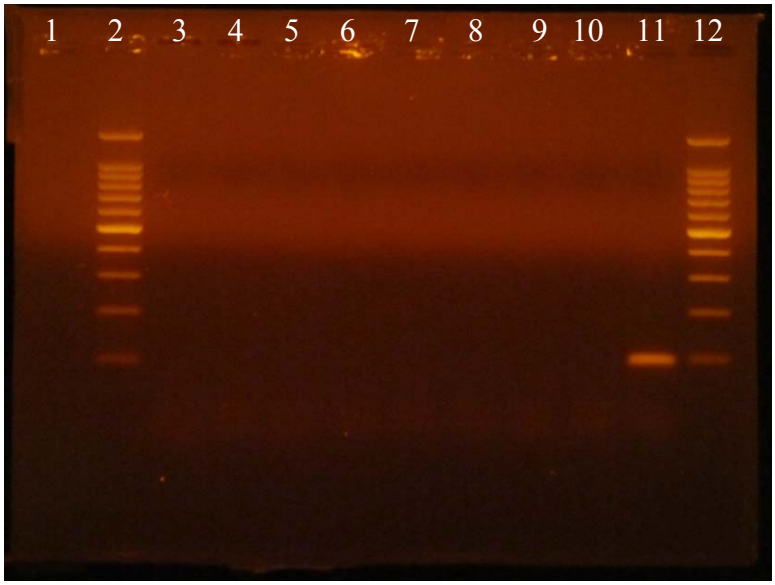


Figure 47: *Francisella tularensis* screening of *D. variabilis* using primer set ISFtu2F/ISFtu2R. 2% agarose gel run for 1hour at 100V. Each lane is an individual tick. Lanes: 1) blank, 2) 100bp DNA ladder, 3) group 25, 4-7) group 26, 8) group 8, 9) group 9, 10) negative, 11) *F. tularensis* positive control, 12) 100bp DNA ladder.

CHAPTER V

DISCUSSION

The objectives of this research were to establish PCR protocols for detection of tick-borne pathogens in lab reared and wild ticks, generate stock positive controls and storage system, and screen ticks from the Oklahoma State University Tick Rearing Facility and wild populations. The establishment of these protocols is important due to the quantity of pathogens ticks carry and the use of ticks in research. It is important to be able to determine if the ticks at the rearing facility are free of pathogens that researchers might be using.

A new DNA extraction method for ticks was developed with the combination of DNAzol® Direct (Molecular Research Center, Inc., Cincinnati, OH) with the use of microbeads and mini-beadbeater (Biospec Products, Bartlesville, OK). The literature has shown that the use of microbeads and mini-beadbeater are excellent for homogenizing and obtaining tick tissues (Long et al., 2003). However, the use of DNAzol® Direct (Molecular Research Center, Inc., Cincinnati, OH) has not been published for tick DNA extractions prior to this project. The combination of the two was found to not only be successful at extracting high quality DNA, but was also performed in only 30min – 1hour.

Six PCR protocols were successfully established for detecting one tick gene and five pathogens. The use of a primer set for a tick gene allowed for extraction quality testing ensuring that extraction worked. Without this, it would be unclear if a negative result obtained was truly negative or if the extraction just did not work. The five pathogen protocols were found to be successful with *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* primers detecting down to 1copy/0.5µl and *Borrelia burgdorferi*, *Rickettsia rickettsii*, and *Francisella tularensis* primers down to 100copies/0.5µl in sensitivity testing. However, due to the highly conserved nature of pathogens, a couple of the primer sets were not species specific and depended on sequencing to determine if a positive was the pathogen of interest. This proved to be useful as the large majority of the positive results found were not the pathogen of interest.

Sensitivity of the primer sets down to 1copy/0.5µl is unlikely. The high positive result for 1copy/0.5µl for the *E. chaffeensis* and *A. phagocytophilum* primer set is most likely due to inaccuracies in the measurement of the original DNA concentration. A spectrophotometer using only 2µl of product was used to measure the original concentration of DNA in a plasmid DNA sample. Though PCR theoretically can detect down to one copy of the target sequence, it is unlikely. PCR detection limits are closer to 10 to 10^4copies (Zourob et al., 2008).

For both the lab reared and wild ticks, negative results were found for *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Rickettsia rickettsii,* and *Francisella tularensis*. These results were expected due to the difficulty of transovarial transmission amongst ticks and the infrequency of wild ticks introduced to the reared colonies. The only positive result for the five pathogens of interest was a wild *Amblyomma americanum*

(from Greenleaf State Park) positive for *Ehrlichia chaffeensis*. No positives for *E. chaffeensis* were found within the reared tick colonies. Known positive controls were obtained from outside sources to ensure the accuracy of the negative results. Due to the biosecurity level of the lab, no known positive ticks were used for positive controls.

The obtained positive controls were cloned and stored in two formats, plasmid DNA and glycerol stocks, for easy access in the future. The stored glycerol stocks make it easy to replenish the stocks when needed without having to find a source to provide a positive control. However, the stored positive controls are only small segments of DNA. If the total DNA of one of the organisms is needed, that organism will need to be found elsewhere. All positive controls have been stored at -80°C to ensure the longest life possible. They will stay in the possession of the Tick Rearing Facility. No reagents such as PCR mixes and primer sets were stored due to the ease of ordering them and to ensure that they do not expire.

PCR proved to be successful for detecting pathogens in the lab reared and wild ticks. Other techniques such as Multiplex PCR may be beneficial or unfavorable. Multiplex PCR has the benefit of using all the primer sets in one reaction resulting in large screening amounts in shorter periods of time as it eliminates the necessity of running multiple PCR reactions per DNA extraction (Elnifro et al., 2000). However, Multiplex PCR can result in interactions amongst the different primer sets (Elnifro et al., 2000). Also, the large amount of tick DNA present in the samples may result in false negatives due to more targets being available for the tick 16S rRNA primer set (Edwards and Gibbs, 1994). Once replication of the segment occurs, even more template is

available. This may result in the inability of the other primer sets to find its template even if the pathogen is present.

