

Testing for the Presence of *Borrelia lonestari* and *Ehrlichia chaffeensis* in the tick vector *Amblyomma americanum*.

A THESIS SUBMITTED TO THE HONORS COLLEGE IN
FULFILLMENT OF THE REQUIREMENTS

For the degree of

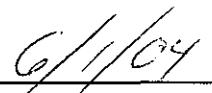
BACHELOR OF SCIENCE

By

Meghan Doolittle



Advisor



Date

BALL STATE UNIVERSITY

MUNCIE, INDIANA

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THESIS ABSTRACT

Thesis: Testing for the presence of *Borrelia lonestari* and *Ehrlichia chaffeensis* in the tick vector *Amblyomma americanum*.

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The purpose of this study was to determine the presence of *Borrelia lonestari* and *Ehrlichia chaffeensis* in the tick vector, *Amblyomma americanum*. Ticks were collected from four different sites located in three counties in southern Indiana. Some ticks were put into pools, while others were tested individually. These ticks were tested by nested PCR. The results show that *B. lonestari* and *E. chaffeensis* are present in the tick vector *A. americanum*.

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Abstract

In this study, *Amblyomma americanum* ticks were tested for the presence of *Ehrlichia chaffeensis* or *Borrelia lonestari*. Ticks were collected from selected sites in three southern Indiana counties: Perry Co., Pike Co., and Spencer Co. in the summer of 2003. The ticks were sorted by site. Some were stored individually; others were placed into 55 pools containing four to five ticks per pool. All the ticks were stored at -80°C. The ticks were homogenized and DNA was extracted. A nested PCR (polymerase chain reaction) followed by an agarose gel electrophoresis was used to check for the presence of *B. lonestari* and *E. chaffeensis* in each sample. *E. chaffeensis* was detected in pools of ticks from all three counties. *B. lonestari* was detected in pools of ticks from Perry and Spencer counties, but not from Pike County. 134 ticks were collected from Old Pigeon Cemetery, Lincoln State Park, and Spencer County. These ticks were tested individually for *E. chaffeensis* and *B. lonestari*. Three of these ticks were positive for *B. lonestari*; none was positive for *E. chaffeensis*.

Introduction

(*Amblyomma americanum*)

During warmer months, more people find themselves venturing outside. This exposes them to annoying pests such as horseflies, mosquitoes, and ticks. These pests look for hosts to feed upon in order to obtain a blood meal. But these insects may leave more than just an itchy bump after they bite. They may leave behind harmful bacterial infections. One such culprit is *Amblyomma americanum*, the lone star tick. Pictures of the male and female lone star tick can be seen in figure 1 and figure 2. *A. americanum* was not proven to be a principle vector for any human disease until the early 1990's. It is aggressive at all three stages of life and is unbiased as to whom or what it bites. *A. americanum* can be found from west-central Texas, northwest to parts of Missouri and Iowa in the southeastern United States, and in areas of New England (4). *A. americanum* can be found in woodland habitats, such as young second-growth forests that contain dense underbrush. This is where the white-tailed deer, one of the main hosts for *A. americanum*, can also be found. Deer are good hosts for *A. americanum* because the ticks can obtain blood meals from deer in all three stages of the tick's life (4). Adult *A. americanum* also feed on other medium to large sized mammals. The larvae and nymphs are attached to ground-feeding birds, medium to large sized mammals, and sometimes small mammals such as rodents (4). Throughout its range, *A. americanum* is the most commonly reported tick to be found on humans. In the months of April through June; the nymphal and adult ticks are the most active. The tick's population and activity decrease after June (4). Several bacteria have been found in *A. americanum*. The focus of our study is on *Borrelia lonestari* and *Ehrlichia chaffeensis*, two bacteria species harbored by the lone star tick. *E. chaffeensis* causes human monocytotropic ehrlichiosis (HME), while the spirochete, *B. lonestari* has been associated with a newly recognized disease, Southern tick-associated rash illness (STARI). The objectives of our study were to determine the presence of *E. chaffeensis* and *B. lonestari* in pools of ticks and in ticks tested individually and to determine an infection rate for each bacterium, to further establishing the geographic distribution of *B. lonestari*, and to find out whether we can detect one or more co-infections within any of the ticks specimens.

