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THE PREVALENCE OF HUMAN GRANULOCYTIC EHRLICHIAE IN IXODES SCAPULARIS TICKS IN SOUTHEASTERN GEORGIA

Heather Anne Merten

Georgia Southern University
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THE PREVALENCE OF HUMAN GRANULOCYTIC EHRLICHIAE IN *IXODES SCAPULARIS* TICKS IN SOUTHEASTERN GEORGIA

A Thesis

Presented to

The College of Graduate Studies of

Georgia Southern University

In Partial Fulfillment

of the Requirements for the Degree of

Master's of Science

In the Department of Biology

---

by

Heather Anne Merten

May 2001

April 18, 2001

To the Graduate School:

This thesis, entitled "The Prevalence of Human Granulocytic Ehrlichiae in *Ixodes scapularis* Ticks Collected in Southeastern Georgia" and written by Heather Anne Merten is presented to the College of Graduate Studies of Georgia Southern University. I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master's of Science in the Department of Biology.

Lance A. Durden

Lance A. Durden, Supervising Committee Chair

We have reviewed this thesis
and recommend its acceptance:

Quentin Q. Fang, Co-advisor

James H. Oliver, Jr, Committee Member

John E. Averett, Department Chair

Accepted for the College of Graduate Studies

G. Lane Van Tassell
Dean, College of Graduate Studies

Dedication

This thesis is dedicated to my parents, Patricia A Merten, and in loving memory of my father, Robert R. Merten, who have always encouraged me to take risks, and have known all along that with enough determination, any dream can become a reality.

Acknowledgments

I would like to thank my parents, Robert and Patricia Merten, for all of their encouragement, support, and dedication and for contributing all of those wonderful genes that make me the person I am today. Had it not been for their support throughout my college career, I certainly would not have made it this far. Thanks also to my big brother, Rod Merten, whose sensible advice has kept me focused on my future and who has given me the courage to take on new challenges. I would also like to thank Justin Hodges for his continuing support, commitment, and guidance throughout the last two years and for being an invaluable part of my life.

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VITA

I completed my Bachelor of Science degree at Georgia Southern University in May of 1998. In December of 1999, I entered graduate school at Georgia Southern and began to work under the supervision of Dr. Lance Durden and Dr. Quentin Fang. During my graduate career, I published a paper in the Journal of Vector Ecology entitled, "A State by State Survey of Ticks Recorded From Humans in the United States". I spent many hours in the lab and found myself drawn to molecular biology. I received an Academic Excellence grant in March 2000 for the amount of $250 which was used for supplies. I plan to receive my Master's of Science degree in May 2001 and go on to do research in the field of molecular biology.

ABSTRACT

THE PREVALENCE OF HUMAN GRANULOCYTIC EHRLICHIAE IN *IXODES SCAPULARIS* TICKS IN SOUTHEASTERN GEORGIA

May 2001

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Directed by: Drs. Lance A. Durden and Quentin Q. Fang

Human granulocytic ehrlichiosis (HGE) is a newly emerging, tick-borne zoonosis in North America. The agent that causes HGE is very similar to *Ehrlichia phagocytophilia* and *Ehrlichia equi*, but has yet to be classified as a separate species.

This study was carried out in order to determine the prevalence rate of HGE infection in blacklegged ticks (*Ixodes scapularis*) in insular and mainland sites in southeastern Georgia using nested PCR techniques. Four locations were studied representing 2 barrier islands and 2 mainland sites. We hypothesized that areas that have low levels of human disturbance and are somewhat isolated will have a higher prevalence of infected ticks than areas where there is more disturbance and no geographical barriers. Ticks were collected from each of the four sites and DNA was extracted from them in the laboratory. The DNA was amplified using a nested PCR assay and then visualized on a 1% agarose gel. All positive samples were re-tested in order to confirm that there were

no false positives. Selected products were then sequenced. Our hypothesis was supported by the results, which showed the island populations of ticks to have similar prevalence rates, but when compared to the mainland sites, there was a significant difference in prevalence. On Sapelo Island, 25 of 151 (16.6%) ticks were positive, and on Jekyll Island, 6 of 382 (1.6%) ticks were positive. On both mainland sites, there were no positive ticks screened. In Bulloch County, 112 ticks were sampled and 0 were positive (0.0%), and in Emanuel County, 18 ticks were screened and 0 were positive (0.0%) for the HGE agent. Human disturbance or the availability of competent reservoir hosts may play an important role in these results, as well as low levels of bacteremia in the reservoir ticks.

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CHAPTER I

Introduction

Many tick-borne pathogens and their associated diseases have recently emerged or re-emerged in the United States, and have become a threat to the public. Some of these diseases include: Rocky Mountain spotted fever, Lyme disease, and Human Monocytic Ehrlichiosis. Another human pathogen, the agent of Human Granulocytic Ehrlichiosis (HGE), has recently been recognized in North America as the cause of a zoonotic disease. The etiological agent of HGE is a bacterium in the genus *Ehrlichia*, family Rickettsiaceae. This bacterium, or a very close relative of it, is well established as a veterinary pathogen of horses and other livestock, but is now, like many other pathogens, threatening the health of humans in the U.S.