Positives were found for the *Rickettsia* species *R. amblyommii* in *A. americanum* (both reared colonies and wild) and *R. montanensis* in *D. variabilis* using the primer set TZ15/TZ16. The presence of *R. amblyommii* in *A. americanum* is due to their symbiotic relationship (Jiang et al., 2010). It is transmitted both transstadially and transovarially within tick populations (Jiang et al., 2010). This makes it unlikely that it would be removed from the reared tick colonies. It has also been found present in both male and female ticks (Jiang et al., 2010). Saraiva et al. (2013) found that *R. amblyommii* can be transmitted to laboratory rabbits, but was found to be non-pathogenic. The literature was unclear if it is carried by sheep. It has recently been suggested that *R. amblyommii* may cause rickettsiosis in humans (Vaughn et al., 2014), as well as infection in dogs and other animals (Paddock et al., 2015). Vaughn et al. (2014) identified some patients with Rocky Mountain spotted fever had antibodies for *R. amblyommii* but not *R. rickettsii*.

A review of the literature was unclear about the type of transmission performed by *R. montanensis* within tick populations and whether or not there might be a symbiotic relationship with the tick. *R. montanensis* is still in the process of being studied therefore, it is unclear whether it is found in sheep or rabbits. It has been suggested that it causes infection in humans, dogs, and other animals (Paddock et al., 2015).

The presence of either *R. amblyommii* or *R. montanensis* in the lab reared tick colonies and wild populations may prevent the presence of *R. rickettsii* due to rickettsial interference (Paddock et al., 2015). Their presence in the lab reared ticks may affect

future research. Researchers studying *R. rickettsii* infections and transmission via ticks may need Rickettsia free ticks. This, however, may not be possible due to the transtadial and transovarial transmission of *R. amblyommii* within *A. americanum* populations (Jiang et al., 2010). A higher number of ticks from the lab reared colonies need to be screened in order to establish the true prevalence of infection in the ticks. It is important to continue following the literature for new information about *R. amblyommii* and *R. montanensis*.

With the observation of multiple *Rickettsia* species in both the lab reared and wild ticks, it may be beneficial to screen for other pathogens as well. However, not all tick-borne diseases are found in Oklahoma. Wild ticks are only introduced to the lab reared colonies once a year and are collected from only a few locations. It will be beneficial to monitor those locations for tick-borne pathogens in case of the introduction of a pathogen into these specific populations. It is also important to monitor other wild populations in Oklahoma to detect any emergence of pathogens, such as *Borrelia burgdorferi* and *Anaplasma phagocytophilum* which are not commonly found in Oklahoma.

It may be beneficial to develop a species specific primer set for *R. rickettsii*. With the current primer set being used, verification of positives was done with the use of sequencing. However, genes are highly conserved amongst the different species of *Rickettsia* and may only vary by one or two nucleotides. This can result in an inaccurate verification if a mistake occurred during the annealing process in the PCR reaction. A specific primer set would help eliminate that probability.

A routine schedule for monitoring the lab reared ticks is essential. Screening the wild ticks before introducing them to the lab reared colonies would decrease the chance

that pathogens could be introduced into the colonies. Since it is not possible to test all ticks because the tick must be killed during the screening process, it is still possible to introduce a pathogen to the colonies with the introduction of wild ticks. This makes it important to also screen the tick colonies routinely, especially since the pathogens can be transmitted either transstadially or transovarially within the ticks. Ticks can also transmit pathogens amongst each other when co-feeding (Sonenshine and Roe, 2014). This is a result of ticks being pool feeders (Capinera, 2008) allowing co-feeding ticks in close proximity to exchange blood. So it may be necessary to feed wild ticks on different animals than the lab reared ticks. This would help ensure that if an infected tick was present it wouldn't infect other, non-wild ticks.

The commercial aspect of testing these ticks means that the ticks in the colonies must be certified free (below a certain percentage) of certain pathogens. Due to the nature of the testing involved, it is not possible to screen all tick species for all the possible pathogens. It will be important to determine the quantity of ticks to be tested to ensure a large enough sample to accurately say that they are free or below the desired percentage. While important to be able to certify the ticks are pathogen free, they will only be able to be certified to be free (below a certain percentage) of pathogens that are normally associated with the tick species as per the protocol described in this study. If customers wish to know about other pathogens, specific protocols will need to be developed for those specific pathogens. There are many symbionts that live with ticks, so it is unlikely that there will ever be a tick free of other organisms.

In conclusion, this study has demonstrated the importance of screening lab reared tick colonies and wild populations. While some important pathogens, such as *Francisella*

tularensis, *Rickettsia rickettsii*, *Borrelia burgdorferi*, and *Anaplasma phagocytophilum*, were not detected in either the lab reared or wild ticks, another pathogen, *Ehrlichia chaffeensis*, and a few rickettsial organisms, *Rickettsia amblyommii* and *Rickettsia montanensis*, were detected in the lab reared and/or wild ticks. Their presence in the lab reared tick colonies illustrates the importance of monitoring the colonies routinely.

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APPENDICES

Appendix A: Protocol for tick DNA extraction and PCR Detection of *Ehrlichia chaffeensis* in ticks

Description: *Ehrlichia chaffeensis* is a tick-borne pathogen found in *Amblyomma americanum*. It can be detected in a tick DNA extraction with the use of PCR. The whole tick can be used, no dissection required. Any life stage of the tick can be used.