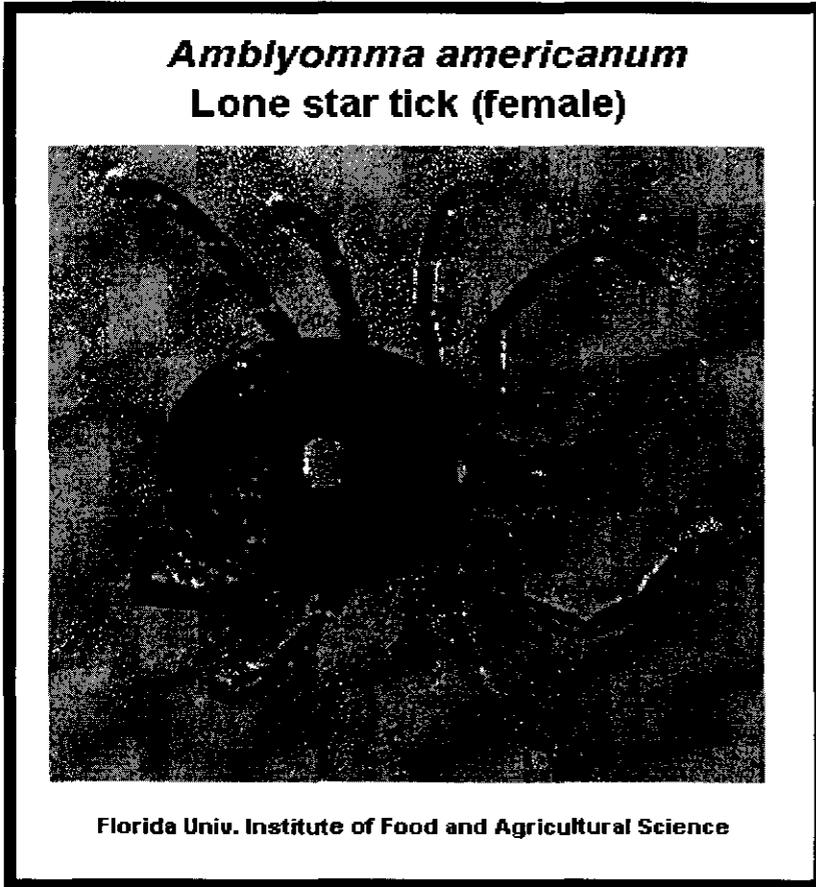


Figure 1



FIGURE 2

Review of Literature

Borrelia lonestari

Lyme disease is a nationally recognized disease. *Borrelia burgdorferi*, the bacterium which causes Lyme disease, is transmitted by the bite of its tick vector, *Ixodes scapularis*. The white-footed mouse and other rodents are the primary hosts for the blacklegged tick, *I. scapularis*. While these serve as reservoirs for *B. burgdorferi* as well as hosts for the bacteria and for the blacklegged tick, white-tailed deer, do not (9). White-tailed deer are good hosts for adult *I. scapularis* ticks. They develop an antibody response following an infection with *B. burgdorferi*. Because of this, the deer are unable to serve as a reservoir for *B. burgdorferi* and are unable to infect ticks. Thus the primary role of the white-tailed deer is to promote the growth and spread of the vector tick population (9).

B. burgdorferi occurs in the northeastern United State (9). In some of these states, along with several southeastern and south central states, a Lyme disease like illness has been reported. At first, the illness was thought to be caused by *B. burgdorferi*, but the illness occurred following the bite of *A. americanum* (1). There were cases where many patients were diagnosed with Lyme disease. Their diagnosis was determined from the presence of a Lyme disease like rash and exposure to *A. americanum*. However, the bacterial spirochete, *B. burgdorferi*, could not be cultured from these patients and *A. americanum*, is unable to transmit *B. burgdorferi*. This meant that some other bacteria had to be causing the illness. This also ruled out *B. burgdorferi* as being a cause. The Lyme disease like illness became referred to as STARI, southern tick associated rash illness, or Master's disease. Characteristic of STARI is a localized expanding circular skin rash (erythema migrans) originating at the site of the tick bite. This is very similar to the rash that is characteristic of Lyme disease. Along with the rash, general fatigue, headache, stiff neck and occasional fever also occur. *B. lonestari* bacteria have been seen in *A. americanum* ticks and in samples taken from STARI rashes. Thus, *B. lonestari* is a candidate for the causative agent of STARI (2).