Ehrlichiae are obligately intracellular, non-motile, gram negative cocci averaging 0.5-1.5 μm in length that develop within an endosome (the central body of a nucleus) of a host cell and cause infection. Ehrlichiosis first attracted attention in 1935 as a pathogenic infection of dogs. The index case of human monocytic ehrlichiosis in North America was first discovered in 1986 in an Arkansas resident who had been exposed to ticks (Telford et al. 1995). Worldwide, five species of *Ehrlichia* have been identified as pathogens of humans: *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia canis*, *Ehrlichia sennetsu*, and the agent of human granulocytic ehrlichiosis (Buller et al. 1999). Human granulocytic ehrlichiosis results from infection with a bacterium that is nearly indistinguishable from the veterinary pathogens *Ehrlichia equi* and *E. phagocytophila*

(McQuisten et al. 1999). The main vector responsible for the transmission of these pathogens is the blacklegged tick, *Ixodes scapularis* in the eastern U.S., and the western blacklegged tick, *I. pacificus* in the western U.S. However, most HGE cases are reported from the midwestern and northeastern states (Kramer et al. 1999). HGE was first discovered in 1994 among residents of Wisconsin and Minnesota (Comer et al. 1999). This tick-transmitted pathogen appears to require a period of reactivation and replication during the tick's blood meal before it is able to infect a host. In a study done by Katavolos et al. (1998), it was shown that the duration of tick attachment required for transmission of the HGE agent is at least one day. Human granulocytic ehrlichiosis is a tick-borne febrile illness, which ranges from mild to severe, where over 50% of those infected require hospitalization and case fatality ratios are nearly 10% (McQuisten et al. 1999). Symptoms resemble those of Lyme disease (Peterson et al. 1989) and can be complicated by pre-existing conditions. The most common clinical manifestations of this disease include: myalgia, headache, malaise, chills, fever, nausea, thrombocytopenia, and leukopenia. The agent of HGE is capable of establishing infection in many tissues and organs. However, the heaviest burden of infection is typically seen in the spleen, liver, lungs, and on some occasions, the heart and kidneys (Lepidi et al. 2000). The bacteria infect leukocytes upon introduction into the mammalian host and then lyse the host cells to release infectious ehrlichiae, which then infect neighboring phagocytic cells (Dumler 1997). Only in the most severe cases does death occur, but this can usually be prevented by prompt clinical diagnosis and antibiotic therapy.

Since 1994, more than 200 human cases of HGE have been diagnosed in the U.S. (Hodzic et al. 1998). The first human case of HGE diagnosed in Europe was in 1995, and

it continues to affect livestock in the United Kingdom, where *E. phagocytophila* is the most common tick-borne pathogen, affecting 300,000 sheep annually (Ogden et al. 1998). Since 1995, there have been documented cases of HGE in Switzerland also, where the vector, *Ixodes ricinus,* is responsible for transmitting the pathogen to cattle. This tick plays an important role as the vector of *E. phagocytophilia*, *Borrelia burgdorferi*, *Rickettsia helvetica*, *Coxiella burnetii* as well as protozoans and trypanosomes in Switzerland (Liz et al. 2000). Serologic evidence suggests that the HGE agent is also present in Israel, and should be included in diagnoses of persons who have had tick exposure and possess influenza like symptoms (Keysary et al. 1999). Transmission of the HGE agent by ticks relies on successful acquisition of the pathogen from the reservoir host. If these hosts have a low level of bacteremia, transmission may be unsuccessful due to a low percentage of granulocytes with morulae (clusters of membrane-bound, intracytoplasmic ehrlichiae) (Hodzic et al. 1998). It is suspected that humans who have come in contact with ehrlichiae via tick bites may develop antibodies to these bacteria without ever showing symptoms of the disease (Magnarelli and Dumler 1996), suggesting that the prevalence of the disease may be much greater than previously thought.

The HGE pathogen has been detected in many small mammals and a laboratory study has confirmed that *I. scapularis* is a competent vector (Telford et al. 1996). There is little information available on the natural history of the HGE agent, but scientists expect that its epidemiology may be similar to that of Lyme borreliosis due to the fact that there are similarities in the distribution of the pathogens (Nicholson et al. 1998). The white-footed mouse, *Peromyscus leucopus*, is a primary host for sub-adult *I. scapularis*

ticks in the northeastern United States (Schauber et al. 1998). A study conducted in Maryland showed strong evidence that *P. leucopus* contained antibodies to the HGE agent. The authors suggested that the HGE agent (or an antigenically related organism) had been present for over a decade in local rodent populations (Bunnell et al. 1998). A more recent study done in the eastern U.S. (Georgia, Connecticut, Maryland, Florida) showed that *Peromyscus gossypinus*, the cotton mouse, may also be a potential reservoir host in nature. In that study, both the cotton mouse and the white-footed mouse were collected from several areas where seropositive mammals had previously been collected. However, there were much higher numbers of seropositive *P. leucopus*, making the white-footed mouse a more logical reservior in those areas where it occurred (Magnarelli et al. 1999). In northern California, the likely vector for granulocytic ehrlichiae is the western blacklegged tick, *Ixodes pacificus*. Immature stages of this tick prefer to feed on lizards, but will occasionally feed on small rodents, and this tick has been shown to be an efficient vector of *E. equi* in transmission studies with horses (Nicholson et al. 1999). However, the reservoir hosts for HGE are not known in northern California. The dusky-footed rat (*Neotoma fuscipes*) is a primary reservoir host of *Borrelia burgdorferi* in this area, and specimens of *N. fuscipes* have been previously identified that were seropositive for the HGE agent, which suggests that these rodents are capable of carrying both agents simultaneously (Levin et al. 1999). A seroprevalence of >30% in samples of this rodent provide evidence supporting the role of *N. fuscipes* in maintaining a cycle of HGE in this area. Also, different species of *Peromyscus* may be reservoirs for HGE in California. It has also been shown that the white-tailed deer, *Odocoileus virginianus*, is a reliable reservoir host for HGE. This particular species of deer is a major host for *Amblyomma*

americanum, and has been identified as a reservoir host for *Ehrlichia chaffeensis*, the agent of human monocytic ehrlichiosis, through positive polymerase chain reaction and serological results (Little et al. 1998). In Wisconsin, *E. equi* infection is widely distributed among white-tailed deer within the geographic range of *I. scapularis*, but the deer may only be incidental hosts, along with humans (Belongia et al. 1997). Human exposure to deer blood (if blood enters a cut while dressing the deer) may be a possible mechanism of HGE transmission from deer to humans. The sudden emergence of HGE in the United States may be due to the repopulation of areas which were once absent of ticks and other reservoir host animal species of HGE according to Dumler (1997). During the past two decades, there has been an increase of microbe-infected reservoirs, and infected ticks have contributed to the spread of disease in several areas of the country. Humans have had the desire to live in more rural areas and spend much of their time outdoors, which has permitted them to have a greater exposure to infected vectors (Mitchell et al. 1996). Also, bacterial pathogens have become easier to detect in recent years due to advancements in biomolecular screening techniques (Dumler 1997).