Extraction Reagents and Materials: DNAzol® Direct (Molecular Research Center, Inc., Cincinnati, OH), 2ml sterile bead beater tubes, 1.5ml nuclease free microcentrifuge tubes, 1.0mm zirconium/silica beads, 2.5mm zirconium/silica beads

Extraction Procedure: Weigh empty bead beater tube (label before weighing), add 1 tick then reweigh tube, add 100µl DNAzol per 0-10mg tick weight, incubate at 80-90°C for 15min, add 20 1.0mm z/s beads and 4 2.5mm z/s beads, bead beat 2-3 times at 42 (or 4200rpm) (speed may vary by machine, adjust number of cycles as needed) for 1.5min each, centrifuge tube at 13000xg for 1min, transfer as much liquid as possible without tick parts to a 1.5ml nuclease free tube. This is ready for PCR.

PCR Reagent: Hotstart Taq Master Mix, 2x (Amresco, LLC., Solon, OH) (contains Mg^{++}, dNTPs, and Taq DNA polymerase)

Primer Sequences:

Ehrlichia chaffeensis

1st set

ECB 5'- AGAACGAACGCTGGCGGCAAGCC -3'

ECC 5'- CGTATTACCGCGGCTGCTGGCA -3'

Nested set

HE1 5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3'

HE3 5'- TATAGGTACCGTCATTATCTTCCCTAT-3'

Tick Gene (works with multiple species) (used to ensure extraction worked)

TQ16S+1F 5'- CTGCTCAATGATTTTTTAAATTGCTGTGG-3'

TQ16S-2R 5'- ACGCTGTTATCCCTAGAG-3'

PCR Protocol:

Combine the following:

	1vol(µl)
Hotstart	12.5
Forward primer	0.5
Reverse primer	0.5
Water (nuclease free)	11
Template	0.5
Total	25

Thermal Cycler Parameters:

ECB/ECC cycle

1) Initial denaturation	95°C for 5min	
2) Denaturation	94°C for 1min	30 cycles
3) Annealing	65°C for 1min	
4) Extension	72°C for 1min	
5) Final extension	72°C for 5min	
6) Hold	4°C for ∞	

HE1/HE3

1) Initial denaturation	95°C for 5min	
2) Denaturation	94°C for 1min	30 cycles
3) Annealing	58°C for 1min	
4) Extension	72°C for 1min	
5) Final extension	72°C for 5min	
6) Hold	4°C for ∞	

TQ16S+1F/TQ6S-2R

1) Initial denaturation	95°C for 5min	
2) Denaturation	94°C for 1min	30 cycles
3) Annealing	55°C for 1min	
4) Extension	72°C for 1min	
5) Final extension	72°C for 5min	
6) Hold	4°C for ∞	

Positive Control: *Ehrlichia chaffeensis* Plasmid DNA stored in a concentration of 1pg/µl in -80°C in a box labeled: *Ehrlichia chaffeensis*, Plasmid DNA, 1pg/µl at 50pg per tube, 0.1X TE buffer 01/08/2014.

Gel Parameters: 1X TBE buffer (both for making and running gel), 2% agarose, 0.5µl ethidium bromide for every 10ml of 1X TBE buffer, 100V, 60-70min. Nested primer set

HE1/HE3 results in a 390bp long amplicon. Primer set TQ16S+1F/TQ16S-2R results in a 310bp long amplicon.

Example gels:

Figure A1: *Ehrlichia chaffeensis* screening of *A. americanum* using nested primer sets ECB/ECC and HE1/HE3. 2% agarose gel run for 1hour 10min at 100V. Each lane is an individual tick. Lanes: 1) blank, 2) 100bp DNA ladder, 3 and 4) group 22, 5-8) group 23, 9) group W2 (positive result), 10-12) group W2, 13) negative nested, 14) *E. chaffeensis* positive control nested, 15) negative not nested, 16) *E. chaffeensis* positive control not nested, 17) 100bp DNA ladder.

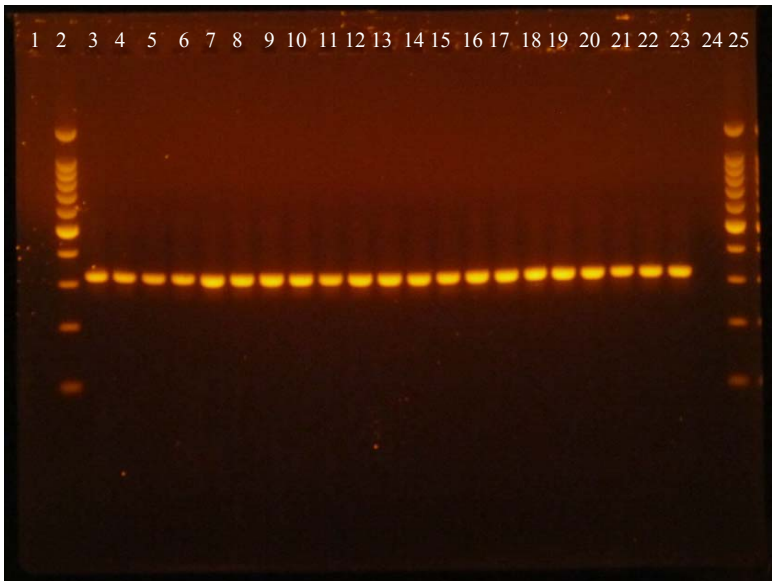

Figure A2: Tick 16S rRNA screening using primer set TQ16S+1F/TQ16S-2R. 2% agarose gel run at 1hour 10min at 100V. Each lane is an individual tick. Lanes: 1) blank, 2) 100bp DNA ladder, 3) group 2, 4) group 3, 5) group 19, 6) group 20, 7) group W2, 8-12) group W1, 13-17) group W3, 18-20) group W4, 21-23) group 35, 24) negative, 25) 100bp DNA ladder.