When people first began reporting symptoms of this illness, the cause was uncertain since *B. lonestari* had not been discovered. Since the actual cause of STARI was uncertain, testing needed to be done. A study was done on *A. americanum* ticks collected from New Jersey, New York, Missouri, and Texas. These ticks were homogenized, the DNA stabilized, and PCRs were performed. The primers used in the PCR were based from the same sequences in flagellin and 16S rRNA genes of the *Borrelia* species. The primer pairs used were FlaLL FlaLS and FlaRS FlaRL (2) The nested PCR amplified a 350-bp region on the flagellin gene (fla B) (2). The bacteria found in *A. americanum* was a *Borrelia* species, but it had different sequences from other known *Borrelia*. There were also some biological differences. The spirochete found within the *A. americanum* tick would not grow in media that would support the growth of other *Borrelia* species. A new bacterium had been discovered. It became known as *Borrelia lonestari* (2).

Until recently, *B. lonestari* had not been isolated in culture, so the PCR of 16S ribosomal DNA(rDNA) and the flagellin gene are used to determine whether or not a human or tick is infected with *B. lonestari* (10). In a study done by Moore, whole blood samples were taken from white tail deer. DNA was extracted by using the GFX genomic blood DNA purification kit. Nested PCRs that used the primer pairs FlaLL and FlaRL and FlaLS and FlaRS were performed to identify any positive samples. *B. lonestari* infection was found in 7 out of 80 deer (8.7%). This was the first reported evidence for the presence of *B. lonestari* to be found in a vertebrate other than a human (10).

In Missouri, and other south central and southeastern states, many Lyme disease cases have been reported. In a study done by Bacon, ticks were collected by a white drag cloth and sorted into pools of 1-25 ticks. The ticks were homogenized in 1 ml of phosphate buffer saline and a pellet was obtained. The QIAamp DNA mini kit was used to extract DNA, and a nested PCR was done on the samples. The primers described by Barbour were used to amplify the 16S rRNA gene fragment (2). This experiment was the first time *B. lonestari* was shown to be present in ticks from Missouri. They were confirmed by 16SrRNA and flaB gene sequence analyses. One 16SrRNA (1330bp) and two flaB (640 and 1,600 bp) PCR products were obtained from the DNA from the pools of ticks and the DNA was sequenced. This sequencing managed to extend the 1996 published sequence of *B. lonestari* flaB by 920 nucleotides. This sequencing gave a 1611-nucleotide fla B product (2).

There is a low prevalence of *B. lonestari* in *A. americanum* (1-2%), but the chance of being infected is still high. This is due to the large quantity of the tick *A. americanum* and its aggressive nature. Because the tick can bite multiple times, the chance of infection is increased (8). In a recent study done in 2003 by Varela, the first culture of *Borrelia lonestari* was grown. This was done by feeding the culture every 3 to 4 days with a mixture that contained ISE6 cells. The ISE6 cells are an embryonic *Ixodes scapularis* tick cell line (11). This will hopefully lead to a new understanding of the bacteria, *B. lonestari*.

Ehrlichia Chaffeensis

Ehrlichia chaffeensis is an obligatory intracellular gram negative bacterium that grows within the endosomes of the host cell as clusters named morulae. *E. chaffeensis* causes the disease HME or human monocytotropic ehrlichiosis (5).

It was observed that the cases of HME overlap with the geographic range of *A. americanum*. The lone star tick was suspected to be the vector of *E. chaffeensis*. PCR testing done with pools and individual ticks collected in endemic areas shows 1% to 5% minimum infection rates for pools and 5% to 15% for individual ticks (4). Fifteen states in the northeast, southeast, and Midwest parts of America have had positive results for the presence of *E. chaffeensis* in the DNA of *A. americanum* were both males and female lone star ticks were infected (4). The cycles of replication, growth, and development of the bacteria *E. chaffeensis* in the lone star tick is uncertain along with the mechanism that allows the vertebrate host to become infected during the ticks feeding (4).