Detection of HGE was once quite difficult, but simple laboratory procedures have now made it easier to identify. Serologic testing has been made available for state health departments by the Centers for Disease Control and Prevention since August of 1995, after there were 29 confirmed or probable cases of HGE reported in Westchester County, N.Y. (Comer et al. 1999). The serological test of choice is the indirect immunofluorescence antibody (IFA) assay, where recognition of infected cells is recorded (Massung et al. 1998). Laboratory confirmation using this test requires a fourfold change in IFA titer (seroconversion) between acute-phase and convalescent sera

of an antibody to *Ehrlichia* species antigen (Comer et al. 1999). Before 1996, several strains of granulocytic ehrlichiae grown in horse neutrophils were used as antigens to test for HGE antibody by IFA (Comer et al. 1999). Today, since the HGE agent has been isolated and adapted to cell culture, IFA's that use cell culture-derived antigens are being used. In many instances, the patients will have antibodies to two or more tick-borne agents (Magnarelli et al. 1998). This suggests that when one of these diseases is diagnosed or suspected, clinicians should consider the possibility of other past or current tick-borne infections. Unfortunately, serologic tests can be negative during the acute phase of HGE infection, making diagnosis difficult. Also, it may take up to one month or longer to obtain an adequate rise in antibody titer to confirm exposure to HGE, and it is often difficult to obtain convalescent-phase serum samples. Western blotting can also be used to test sera for antibodies, but this test is not as accurate as IFA in diagnosing HGE (Magnarelli and Dumler 1996). A test that is being used more frequently for diagnosis is Polymerase Chain Reaction (PCR) assay. Molecular identification of pathogens using PCR provides a rapid, sensitive, and cost-effective alternative to traditional detection methods (Mauel et al. 1999). This technique is used to amplify a number of copies of a specific region of DNA, in order to produce enough DNA to adequately be tested. A nested PCR assay is used to identify *E. equi* and *E. phagocytophila* species (although it is not entirely specific) on the nucleotide sequence of the 16S rRNA gene in acute phase blood or serum (Massung et al. 1998). Specific oligonucleotide primers used in PCR can indicate the presence or absence of ehrlichial template DNA (Munderloh et al. 1996). There have been several primers developed that aid in the detection of HGE. PCR assay can also yield a DNA product suitable for

sequencing , which is important in confirming detection of the agent. A recently developed technique known as repetitive element polymerase chain reaction (rep-PCR) generates DNA fingerprints that discriminate bacterial species. This method has been used to differentiate a variety of clinical bacterial isolates at the genus, species, and strain levels (Dawson et al. 1997). Sequencing techniques and PCR amplification of 16S rDNA form the basis for detection and possibly identification of ehrlichiae (Whitlock et al. 2000). This method of testing may become the most widely used and accurate identification procedure available. Unfortunately, as with all tests, the success of PCR is dependent on the quality of the equipment, the sterility of the laboratory, and the skill of the technician. Contamination of the samples is a common problem in PCR reactions and results in the appearance of bands that do not contain the target DNA.

More research must be done in order to determine whether or not there are three different strains of ehrlichiae involved in HGE infections (i.e., *E. equi*, *E. phagocytophilia*, and the HGE agent), or whether they represent a single strain/species. Serologic genetic data suggest that they are all the same species, but further phenotypic and biological research may reveal distinguishing features (Chen et al. 1994). Because some individuals do not report their illness, or the disease is misdiagnosed, it is impossible to accurately determine the prevalence of HGE in North America. With advances being made in culturing the HGE agent from humans, we may soon begin to understand the reason for its prevalence in the United States and to determine areas that should be considered high-risk for human infection. This information will be important in the development of vaccines for the disease.

Significance

The biological significance of this research is to provide data on the proportion of ticks that are infected with the HGE agent in southeastern Georgia, and to determine if there is a significant difference in the prevalence of HGE on the barrier islands and inland locations. This study will provide an indication of how prevalent this bacterium is in populations of ticks in southern Georgia, and whether methods should be taken to control the spread of this zoonotic agent. Over the years, many tick-borne pathogens, such as those that cause Lyme disease and Rocky Mountain spotted fever, have increased in prevalence. These debilitating diseases can be fatal, but researchers have developed vaccines and antibiotic therapies to help prevent further spread. The ticks that serve as vectors, as well as the wild mammals that serve as reservoirs should be of particular interest because they are the link to better understand how to treat and prevent this disease effectively. Unfortunately, there are little data on ehrlichiosis outbreaks in this area, but it may soon become a common concern in all households. It is important to determine how serious of a problem ehrlichiosis is or may become, so that researchers can develop ways to control these bacteria from spreading into populations of many species.

Objectives

The overall objectives of this research are to determine the prevalence of the HGE agent in *I. scapularis* ticks in southeast Georgia, and to determine if there is a significant difference of prevalence between mainland and isolated island areas.

CHAPTER II

Materials and Methods

Collection of Ticks

Ticks were collected at four southeastern Georgia sites; Sapelo Island (McIntosh County), Jekyll Island (Glynn County), Bulloch County, and George L. Smith State Park (Emanuel County) (Figure 1). Questing *Ixodes scapularis* ticks were collected from vegetation during the months of November – March in 2000-2001, corresponding to the season of peak activity of the tick (Barlough et al. 1997). Ticks were collected by dragging a soft 1 x 1 m white cloth over the vegetation where questing ticks are commonly found. The ticks were carefully picked off of the cloth using forceps and placed into a vial. Male and female ticks were placed in separate vials to prevent any possible transfer of ehrlichiae between mating pairs. Ticks were then identified in the laboratory, and put into storage at -80° C until further use.