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Appendix B: Protocol for tick DNA extraction and PCR Detection of *Borrelia burgdorferi* in ticks

Description: *Borrelia burgdorferi* is a tick-borne pathogen found in *Ixodes scapularis*. It can be detected in a tick DNA extraction with the use of PCR. The whole tick can be used, no dissection required. Any life stage of the tick can be used.

Extraction Reagents and Materials: DNAzol® Direct (Molecular Research Center, Inc., Cincinnati, OH), 2ml sterile bead beater tubes, 1.5ml nuclease free microcentrifuge tubes, 1.0mm zirconium/silica beads, 2.5mm zirconium/silica beads

Extraction Procedure: Weigh empty bead beater tube (label before weighing), add 1 tick then reweigh tube, add 100µl DNAzol per 0-10mg tick weight, incubate at 80-90°C for 15min, add 20 1.0mm z/s beads and 4 2.5mm z/s beads, bead beat 2-3 times at 42 (or 4200rpm) (speed may vary by machine, adjust number of cycles as needed) for 1.5min each, centrifuge tube at 13000xg for 1min, transfer as much liquid as possible without tick parts to a 1.5ml nuclease free tube. This is ready for PCR.

PCR Reagent: Hotstart PCR-To-Gel Master Mix, 2x (Amresco, LLC., Solon, OH) (contains Mg^{++}, dNTPs, Taq DNA polymerase, and loading dye)

Primer Sequences:

Borrelia burgdorferi

ospA2	5'- GTTTTGTAATTTCAACTGCTGACC-3'
ospA4	5'- CTGCAGCTTGGAATTCAGGCACTTC-3'

Tick Gene (works with multiple species) (used to ensure extraction worked)

TQ16S+1F	5'- CTGCTCAATGATTTTTTAAATTGCTGTGG-3'
TQ16S-2R	5'- ACGCTGTTATCCCTAGAG-3'

PCR Protocol:

Combine the following:

	1vol(µl)
Hotstart	12.5
Forward primer	0.5
Reverse primer	0.5
Water (nuclease free)	11
Template	0.5
Total	25

Thermal Cycler Parameters:

1) Initial denaturation	95°C for 5min	
2) Denaturation	94°C for 1min	⎫
3) Annealing	55°C for 1min	⎬ 30 cycles
4) Extension	72°C for 1min	⎭
5) Final extension	72°C for 5min	
6) Hold	4°C for ∞	

Positive Control: *Borrelia burgdorferi* Plasmid DNA stored in a concentration of 1pg/µl in -80°C in a box labeled: *Borrelia burgdorferi*, Plasmid DNA, 1pg/µl at 50pg per tube, 0.1X TE buffer 09/03/2014.

Gel Parameters: 1X TBE buffer (both for making and running gel), 2% agarose, 0.5µl ethidium bromide for every 10ml of 1X TBE buffer, 100V, 60-70min. Primer set ospA2/ospA4 results in a 196bp long amplicon. Primer set TQ16S+1F/TQ16S-2R results in a 310bp long amplicon. Primer set ospA2/opsA4 amplifies all *Borrelia* species, sequences must be obtained for any positive result.

Example gels:

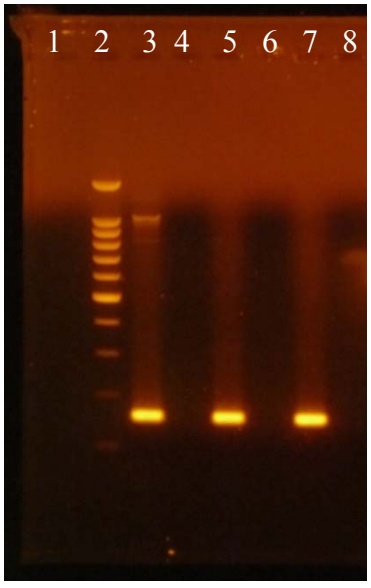

Figure B1: *Borrelia burgdorferi* with primer set ospA2/ospA4. 2% agarose gel run for 1hour at 100V. Different annealing temperatures. Lanes: 1) blank, 2) 100bp DNA ladder, 3) 45°C, 4) negative 45°C, 5) 50°C, 6) negative 50°C, 7) 55°C, 8) negative 55°C

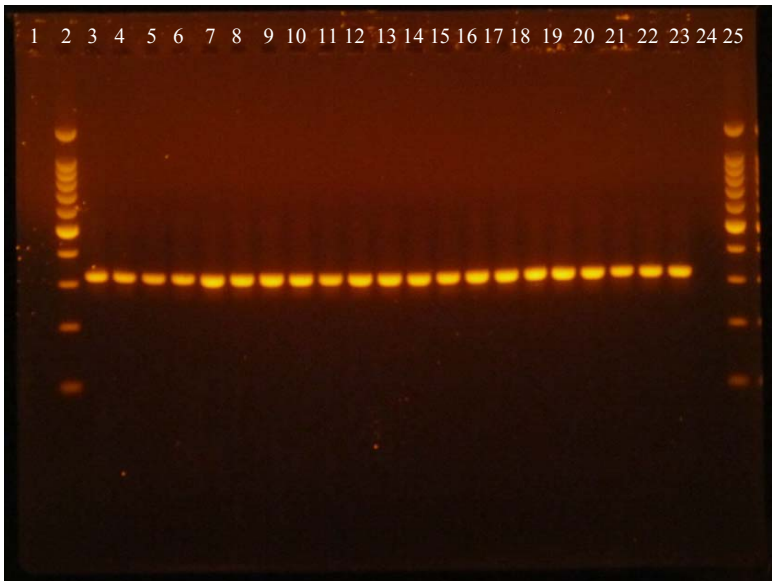

Figure B2: Tick 16S rRNA screening using primer set TQ16S+1F/TQ16S-2R. 2% agarose gel run at 1hour 10min at 100V. Each lane is an individual tick. Lanes: 1) blank, 2) 100bp DNA ladder, 3) group 2, 4) group 3, 5) group 19, 6) group 20, 7) group W2, 8-12) group W1, 13-17) group W3, 18-20) group W4, 21-23) group 35, 24) negative, 25) 100bp DNA ladder.