According to Childs and Paddock, the white-tailed deer is the only vertebrate species known to be a complete and sufficient host for maintaining the transmission cycle of *E. chaffeensis* (4). In testing done by Bolte and Brennan, 80% to 100% of white-tailed deer tested in endemic areas were infested with all three stages of *A. americanum* (4). In the larval and nymphal stages, the ticks feed on deer. If a deer is infected with the bacteria, the feeding larva and nymphs could also become infected. While the ticks undergo molting and develop into adults, the bacteria, *E. chaffeensis* remain present in the tick. During a blood meal, nymphal or adult ticks can infect people, dogs, and deer. Ticks cannot pass on *E. chaffeensis*

to their offspring through transovarian transmission. The larva must acquire the bacteria by feeding from an infected host. If a larva becomes infected, it can transmit *E. chaffeensis* to humans. This can then lead to HME.

HME is more likely to infect people who live in suburbs or rural areas, where 66% of infection occurs. HME usually occurs in the months of April through September, making it a seasonal disease. About 68% of the cases take place in the months of May through July. (5) There is an incubation period of 7 to 10 days after transmission of *E. chaffeensis* has occurred. After the incubation period, symptoms start to show up which generally include fever, malaise, and headache. Myalgia, chills, diaphoresis, nausea, and anorexia are less common symptoms. Forty percent to sixty-two percent of patients need to be hospitalized due to an increase in the intensity of the illness. Intensive care may be needed. The illness can last around 23 days. Even though there is effective antimicrobial therapy, 2% to 7 % of people still die (5). HME is life-threatening and should be taken seriously. It leads to hypotension, respiratory failure, meningoencephalitis, coagulopathy, acute renal failure, hemorrhagic manifestations and possibly cardiac failure (5). The more severe infections involve the central nervous system (5). Patients may experience hallucinations, confusion, photophobia, and stupor. Seizures and comas can also occur, although these are seen in more severe cases. In diagnosing someone with HME, their symptoms should be taken into consideration. If the symptoms of an illness would include those of HME, and if someone is suffering for more than 3 days from fever, headache, and malaise and have possibly been exposed to *A. americanum*, HME should be considered as a diagnosis. A blood count should be taken and the presence of leucopenia or thrombocytopenic should be determined. If *E. chaffeensis* is present, doxycycline should be given to the patient. When HME is diagnosed, it is usually confirmed with four-fold or greater rise in IFA antibody titre to *E. chaffeensis*. There is concern that human ehrlichioses will possibly increase over the next 10 years. This is because more people will be moving into or near areas with infected ticks. There will also be an increase in the number of active older people and immunocomprised people. With better ways to diagnose infected patients, the actual number of infected people will also rise. This is why it is important to study the disease and know the percentage of ticks infected (5).

Procedures

Tick Collection

Tick samples were collected during the months of May and June of 2003 by dragging a 1m² corduroyed flag, on the ground, through fields and wooded areas. Ticks were collected from Perry, Pike, and Spencer counties, known to have higher levels of *Ehrlichia chaffeensis* present in the tick vector, *Amblyomma americanum*. A map of Indiana with the designated counties marked can be seen in figure 3. The ticks were then removed from the corduroyed flag with forceps and placed in a small epp tube. A blade of grass was also placed in the vials. The vials were then put in a cooler and transported to the lab where the ticks were sorted, surface sterilized with ethanol, and put into pools of four to five ticks. This was done without discrimination between males and females. Individual ticks were placed in epp tubes and labeled male or female. Pools of ticks and individual ticks were stored at -80°C

MAP OF INDIANA



KEY

Perry Co.

Pike Co.

Spencer Co.

Template Preparation

To prepare a template for the PCR, DNA was extracted from the samples. Individual ticks were ground with a pestle with the presence of liquid Nitrogen in order to homogenize it. Then, DNA was extracted by using the DNA Extraction GentraKit (7). The DNA from the pools of ticks was extracted by using the Phenol-Chloroform DNA extraction procedure. The pools of ticks were smashed with a hammer and a mixture of CTAB and EtSH was added to stabilize the DNA. After homogenization, the DNA needed to be extracted.