Nucleic Acid Isolation

The DNA extraction method used for the ticks was a modified version of a previous protocol (Doyle and Doyle 1990). Each tick was placed in a 1.5 ml microcentrifuge tube with 250 µl of lysis buffer (4.5 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% lauryl sarcosine). Then, the ticks were pulverized using a sterile pestle and hammer. After the ticks were thoroughly dismembered, 250 µl of 2% (w/v)

Cetyltrimethylammoniumbromide (CTAB) buffer solution was added to each sample which was then vortexed for 20 seconds. Samples were then incubated at 65°C for 15 minutes, and vortexed every 5 minutes. Five hundred µl of chloroform: isoamyl alcohol (24:1) placed in each of the tubes and then the samples were centrifuged at 15,800 g for 5 minutes in order to separate the phases. The supernatant was removed and placed into new 1.5 ml microcentrifuge tubes where 500 µl of chloroform was added to the samples before they were centrifuged at 15,000 g rpm for five minutes. The supernatant was again removed and transferred to a new tube where 1/10 volume of 3M sodium acetate and an equal volume of cold isopropanol was added in order to precipitate the DNA. For a third time, the samples were centrifuged at 15,800 g for 15 minutes, which resulted in a pellet of DNA at the bottom of the tube. The supernatant was removed and the pellet was washed with 70% ethanol and then 100% ethanol. The DNA pellet was then dried using a DNA speed vac, and the pellet was then resuspended in 30µl of 10mM Tris-HCL (pH 8.0). Gel electrophoresis was performed in order to verify the presence of high molecular weight DNA. Each sample was stored at -20°C until PCR was performed.

Nested Polymerase Chain Reaction

The PCR was assembled under an isolated hood and disposable gloves were worn in order to prevent contamination. The hood was used only for PCR purposes and the instruments used were regularly treated with UV light. A negative control was run with each PCR reaction in order to verify that there was no contamination of the samples.

The PRC reactions were carried out using 25 µl of reaction mixture that consisted of reactants in the following concentrations: sterilized water, 2.5mM MgCl2, 1 x *Taq* buffer (10 mM Tris-HCl, pH 8.5, 50 mM Kcl), 200µM of each dNTP, 0.2µM of each

primer, and *Taq* DNA polymerase. One µl of template DNA was added to each tube and a negative control (no template added) and positive control which consisted of genomic DNA of the HGE agent were used. The positive control consisted of DNA from cultured HGE agent and was supplied by W. A. Nicholson, CDC, Atlanta, Georgia. The PCR was done using a Perkin-Elmer 9600 thermal cycler.

The initial reaction (outside) of the nested PCR consisted of the primers Ehr 26F: 5' TTA CAC ATG CAA GTC GRA CG and Ehr 1430RC: 5' AGT CAC TRA CCC AAC CTG AAA, which amplify 1404bp product from many *Ehrlichia* species and target conserved regions of the 16S rRNA gene. For the nested (inside) reaction, the primers GE 9F: 5' AAC GGA TTA TTC TTT ACA GCT TGC T and GE 2: 5' GGC AGT ATT AAA AGC AGC TCC AGG were used which target regions of the 16S rRNA gene that are inconstant among rickettsial species. These primers are specific to the HGE agent and amplify a 546bp product. The condition of the initial was 40 cycles of denaturation at 94°C for 35 seconds, annealing of primers at 55°C for 45 seconds, and extension at 72°C for 2 minutes. The nested reaction consisted of 40 cycles of denaturation at 94°C for 35 seconds, primer annealing for 45 seconds at 55°C, and extension for 1 minute at 72°C. All PCR products were visualized on a 1% agarose gel using 5µl of template mixed with 5µl of loading buffer, with a 1kb standard marker.

All positive samples were re-tested using the same primers and were run on a 1% agarose gel to ensure that there were no false positives due to contamination, etc. All samples that had identical banding patterns were assumed to have analogous sequences. However, even slight shifts in banding patterns indicated a different genotype, and to confirm the positives, four positive samples from each site were chosen to be sequenced.

The samples were prepared for sequencing by running 40µl of the inside product on a 7% agarose gel for 2 hours at 40 volts. The DNA was then excised from the gel using spatulas that had been washed, sterilized with 100% alcohol, and heat flamed so that no contamination would occur. The excision was done using a long wavelength UV light and the slice of agarose containing the DNA was placed into a 1.5 ml microcentrifuge tube. The sample was incubated at 75° C for 5 minutes so that the agarose was completely melted. Next, 1 ml of Magic PCR Prep Resin was added to the tubes, and the solution was pipetted several times in order to thoroughly mix the DNA with the resin. A minicolumn was connected to a 3cc syringe barrel and the mixture was carefully pipetted into the barrel. The minicolumn was washed with 2ml of 80% isopropanol by gently pushing it through the column. The syringe and barrel were then removed and discarded, and the minicolumn was placed in the centrifuge for 30 seconds at 15,800 g to dry the resin from the column. The minicolumn was then transferred to a new 1.5ml tube where 35µl of 2mM Tris-HCl was added to elute the DNA for sequencing. The tube was once again placed into the centrifuge at 15,800 g for 30 seconds and the minicolumn was then discarded and the DNA was stored in the freezer until it was sent to be sequenced.

CHAPTER III

Results

PCR amplification of the 546 bp, 16S rRNA gene fragment from tick nucleic acid samples

High molecular weight DNA was extracted from a total of 663 *Ixodes scapularis*, and all were screened by PCR (Table 1, Figure 2). The 546 bp PCR product which characterized the HGE agent was successfully amplified from six ticks from Jekyll Island and twenty five ticks from Sapelo Island. Eight of the original 382 tick samples from Jekyll Island and 25 of the original 151 samples from Sapelo Island were positive and were re-tested. All 25 of the of the positive results were reproduced from the original nucleic acid samples from Sapelo Island, but only five were reproduced from the Jekyll Island site. The three samples that were not successfully reproduced were screened again and were negative. Perhaps due to human error or contamination, these samples were positive during the initial PCR amplification, but were consistently negative in the latter two PCR amplifications. All of the remaining 31 samples were consistently reproduced with each succeeding PCR reaction.