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Appendix C: Protocol for tick DNA extraction and PCR Detection of *Anaplasma phagocytophilum* in ticks

Description: *Anaplasma phagocytophilum* is a tick-borne pathogen found in *Ixodes scapulris*. It can be detected in a tick DNA extraction with the use of PCR. The whole tick can be used, no dissection required. Any life stage of the tick can be used.

Extraction Reagents and Materials: DNAzol® Direct (Molecular Research Center, Inc., Cincinnati, OH), 2ml sterile bead beater tubes, 1.5ml nuclease free microcentrifuge tubes, 1.0mm zirconium/silica beads, 2.5mm zirconium/silica beads

Extraction Procedure: Weigh empty bead beater tube (label before weighing), add 1 tick then reweigh tube, add 100µl DNAzol per 0-10mg tick weight, incubate at 80-90°C for 15min, add 20 1.0mm z/s beads and 4 2.5mm z/s beads, bead beat 2-3 times at 42 (or 4200rpm) (speed may vary by machine, adjust number of cycles as needed) for 1.5min each, centrifuge tube at 13000xg for 1min, transfer as much liquid as possible without tick parts to a 1.5ml nuclease free tube. This is ready for PCR.

PCR Reagent: Hotstart Taq Master Mix, 2x (Amresco, LLC., Solon, OH) (contains Mg^{++}, dNTPs, and Taq DNA polymerase)

Primer Sequences:

Anaplasma phagocytophilum

1st set

GE3A	5'- CACATGCAAGTCGAACGGATTATTC -3'
GE10R	5'- TTCCGTTAAGAAGGATCTAATCTCC -3'

Nested set

GE9F	5'- AACGGATTATTCTTTATAGCTTGCT -3'
GGE2	5'- GGCAGTATTAAAAGCAGCTCCAGG -3'

Tick Gene (works with multiple species) (used to ensure extraction worked)

TQ16S+1F	5'- CTGCTCAATGATTTTTTAAATTGCTGTGG-3'
TQ16S-2R	5'- ACGCTGTTATCCCTAGAG-3'

PCR Protocol:

Combine the following:

	1vol(µl)
Hotstart	12.5
Forward primer	0.5
Reverse primer	0.5
Water (nuclease free)	11
Template	0.5
Total	25

Thermal Cycler Parameters:

ECB/ECC cycle

1) Initial denaturation	95°C for 5min	
2) Denaturation	94°C for 30sec	⎫
3) Annealing	55°C for 30sec	⎬ 40 cycles
4) Extension	72°C for 1min	⎭
5) Final extension	72°C for 5min	
6) Hold	4°C for ∞	

HE1/HE3

1) Initial denaturation	95°C for 5min	
2) Denaturation	94°C for 30sec	⎫
3) Annealing	54°C for 30sec	⎬ 30 cycles
4) Extension	72°C for 1min	⎭
5) Final extension	72°C for 5min	
6) Hold	4°C for ∞	

TQ16S+1F/TQ6S-2R

1) Initial denaturation	95°C for 5min	
2) Denaturation	94°C for 1min	⎫
3) Annealing	55°C for 1min	⎬ 30 cycles
4) Extension	72°C for 1min	⎭
5) Final extension	72°C for 5min	
6) Hold	4°C for ∞	

Positive Control: For primer set GE3a/GE10R, *Anaplasma phagocytophilum* Plasmid DNA stored in a concentration of 1pg/µl in -80°C in a box labeled: *Anaplasma phagocytophilum*, Plasmid DNA, 1pg/µl at 50pg per tube, 0.1X TE buffer 09/12/2014. For primer set GE9F/GE2, *Anaplasma phagocytophilum* Plasmid DNA stored in a concentration of 1pg/µl in -80°C in a box labeled: *Anaplasma phagocytophilum*, Plasmid DNA, 1pg/µl at 50pg per tube, 0.1X TE buffer 01/08/2014

Gel Parameters: 1X TBE buffer (both for making and running gel), 2% agarose, 0.5µl ethidium bromide for every 10ml of 1X TBE buffer, 100V, 60-70min. Nested primer set GE9F/GE2 results in a 546bp long amplicon. Primer set TQ16S+1F/TQ16S-2R results in a 310bp long amplicon.

Example gels:

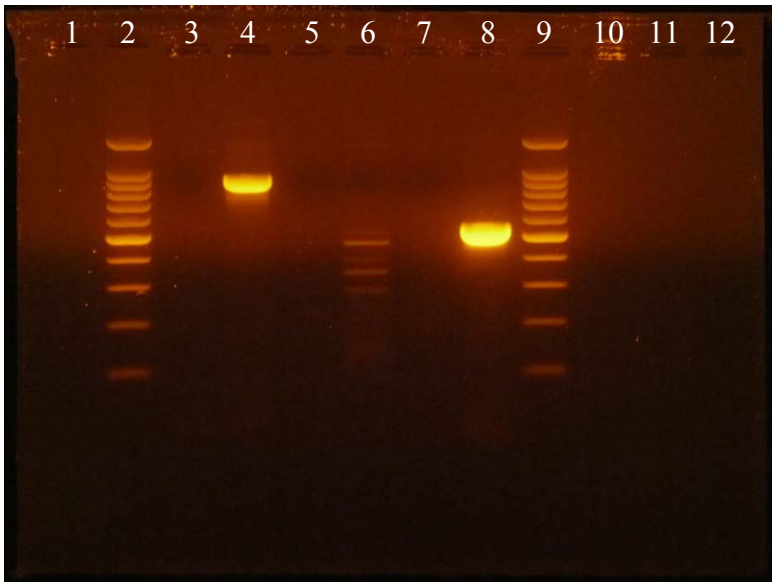

Figure C1: *Anaplasma phagocytophilum* with primer sets GE3a/GE10R and GE9F/GE2. 2% agarose gel run for 1hour 10min at 100V. Positive primer test with *A. phagocytophilum*. Lanes: 1) blank, 2) 100bp DNA ladder, 3) GE3a/GE10R negative, 4) GE3a/GE10R positive , 5) GE9F/GE2 not nested negative, 6) GE9F/GE2 not nested positive, 7) GE9F/GE2 nested negative, 8) GE9F/GE2 nested positive, 9)100bp DNA ladder, 10-12) blank

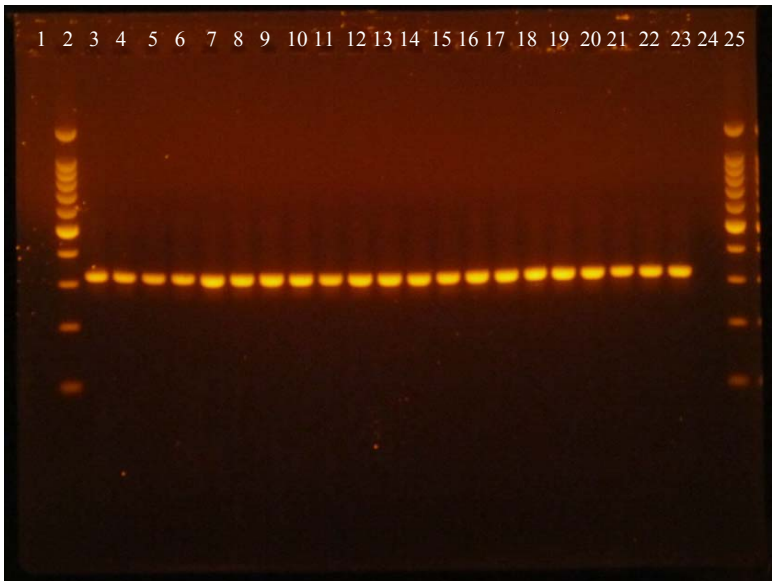

Figure C2: Tick 16S rRNA screening using primer set TQ16S+1F/TQ16S-2R. 2% agarose gel run at 1hour 10min at 100V. Each lane is an individual tick. Lanes: 1) blank, 2) 100bp DNA ladder, 3) group 2, 4) group 3, 5) group 19, 6) group 20, 7) group W2, 8-12) group W1, 13-17) group W3, 18-20) group W4, 21-23) group 35, 24) negative, 25) 100bp DNA ladder.

References:

CDC, 2010. PCR Procedure Manual, Rickettsial Zoonoses Branch. Crowder, **C.D., M.A. Rounds, C.A. Phillipson, J.M. Picuri, H.E. Mathews, J. Halverson,S.E. Schutzer, D.J. Echer, and M.W. Eshoo, 2010.** Extraction of Total Nucleic Acids from Ticks for the Detection of Bacterial and Viral Pathogens. Journal of Medical Entomology 47: 89-94.

Halos, L., T. Jamal, L. Vial, R. Maillard, A. Suau, A. Le Menach, H. Boulouis and M. Vayssier-Taussat, 2004. Determination of an efficient and reliable method for DNA extraction from ticks. Veterinary Research 35: 709-713.

Salazar, J.L, 2015. Detection of Tick-Borne Pathogens in Lab Reared Tick Colonies and Wild Population. Master's Thesis, Oklahoma State University.

Appendix D: Protocol for tick DNA extraction and PCR Detection of *Rickettsia rickettsii* in ticks

Description: *Rickettsia rickettsii* is a tick-borne pathogen found in *Dermacentor variabilis* and *Amblyomma americanum*. It can be detected in a tick DNA extraction with the use of PCR. The whole tick can be used, no dissection required. Any life stage of the tick can be used.

Extraction Reagents and Materials: DNAzol® Direct (Molecular Research Center, Inc., Cincinnati, OH), 2ml sterile bead beater tubes, 1.5ml nuclease free microcentrifuge tubes, 1.0mm zirconium/silica beads, 2.5mm zirconium/silica beads

Extraction Procedure: Weigh empty bead beater tube (label before weighing), add 1 tick then reweigh tube, add 100µl DNAzol per 0-10mg tick weight, incubate at 80-90°C for 15min, add 20 1.0mm z/s beads and 4 2.5mm z/s beads, bead beat 2-3 times at 42 (or 4200rpm) (speed may vary by machine, adjust number of cycles as needed) for 1.5min each, centrifuge tube at 13000xg for 1min, transfer as much liquid as possible without tick parts to a 1.5ml nuclease free tube. This is ready for PCR.

PCR Reagent: Hotstart PCR-To-Gel Master Mix, 2x (Amresco, LLC., Solon, OH) (contains Mg^{++}, dNTPs, Taq DNA polymerase, and loading dye)

Primer Sequences:

Rickettsia rickettsii

TZ15 5'- TTCTCAATTCGGTAAGGGC -3'
TZ16 5'- ATATTGACCAGTGCTATTTC -3'

Tick Gene (works with multiple species) (used to ensure extraction worked)

TQ16S+1F 5'- CTGCTCAATGATTTTTTAAATTGCTGTGG-3'
TQ16S-2R 5'- ACGCTGTTATCCCTAGAG-3'

PCR Protocol:

Combine the following:

	1vol(µl)
Hotstart	12.5
Forward primer	0.5
Reverse primer	0.5
Water (nuclease free)	11
Template	0.5
Total	25

Thermal Cycler Parameters:

1) Initial denaturation	95°C for 5min	
2) Denaturation	95°C for 1min	⎫
3) Annealing	55°C for 1min	⎬ 30 cycles
4) Extension	72°C for 1min	⎭
5) Final extension	72°C for 5min	
6) Hold	4°C for ∞	

Positive Control: *Rickettsia* sp. Plasmid DNA stored in a concentration of 1pg/µl in -80°C in a box labeled: *Rickettsia* species, Plasmid DNA, 1pg/µl at 50pg per tube, 0.1X TE buffer 01/10/2014.