DNA Extraction from individual ticks

For individual ticks, 300 μ l of cell lysis solution were added to each tube along with 1.5 μ l Proteinase K. The contents in the centrifuge tube were mixed by inverting them twenty five times. These samples were placed in a Fisher Scientific Dry Bath Incubator at 55°C for 30 minutes and at 65° C for 20 minutes. After incubation, 1.5 μ l of RNAase A solution were added to each centrifuge tube. The tubes were inverted 25 times. Another incubation period of 30 minutes was required. This was done in an ISOTEMP 202S Fisher Scientific dry bath at 37°C. The samples were then allowed to cool to room temperature and 100 μ l of Protein Precipitation Solution was added. After adding the solution, each sample was required to be vortexed for 20 seconds, and centrifuged for 3 minutes at (13,000-16,000g). Next, clean centrifuge tubes were labeled and dated. The supernatant was then added to each tube and the pellet was discarded. Next, to each tube 300 μ l of 100% Isopropanol and .5 μ l of glycogen were added. The centrifuge tubes were inverted 50 times in order to mix. The samples were then centrifuged for 1 minute at (13,000-16,000g). The supernatant was discarded. The pellet was cleaned with 70% ethanol and centrifuged for 1 minute. The supernatant was again discarded, and the pellet was required to air dry for 10 to 15 minutes. After drying, 50 μ l of DNA hydration solution was added to each centrifuge tube. The samples were left at room temperature overnight. Later they were stored at -80° C.

DNA Extraction from pool of ticks

The pools of ticks underwent the Phenol-Chloroform DNA Extraction Procedure. This procedure started out by smashing the ticks with a hammer. Then a mixture of .4 ml CTAB and .5 μ l EtSH was added to each bag (3). This mixture was transferred to a centrifuge tube and incubated at 65°C for 30 minutes in a water bath. Next .4 ml Phenol-Chloroform:Isoamyl was added. Each centrifuge tube was vortexed and centrifuged at 10,000g for 10 minutes. The aqueous solution was transferred to a new clean and properly labeled centrifuge tube. The organic phase had .4 ml Non-CTAB added to it. This mixture was vortex and centrifuged at 10,000g for 10 minutes. The aqueous solution from each centrifuge tube was added to the aqueous solution that was saved from before. The organic phases leftovers were discarded. To the combined aqueous mixture, .8 ml of CHCl₃:IAA was added and centrifuged for 10 minutes at 10,000g. The organic material was again discarded while the aqueous solution and 524 μ l Isopropanol was added to another clean centrifuge tube. This was then incubated at -20° C for 1 hour or overnight. After incubation, the samples were centrifuged at 10,000g for 10 minutes. The pellets were washed with 80% EtOH, centrifuged, and resuspended in 50 μ l TE. Then they were stored at -80° C until a PCR could be performed on them.

PCR ANALYSIS

The PCR, or polymerase chain reaction, is the most commonly used nucleic acid amplification method. In a PCR, the targeted nucleic acid is copied 10⁷ or more times by using the methods of nucleic acid replication and nucleic acid hybridization. This allows for the detection of the target nucleic acid. There are three steps in a PCR that can be repeated 30 to 50 times. These steps are denaturation of the target nucleic acid, primer annealing, and extension of primer target duplex. In the first step; denaturation of the target nucleic acid, the separation of strands occurs due to the increase of temperature. This is called

denaturation of DNA. The target nucleic acid has to be in single stranded form in order for the next step, primer annealing, to take place. After the DNA has been denatured, the second step takes place. Primers, which are short sequences of nucleic acid, are used to anneal the target nucleic acid. The two primers used are paired so that they flank the sequence. One primer will anneal to one target strand at one end of a certain site. The other primer will anneal at the other end of the complementary sequence at a specific site. In the last step, extension of primer-target duplex, the DNA is replicated. Taq polymerase, which can withstand heat, is used to help the primers add nucleotides in the 5'-3' direction. (6) The outcome is a double stranded DNA molecule with the primers present in the product. (7) One PCR cycle is made up of these three steps. The samples are placed in a thermocycler. The thermocycler will carry out the required number of cycles at the designated temperatures. This can produce 10^7 to 10^8 copies of the target nucleic acid, and allow it to be detected later. In this experiment, nested PCRs were performed to optimize the amplification of target DNA base pairs. The Nested PCRs are very sensitive and can increase specificity (6). Two different sets of primers are used in a nested PCR. The first set is used in the first or primary PCR. The product from the primary PCR is used as a template for the second or secondary PCR. In the secondary PCR, the other set of primers are used.