Prevalence of infection in *I. scapularis* by the HGE agent

Prevalence of infected ticks was calculated for the four locations based on the repeatability of PCR positives. Only two of the four sites (Jekyll Island, Sapelo Island)

exhibited consistant repeatability. The prevalence was 0.0% for Bulloch County, 0.0% for Emanuel County, 1.6% for Jekyll Island, and 16.6% for Sapelo Island (Table 1).

Statistical Analysis

A G test showed there was a significant difference in the infection prevalence among the four locations ($P<0.0001$), and between Sapelo Island and Jekyll Island ($P<0.0001$), as well as Sapelo Island and Bulloch County ($P<0.0001$). There was no significant difference between Bulloch County and Jekyll Island ($P=0.0780$), Bulloch County and Emanuel County ($P=1.0000$), Emanuel County and Jekyll Island ($P=.4555$), or Emanuel County and Sapelo Island ($P=0.0133$).

CHAPTER IV

Discussion

The overall prevalence of HGE agent infection in adult *I. scapularis* on the barrier islands sampled in this study (Sapelo and Jekyll) differed significantly from that of the mainland sites (Bulloch and Emanuel counties). Jekyll Island is a rather small island that is connected to the mainland by a land bridge, so ticks in this area may not be adequately isolated to represent a distinct population. In the mainland areas near Jekyll Island (ie. Brunswick), there have been suspected cases of HGE in individuals who have been in contact with ticks. This suggests that the ticks on Jekyll Island may represent a focus of infection within a greater mainland population. However, when compared to the Bulloch County and Emanuel County sites, these ticks still habituate a significantly isolated area, as do the ticks from Sapelo Island, where there is no land bridge present.

There must be a certain amount of bacteremia present in the bloodstream to cause infection of HGE in the host. In considerably isolated areas, such as the Sapelo Island and Jekyll Island sites, there is probably little immigration and emigration of the reservoir ticks or their hosts. Bacteremia levels of infected ticks may be substantially higher than they would be at a mainland location because the population density of ticks is smaller. This might account for the higher prevalence rates in these particular areas.

Complex interactions between fauna and flora within an ecosystem may influence vector and reservoir populations and the potential for the disease to reach epizootic

proportions (Jones et al. 1998). Environmental factors, such as reforestation, habitat encroachment, controlled burns, and deforestation may also be associated with how prevalent tick borne diseases may become. The data from Sapelo Island and Jekyll Island may reflect the effects of geographic isolation or human disturbance on infection prevalence, while infection prevalence at the two mainland sites may be affected by factors such as deforestation, controlled burns, and human disturbance. The HGE agent most likely exists in the mainland areas, but it apparently occurs in naturally low levels that are not detectable by PCR or where there is a much lower population density of tick vectors. Further investigation of *I. scapularis* throughout southeastern Georgia will help to identify infection prevalences of HGE and show how these prevalence rates vary throughout geographically distinct populations.

The presence of HGE infection in *I. scapularis* on Sapelo Island was expected because Magnarelli et al. (1999) reported seropositive cotton mice on this island. A study conducted by Whitlock et al. (2000) demonstrated the prevalence of *Ehrlichia chaffeensis*, the agent of human monocytic ehrlichiosis, in similar areas of Georgia. The prevalence of infection in this study was much higher in areas that were not geographically isolated, showing an opposite trend. The prevalence of HME infected ticks reported by Whitlock et al. (2000) was 9.3% at a mainland site (Ft. McAllister), while the 2 island sites, St. Catherine's Island and Sapelo Island, had prevalences of 0.9% and 0.0% respectively. This may reflect that a known reservoir host of HGE (*Peromyscus gossypinus*) is abundant on the Georgia barrier islands, but that some reservoir hosts of HME (foxes, opossums, etc.) are not.

Inconsistent nucleic acid isolation from the ticks may be associated with variations in *I. scapularis* infection rates. The intensities of infections within the tick are likely to be low on occasion, as well as variable. The margin for variation in the DNA isolation may be because the target 16S rRNA gene may not have been successfully recovered from all of the bacteria in each infected tick. To expect repeatable results is to anticipate at least one gene and thus one organism per aliquot of template used in PCR. While 10 to 100 or more organisms may be present in a single morula in the host, the minimum number that may be found in ticks, or the survival rate of potentially large numbers ingested by ticks, is not known (Dawson 1995). Also, the few molecules of target DNA contained in dilute isolation samples may not have been present in the fraction of the isolated DNA that was used for PCR. While techniques such as Southern blotting are useful in confirming specificity of questionable PCR positives, demonstrating repeatability of even distinct positive results is assurance that positives are not a result of cross contamination (Burket et al. 1999). These data suggest that it may be sensible to randomly re-screen negative ticks and increase the amount of template used as much as 10 fold.

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Table 1: The prevalence of HGE agent in *Ixodes scapularis* ticks collected from four locations in southeast Georgia, based on nested PCR of the 16S rRNA gene.

Collection Site	Total # of Ticks	# Positive	Prevalence
Bulloch County	112	0	0.0%
George L. Smith SP	18	0	0.0%
Jekyll Island	382	6	1.6%
Sapelo Island	151	25	16.6%
Total	**663**	**31**	**4.7%**

Figure 1: A map showing the sample collecting sites in Georgia: Bulloch County, Emanuel County, McIntosh County, and Glynn County.