Gel Parameters: 1X TBE buffer (both for making and running gel), 2% agarose, 0.5µl ethidium bromide for every 10ml of 1X TBE buffer, 100V, 60-70min. Primer set TZ15/TZ16 results in a 230bp long amplicon. Primer set TQ16S+1F/TQ16S-2R results in a 310bp long amplicon. Primer set TZ15/TZ16 amplifies all spotted fever Rickettsia species, sequences must be obtained for any positive result.

Example gels:

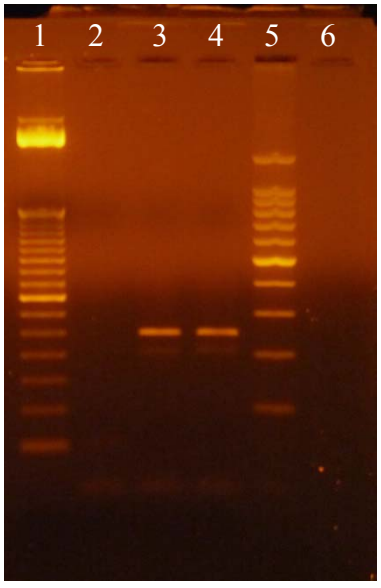

Figure D1: *Rickettsia rickettsii* with primer set TZ15/TZ16. 2% agarose gel run for 1hour at 100V. Different annealing temperatures. Lanes: 1) 50bp DNA ladder, 2) negative, 3) 55°C, 4) 47°C, 5) 100bp DNA ladder, 6) blank

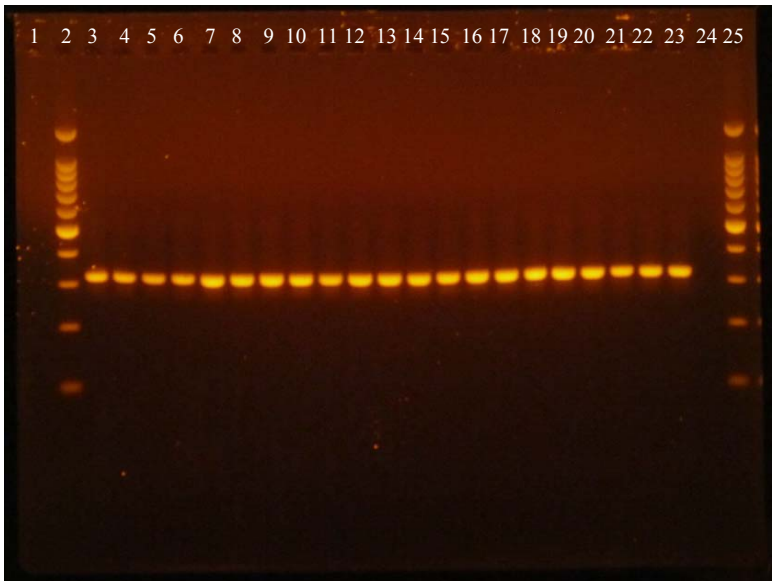

Figure D2: Tick 16S rRNA screening using primer set TQ16S+1F/TQ16S-2R. 2% agarose gel run at 1hour 10min at 100V. Each lane is an individual tick. Lanes: 1) blank, 2) 100bp DNA ladder, 3) group 2, 4) group 3, 5) group 19, 6) group 20, 7) group W2, 8-12) group W1, 13-17) group W3, 18-20) group W4, 21-23) group 35, 24) negative, 25) 100bp DNA ladder.

References:

CDC, 2010. PCR Procedure Manual, Rickettsial Zoonoses Branch. Crowder, **C.D., M.A. Rounds, C.A. Phillipson, J.M. Picuri, H.E. Mathews, J. Halverson,S.E. Schutzer, D.J. Echer, and M.W. Eshoo, 2010.** Extraction of Total Nucleic Acids from Ticks for the Detection of Bacterial and Viral Pathogens. Journal of Medical Entomology 47: 89-94.

Halos, L., T. Jamal, L. Vial, R. Maillard, A. Suau, A. Le Menach, H. Boulouis and M. Vayssier-Taussat, 2004. Determination of an efficient and reliable method for DNA extraction from ticks. Veterinary Research 35: 709-713.

Salazar, J.L, 2015. Detection of Tick-Borne Pathogens in Lab Reared Tick Colonies and Wild Population. Master's Thesis, Oklahoma State University.

Appendix E: Protocol for tick DNA extraction and PCR Detection of *Francisella tularensis* in ticks

Description: *Francisella tularensis* is a tick-borne pathogen found in *Dermacentor variabilis* and *Amblyomma americanum*. It can be detected in a tick DNA extraction with the use of PCR. The whole tick can be used, no dissection required. Any life stage of the tick can be used.