PCR Method used to test for *Ehrlichia chaffeensis*

In the primary PCR used to test for *E. chaffeensis*, 29.5 μ l of water, 5 μ l of 10XBuffer, 5 μ l of MgCl, 1 μ l of each dNTPs, 0.3 μ l of Taq Polymerase, and 0.6 μ l of each Primer, ECC and ECB, were added to each sample. (The information for these primers can be seen in table 1.) To add the required quantity of each reagent, the amount needed was multiplied by the number of samples. This amount was all put in a master mix. After a quick spin in the centrifuge, 45 μ l of the master mix were pipetted into each 0.2 μ l epp tube that was clearly labeled with each specific sample number. Then, 5 μ l of each sample was pipetted into its designated epp tube. After another spin in the centrifuge, the samples were placed in the thermocycler. The samples were denatured for 15 seconds at 94°C, annealed for 30 seconds at 48°C, and extended for 30 seconds at 72°C. This was done for 30 cycles. After the cycles were completed, the samples were extended again at 72°C for 5 minutes, and soaked at 4°C until the samples were removed from the thermocycler. The primary PCR products were used as templates for the secondary PCR. The

reagents used for this master mix were the same except for the pair of primers and the amount of water needed per sample. Each sample required 29.1 μ l of water. The primers that were used this time were HE1 and HE3, which amplified the 389 base pair product. (The information for these primers can be seen in table 1). Each sample required 0.3 μ l of each primer. Three micro-liters of DMSO for each sample was also added in the master mix to the secondary PCR. 48.5 μ l of the master mix was pipeted into the epp tubes, along with 1.5 μ l of each sample. The samples were placed back in the thermocycler. The samples were denatured for 15 seconds at 94°C, annealed for 30 seconds at 55°C, and extended for 30 seconds at 72°C. This was done for 30 cycles. After the cycles, the samples were again extended at 72°C for 5 minutes, and then soaked at 4°C until they were removed from the thermocycler.

Table of Primers used in Nested PCR
For *Ehrlichia chaffeensis*
(TABLE 1)

Primers	ECB	ECC	HE1	HE3
Bases	22	23	29	27
MW	6,727.4	7,092.6	8,855.8	8,175.4

HE1:5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3'

HE3:5'-TATAGGTACCGTCATTATCTTCCTAT-3'

ECB:5'-CGTATTACCGCGGCTGCTGGCA-3'

ECC:5'-AGAACGAACGCTGGCGGCAGCC-3'

PCR Method used to test for *Borrelia lonestari*

In the master mix for *Borrelia lonestari*, the reagents are the same as the ones used in the master mix for *E. chaffeensis*, except for the primers. The water amount was different, and there was no DMSO added to either the primary or secondary PCR. The amount of water needed per sample for the primary PCR was 28.7 μ l. In the secondary PCR, 32.47 μ l of water was required per sample. The primers used in the primary PCR are FlaLL and FlaLR, which amplify the 664 base pair from the flagellin gene. (The information for these primers can be seen in table 2.) One μ l of each primer was needed per sample. Then,

5 μ l of each sample and 45 μ l of the master mix were combined in each epp tube and put in the thermocycler for 35 cycles. The samples were denatured for 3 minutes at 95°C, annealed for 1 minute at 95°C, and extended for 1 minute at 55°C. After the 35 cycles, the samples were extended again at 75°C for 1 minute and soaked at 4°C until they were removed from the thermocycler. The primary PCR product for *B. lonestari* was used as a template for the secondary PCR. The primers used in the secondary PCR were FlaRS and FlaLS, resulting in a 350 base pair product. (The information for these primers can be seen in table 2.) One and a half micro-liters of the template and 48,5 μ l of the master mix were pipetted into each epp tube and put in the thermocycler. The samples were put under the same conditions as the primary PCR. After completion of the second PCR, the samples were ran in an 1.5 % agarose gel electrophoresis's. This allowed us to determine which samples are positive.

Table of Primers used in Nested PCR For *Borrelia lonestari*
(TABLE 2)

Primers	FlaLL	FlaLS	FlaRS	FlaRL
Bases	24	22	26	24
MW	7,409.9	6,832.5	7,870.2	7,351.8

FlaLL:5'-ACATATTCAGATGCAGACAGAGGT-3'

FlaLS:5'-AACAGCTGAAGAGCTTGAATG-3'

FlaRS:5'-CTTTGATCACTTATCATTCTAATAGC-3'

FlaRL:5'-GCAATCATAGCCATTCGAGATTGT-3'