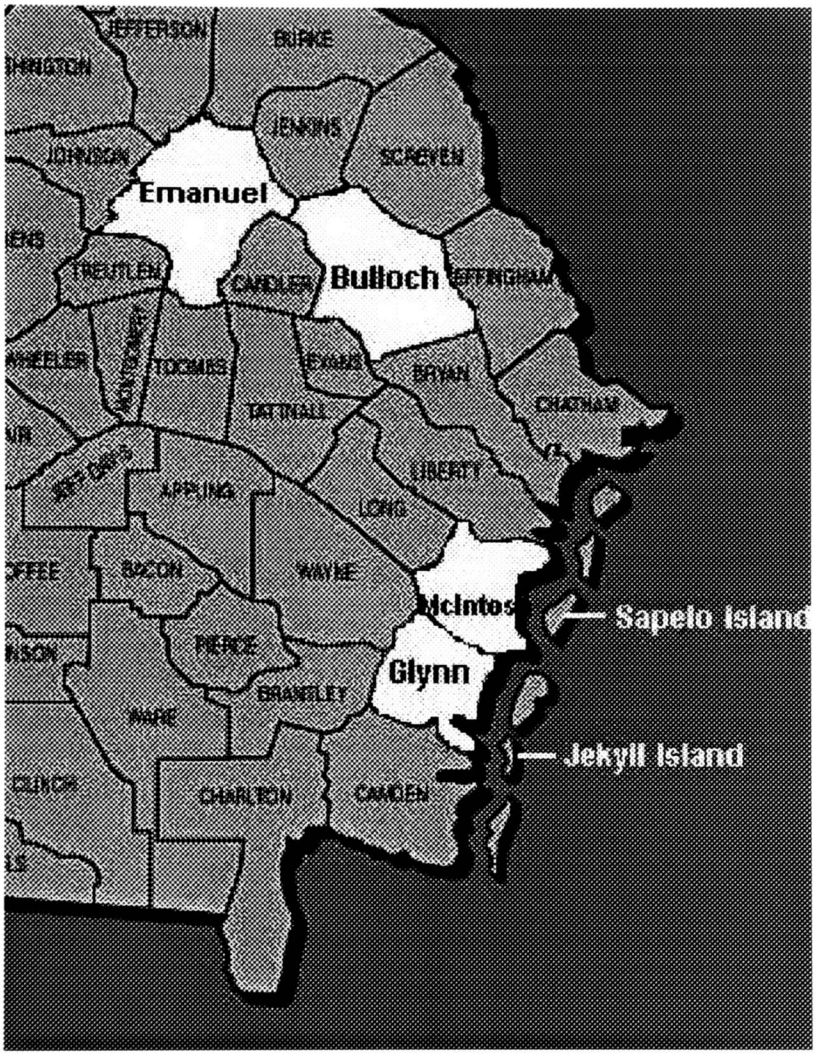
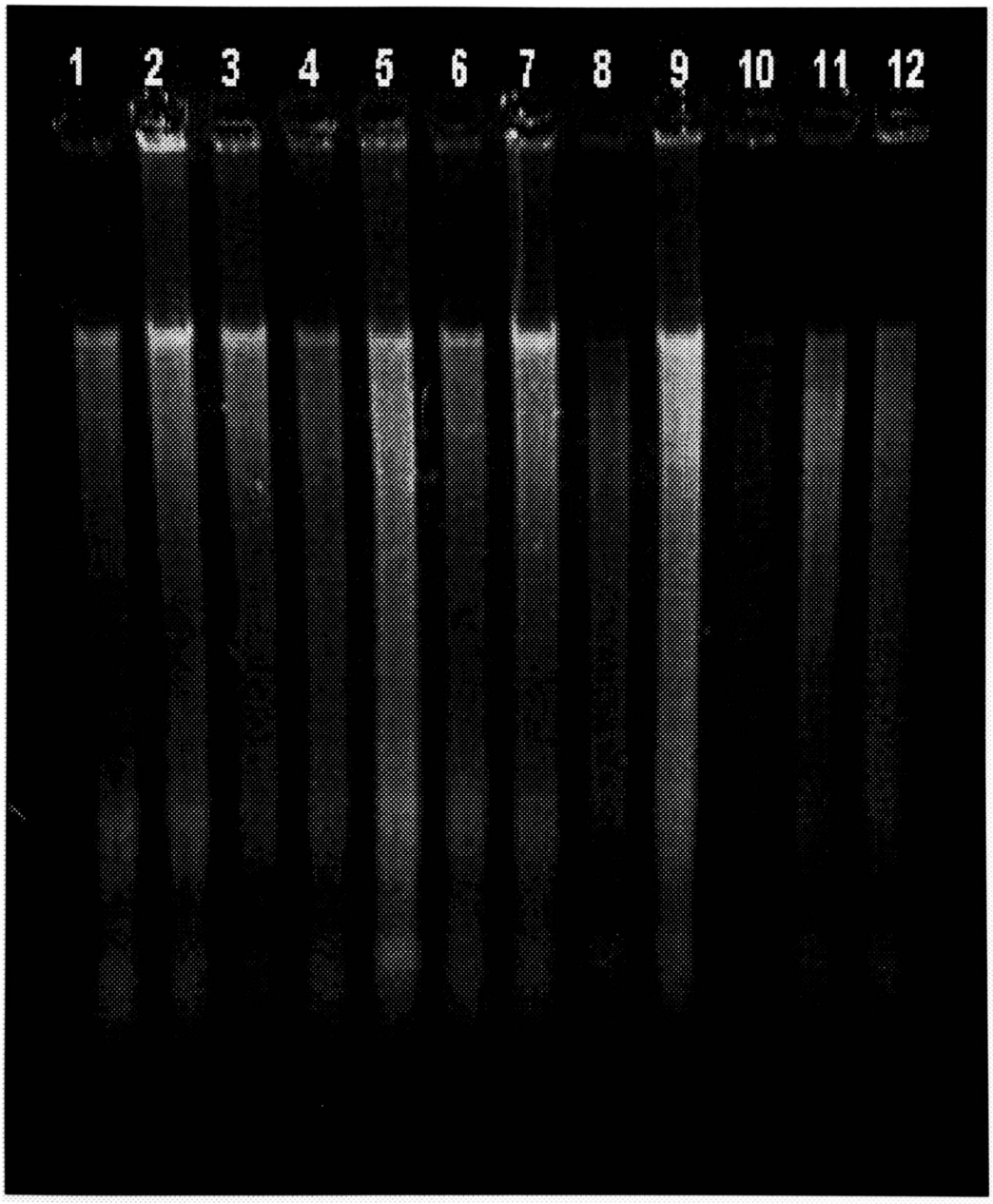