Extraction Reagents and Materials: DNAzol® Direct (Molecular Research Center, Inc., Cincinnati, OH), 2ml sterile bead beater tubes, 1.5ml nuclease free microcentrifuge tubes, 1.0mm zirconium/silica beads, 2.5mm zirconium/silica beads

Extraction Procedure: Weigh empty bead beater tube (label before weighing), add 1 tick then reweigh tube, add 100µl DNAzol per 0-10mg tick weight, incubate at 80-90°C for 15min, add 20 1.0mm z/s beads and 4 2.5mm z/s beads, bead beat 2-3 times at 42 (or 4200rpm) (speed may vary by machine, adjust number of cycles as needed) for 1.5min each, centrifuge tube at 13000xg for 1min, transfer as much liquid as possible without tick parts to a 1.5ml nuclease free tube. This is ready for PCR.

PCR Reagent: Hotstart PCR-To-Gel Master Mix, 2x (Amresco, LLC., Solon, OH) (contains Mg^{++}, dNTPs, Taq DNA polymerase, and loading dye)

Primer Sequences:

Francisella tularensis

ISFtu2F	5'- TTGGTAGATCAGTTGGTGGGATAAC-3'
ISFtu2R	5'- TGAGTTTTACCTTCTGACAACAATATTTC-3'

Tick Gene (works with multiple species) (used to ensure extraction worked)

TQ16S+1F	5'- CTGCTCAATGATTTTTTAAATTGCTGTGG-3'
TQ16S-2R	5'- ACGCTGTTATCCCTAGAG-3'

PCR Protocol:

Combine the following:

	1vol(µl)
Hotstart	12.5
Forward primer	0.5
Reverse primer	0.5
Water (nuclease free)	11
Template	0.5
Total	25

Thermal Cycler Parameters:

1) Initial denaturation	95°C for 5min	
2) Denaturation	95°C for 1min	30 cycles
3) Annealing	55°C for 1min	
4) Extension	72°C for 1min	
5) Final extension	72°C for 5min	
6) Hold	4°C for ∞	

Positive Control: *Francisella tularensis* Plasmid DNA stored in a concentration of 1pg/µl in -80°C in a box labeled: *Francisella tularensis*, Plasmid DNA, 1pg/µl at 50pg per tube, 0.1X TE buffer 06/04/2014.

Gel Parameters: 1X TBE buffer (both for making and running gel), 2% agarose, 0.5µl ethidium bromide for every 10ml of 1X TBE buffer, 100V, 60-70min. Primer set ISFtu2F/ISFtu2R results in a 92bp long amplicon. Primer set TQ16S+1F/TQ16S-2R results in a 310bp long amplicon.

Example gels:

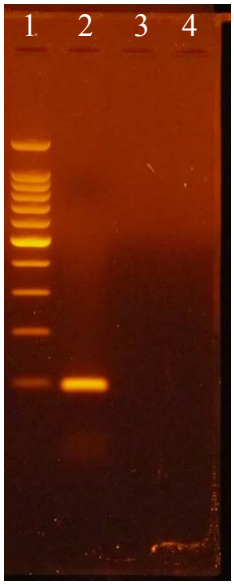

Figure E1: *Francisella tularensis* with primer set ISFtu2F/ISFtu2R. 2% agarose gel run for 1hour at 100V. Lanes: 1) 100bp DNA ladder, 2) *F. tularensis*, 3) negative, 4) blank.

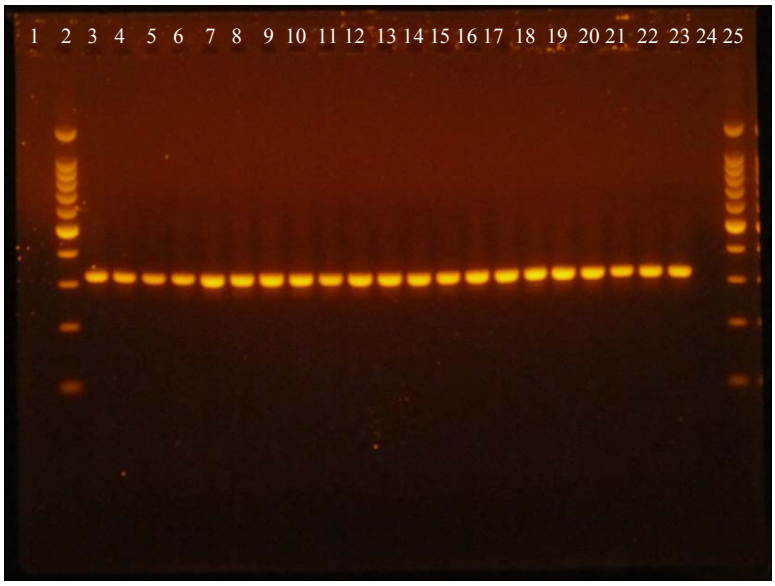

Figure E2: Tick 16S rRNA screening using primer set TQ16S+1F/TQ16S-2R. 2% agarose gel run at 1hour 10min at 100V. Each lane is an individual tick. Lanes: 1) blank, 2) 100bp DNA ladder, 3) group 2, 4) group 3, 5) group 19, 6) group 20, 7) group W2, 8-12) group W1, 13-17) group W3, 18-20) group W4, 21-23) group 35, 24) negative, 25) 100bp DNA ladder.

References:

CDC, 2010. PCR Procedure Manual, Rickettsial Zoonoses Branch. Crowder, **C.D., M.A. Rounds, C.A. Phillipson, J.M. Picuri, H.E. Mathews, J. Halverson,S.E. Schutzer, D.J. Echer, and M.W. Eshoo, 2010.** Extraction of Total Nucleic Acids from Ticks for the Detection of Bacterial and Viral Pathogens. Journal of Medical Entomology 47: 89-94.

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Versage, J.L., D.D.M. Severin, M.C. Chu, and J.M. Petersen, 2003. Development of a Multitarget Real-time Taqman PCR Assay for Enhanced Detection of *Francisella tularensis* in complex Specimens. Journal of Clinical Microbiology 41: 5492-5499.

VITA

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