Gel Electrophoresis

After the PCR is complete, the products need to be visually analyzed in order for positive and negative samples to be determined. This is done by agarose gel electrophoresis. It separates products according to size. The Agarose gel has a wide range of separation. It can disperse DNAs from 200 to 50,000 base pairs. (3) The buffer used to cover the gel was Tris borate EDTA(TBE). TBE has a higher buffering capacity and works better with small molecules. Three to 5mm of the buffer should cover the gel. If there is not enough, the gel could dry out. If there is too much, the buffer can increase the heat levels and cause the motility of the DNA to decrease. Too much buffer can also decrease the amount of current going through the Agarose gel. The current is what helps to move the DNA down the gel. There is

a negative and a positive wire that plugs into the voltage supply. The negative particles flow towards the positive side. Since DNA is negative it moves toward the anode. If the gel is ran for too long, the DNA will run off the gel. The progress of the DNA movement should be monitored. A dye must be added to the gel in order to stain the DNA and allow for its visualization. A loading dye must be added to each DNA sample. The loading dyes are dense. This helps the DNA to settle to the bottom of the wells. The dye also moves with the DNA. This allows the DNA's progress to be monitored so the completion for the electrophoresis will be known. A molecular weight ladder is also added in one of the wells. This ladder will allow the base pair from any of the bands to be measured and positive samples can be identified. After the run has been completed, visualization of the PCR product is done.

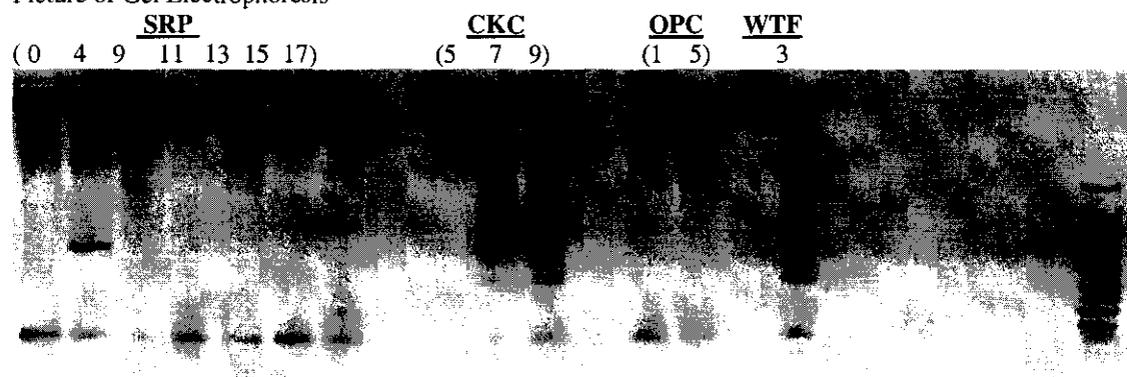
Procedures for Gel Electrophoresis

In order to run the gel electrophoresis, the gel needs to be prepared. The amount of Agarose was weighted out on an electronic scale. Depending on the amount of the gel used, either 1.275 grams or .45 grams of agarose was required. The agarose was added to a sterilized Erlenmeyer flask along with 85 ml or 30 ml of 1X TBE buffer. These volumes correspond with the weight of the agarose. The Erlenmeyer flask was heated in the microwave until the agarose dissolved in the buffer. Ethidium bromide was the dye added to the gel. Either 4.5 μl or 1.5 μl of Ethidium bromide was needed. The gel was allowed to set up in the proper electrophoresis apparatus. While the gel was setting up, the loading dye was pipetted into each sample. Since the samples were at 50 μl in volume, 10 μl of the loading dye was added. When the gel was ready, used 1XTBE buffer was poured over the gel. Then, the samples were pipeted into each well along with a marker. The marker used with the gels was Hyper ladder IV. The ladder creates 9 bands that go from 100 to 1000 base pairs. The 1000 base pair band has a higher intensity in order for it to be distinguished. The gel was run at 60 to 80 milliamps per volts. When it completed running, the gel was placed on a UV box to illuminate the samples and molecular weight band. The Ethidium bromide interacts with the DNA strands and forms a complex that fluoresces when the complex is hit by a UV light. This allows us to visualize the PCR product. A Kodak EDAS 290 camera, which was set up to the computer,

was used to capture the image. The images were properly labeled and saved on the computer for future reference. (An actual picture from one of the gels ran in the lab can be seen in figure 4.)