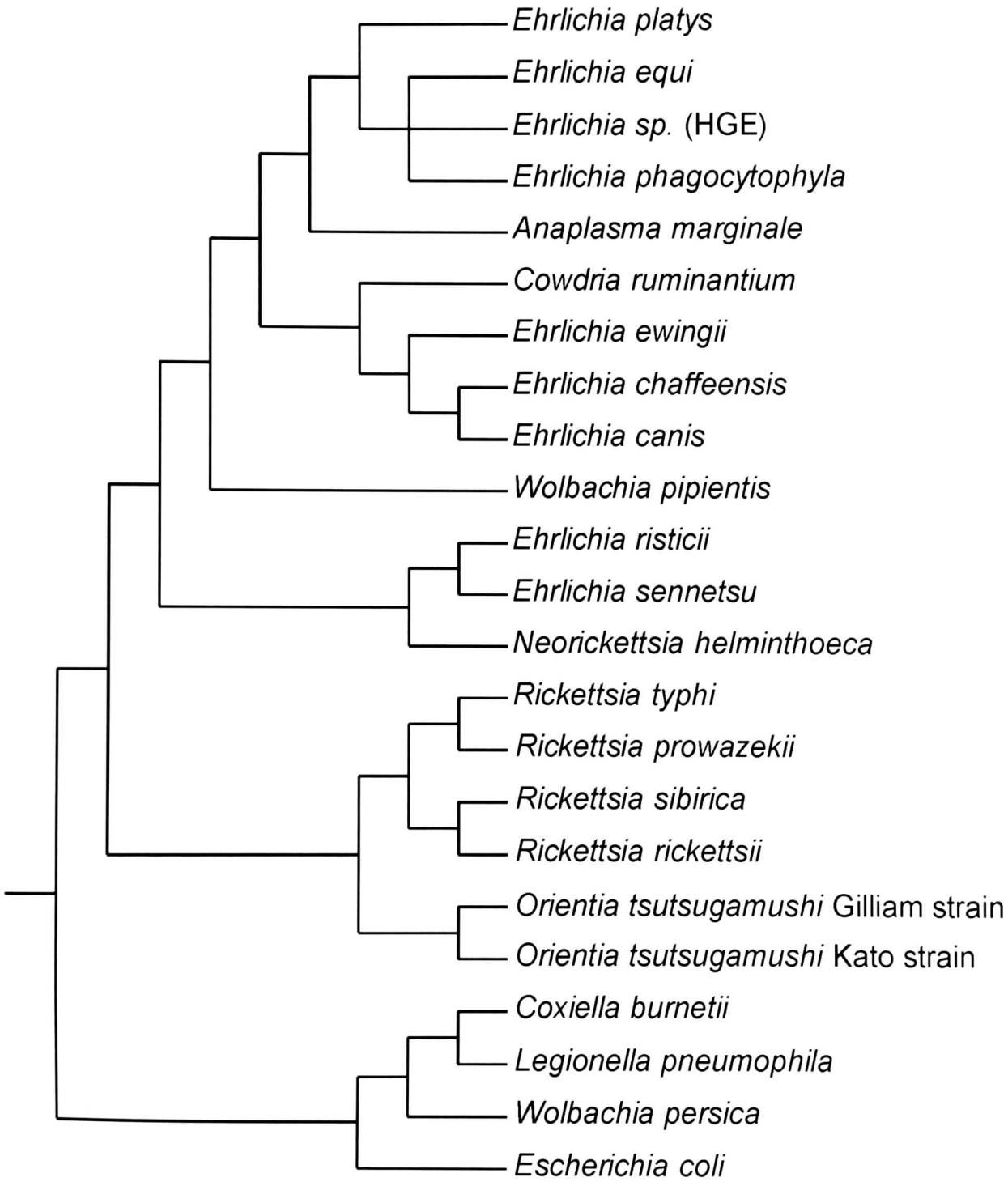