FIGURE 4

Picture of Gel Electrophoresis



Positive PCR products can be seen in sample # 4 from Sugar Ridge Pike, # 7 and # 9 from Camp Kock Cannelton and in # 3 from Whippoorwill Tree Farm

Results

A total of 275 ticks were collected from Perry, Pike, and Spencer counties (Figure 3). There were a total of 55 pools of ticks. Seventeen pools were from Pike Co., 24 pools were from Spencer Co., and 14 pools were from Perry Co. Out of these pools, 3 were positive for *B. lonestari* and 12 were positive for *E. chaffeensis*. In Spencer county, 2 pools were positive for *B. lonestari* and 3 were positive for *E. chaffeensis*. In Pike Co. there were no pools positive for *B. lonestari*, but there were 6 positive pools for *E. chaffeensis*. In Perry Co., 1 pool was positive for *B. lonestari* and 3 pools were positive for *E. chaffeensis*. Table 3 shows the minimal infection rate for each county.

Results from the pools of ticks tested for *E. chaffeensis* and *Borrelia lonestari* And the Minimal Infection Rate in the 55 pools of ticks (total ticks:275)

(TABLE 3)

County	# of Pools	Positive for <i>B. lonestari</i>	MIR for <i>B. lonestari</i>	Positive for <i>E. chaffeensis</i>	MIR for <i>E. chaffeensis</i>
Spencer	24 pools	2 pools	1.67%	3 pools	2.50%
Perry	17 pools	1 pool	1.17%	3 pools	3.53%
Pike	14 pools	0 pools	0%	6 pools	8.57%
Total	55 pools	3 pools	1.09%	12 pools	4.36%

All the ticks tested individually were collected from Old Pigeon Cemetery and Lincoln State Park in Spencer county. A total of 134 ticks were tested, 94 females and 40 males. Three of the males were positive for *B. lonestari*. None of these ticks were positive for *E. chaffeensis*. The infection rate in the ticks tested individually for *B. lonestari* and *E. Chaffeensis* can be seen in table 4.

Results from the Individual ticks from Old Pigeon Cemetery in Spencer County tested for *E. chaffeensis* and *B. Lonestari*

(TABLE 4)

County	Total ticks tested	Positive for <i>B. lonestari</i>	Positive for <i>E. chaffeensis</i>
Spencer	134	3	0
Infection Rate		2.238%	0%

Discussion

The PCR analysis performed on the tick pools and individual ticks was able to detect both *Borrelia lonestari* and *Ehrlichia chaffeensis* in at least some of the ticks tested. *E. chaffeensis* bacteria are present in the ticks collected at Pike, Perry, and Spencer counties. *Borrelia lonestari* was detected in Perry and Spencer Counties only. No samples were positive for both types of bacteria. However, in a previous study done in our lab, there were pools of ticks that shown to be infected with both *E. chaffeensis* and *B. lonestari* (Catalina Griffin). Four DNA samples from tick pools that were positive for *E. chaffeensis* were sequenced by Davis sequencing (Davis, California). They were similar to the DNA strands reported from *E. chaffeensis* found in (Arkansas). In the individual ticks, there were no positive results for *E. chaffeensis*.

However, some samples showed a PCR product. The molecular weight was higher than the 389 base pair seen in *E. chaffeensis*, so the samples were not counted as positive. Due to time constraints, I was unable to perform a reanalysis of the samples to repeat the *E. chaffeensis* PCR in individual ticks. I proposed that sequencing may need to be done to verify the actual identity of the PCR product that is present in some of the individual tick samples, when tested for *E. chaffeensis*.

Conclusion

After analyzing all the information and results, I came to the conclusion that there is a risk of becoming infected with *B. lonestari* or *E. chaffeensis* from lone star tick in south central Indiana because I found evidence of both bacteria in ticks collected there. Since there is a risk of contracting the bacteria, there is also the risk of becoming ill with either STARI or HME. Both of these diseases have a fatality rate, not to mention, unpleasant symptoms.

I was unable to detect evidence of co-infection of a single tick with both bacteria. This does not mean that it cannot occur, but the chance of it happening is low.

Caution should always be taken when there is a chance of being exposed to *Amblyomma americanum* ticks. If symptoms should occur, immediate physician care should be sought. Tick collecting and testing should be done each year. This will allow us to keep track of the infection rate in the ticks and to monitor any changes that may occur. Entomologist can then keep the public informed, and people can take the proper precautions in order to keep themselves STARI and HME free.

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