Figure 2: A typical DNA extraction shown on a 1% agarose gel. Genomic DNA is left near the wells of the gel and mitochondrial DNA appears as a sharp band beneath.

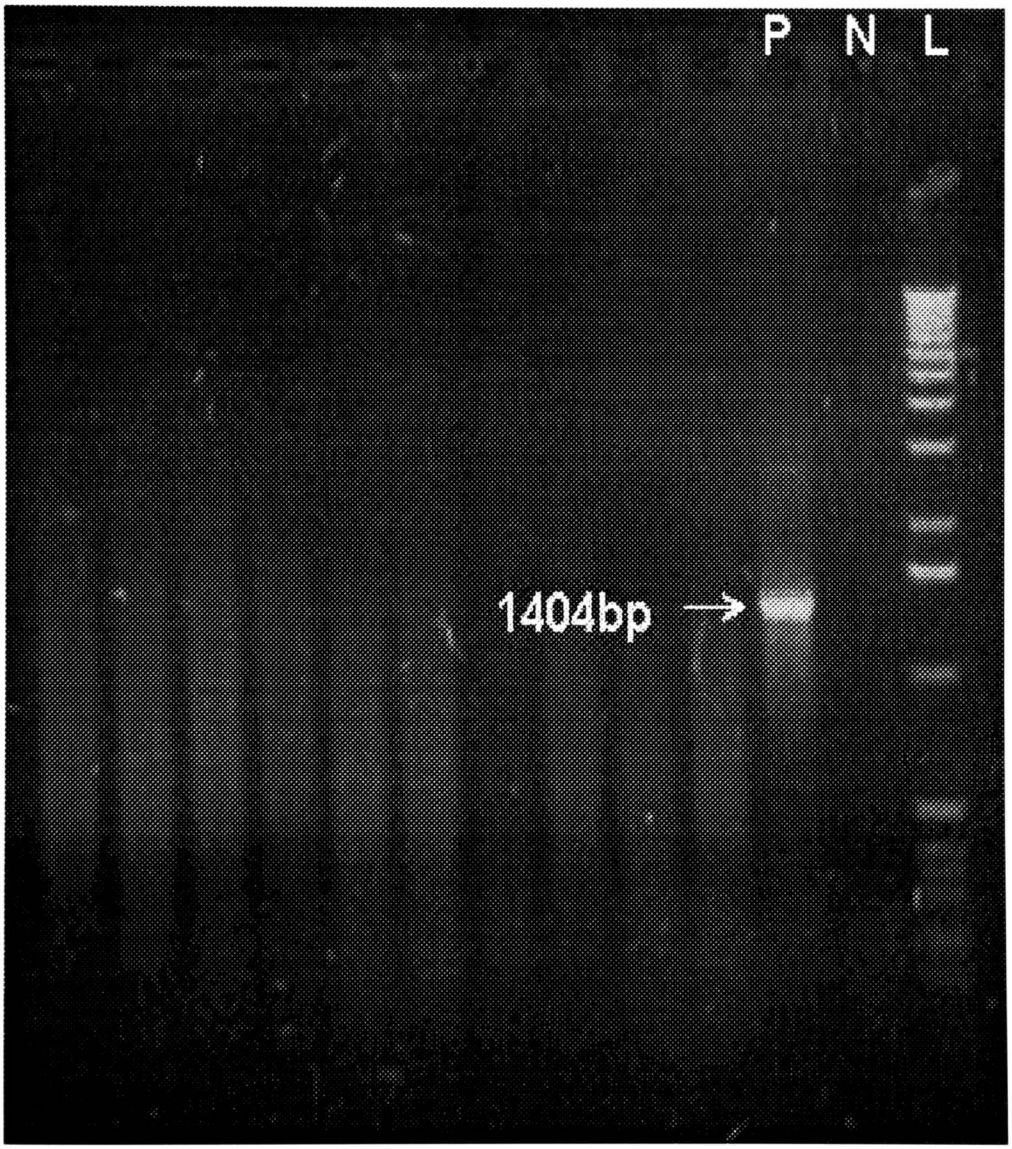
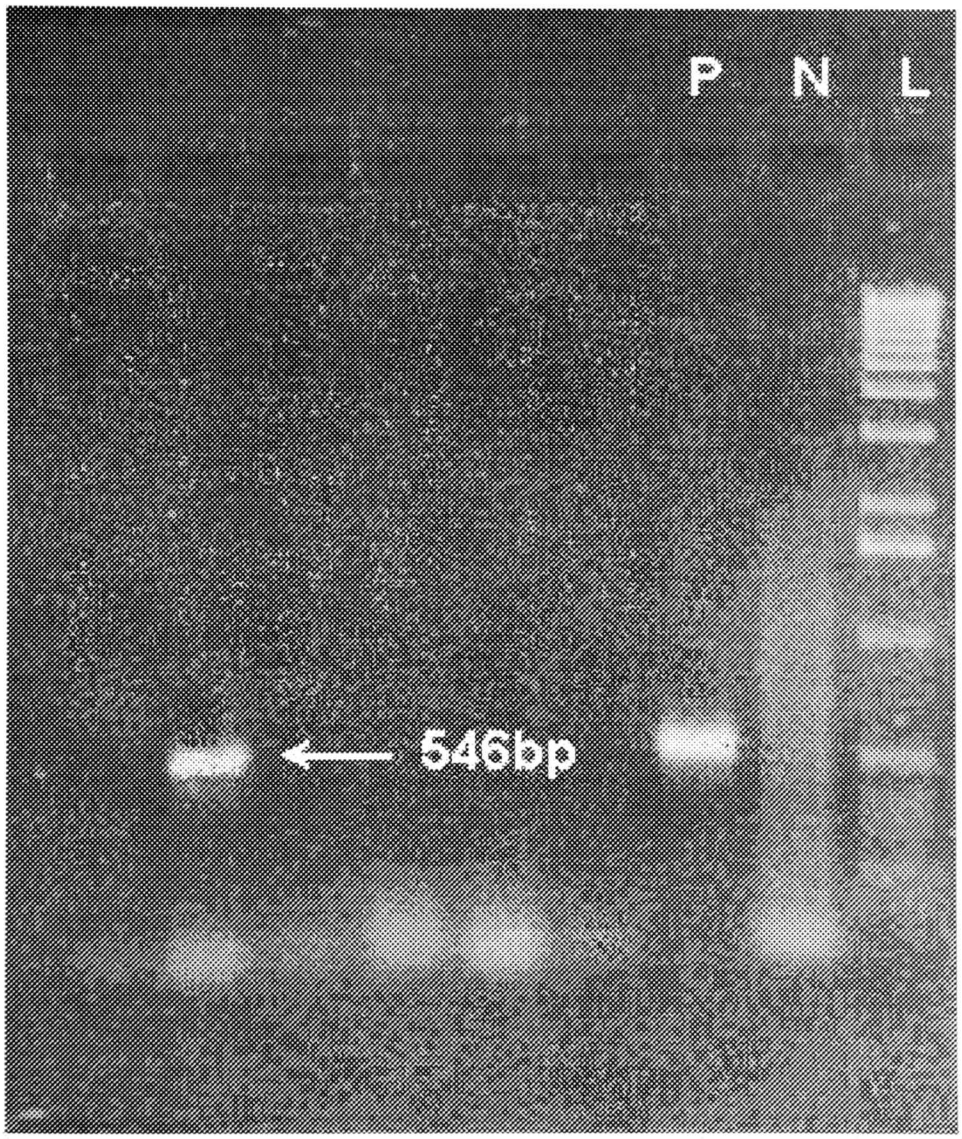


APPENDICES

Appendix A. Phylogenetic tree of *Ehrlichia* spp. in relation to other bacteria based on 16S rRNA gene sequence similarities (after Walker and Dumler, 1996).



Appendix B. Agarose gel image of the initial PCR amplification from a nested PCR assay. Gel lanes (from right to left) contain: molecular weight ladder (L), negative control (N), positive control (P), and 10 tick samples.



Appendix C. Nested PCR reaction that amplified regions from within the 1404bp product produced from the initial PCR. Gel lanes consist of (from right to left): a molecular weight ladder, negative control, positive control, and one positive sample among the 6 screened.