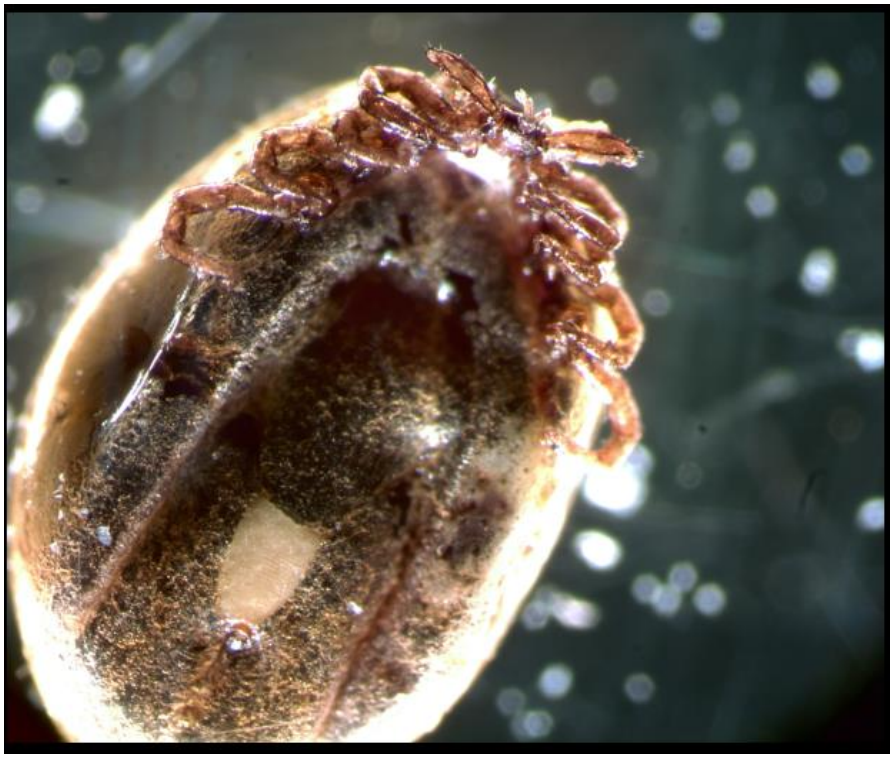
Ticks of the Genus *Ixodes*: Specific identification



Linde Aaftink

Student number 3803473

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Supervisors

J. L. Liotta	Veterinary Parasitology, Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Cornell University
Prof. Dr. D. D. Bowman	
Dr. ing. P. A.M. Overgaauw	Institute for Risk Assessment Sciences, Division Veterinary Public Health, Faculty of Veterinary Medicine, Utrecht University

Table of contents

Abstract

Objectives: Identification of ticks naturally found on domestic cats and thereby confirm that genus *Ixodes* ticks, vectors for *Borrelia burgdorferi*, naturally attach to cats.

Method: The total study group consisted of 54 ticks recovered of domestic housecats collected in the North-East, Southeast and Midwest regions of the United States.

The ticks were morphological determined by microscope and identified as genus *Ixodes* with the presence of anteriorly extending anal groove. DNA extraction took place, followed by amplification of a fragment of the ITS-2 region of rDNA with the use of the polymerase chain reaction. Restriction endonuclease digestion was performed. A molecular genetic key was used to analyze the restriction enzyme digest patterns. At last the samples were sequenced.

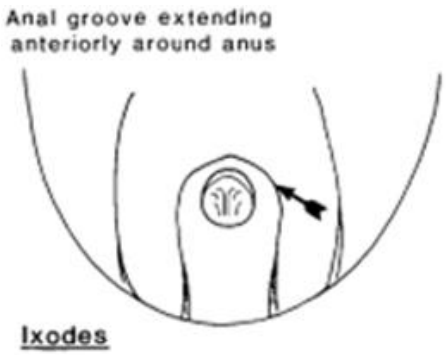
Results: A total of 52 ticks were determined morphologically to be in the genus *Ixodes*, amplification of the ITS-2 region was unsuccessful for 12.2 % of these ticks. A 2 tube nested PCR was also performed on these samples, but again amplification did not occur.

A total of 41 out of 43 ticks restriction endonuclease digested were found to be *Ixodes scapularis.* Very little or no intraspecific variation in restriction fragments banding patterns was detected. Sequencing has shown 2/13 samples were *Ixodes scapulars*, matching parts of the 5.8S rDNA gene. The other 11 samples sequenced gave no unusable sequence data, including the 12.2 % of samples with no amplification. Very little or no intraspecific variation in restriction fragments banding patterns was detected.

Concluded: domestic cats located in the Eastern and Midwest regions and Southeastern parts of the USA, are a natural host for *Ixodes scapularis* ticks.

Introduction

Fig 1: A drawing of the anteriorly anal groove of Ixodes ticks {[1]Kierans]



Ixodidae ticks are hematophagous and are known vectors for pathogens affecting domestic animals, wild animals and people (Bernasconi M.V. et al., 1997)

This family of Ixodidae harbors a total of 14 genera. The genus of interest in this report is genus *Ixodes,* consisting of 246 species. *Ixodes* ticks can be identified morphologically by an anal groove extending anteriorly around the anus, see picture 1.

Although dichotomous morphological keys are available for species identification, separate keys are needed to identify larvae, nymphs, adult females and adult males. In the process of tick removal, structural tick parts used for morphological identification can be damaged, what can make identification of the ticks range from difficult to impossible.

Using a species-specific dichotomous key based on the sizes of digested DNA fragments overcomes the difficulties described above. (Poucher K.L. et al., 1999)

***Ixodes* ticks as a vector**

Ixodes ticks are known to be a vector for multiple bacteria and viruses. Studies demonstrate wild-caught *Ixodes scapularis (I. scapularis)* harbor a wide diversity of zoonotic pathogens including *Borrelia burgdorferi*, *Anaplasma* spp, *Bartonella* spp, *Babesia* spp and *Mycoplasma* spp. (Scott J.D. et al., 2016)

In Europe, the tick-borne encephalitis virus (TBEV) is transmitted by *Ixodes* ticks. This virus consists of three subtypes, each differing in geographical distribution, clinical disease manifestation and tick vector. The western European subtype, formerly known as the Central European encephalitis virus (CEEV), is vectored by *Ixodes ricinus.*

Ixodes persulcatus is a vector for two of the TBEV subtypes: the Seberian sybtype, (formerly known as the West Siberian virus) and the Far Eastern subtype (formerly known as the Russian Spring Summer encephalitis virus (RSSEV)). (Vilibić-Čavlek T, et al., 2014)

In the US *Ixodes* ticks are a vector for two distinct genotypes of the Powassan virus, the deer tick virus (DTV) and the Powassan virus (POW) (Anderson et al., 2012).

Many of the same tick-borne pathogens can affect humans, dogs and other animals. An example of such a pathogen is *Borrelia burgdorferi*; isolates of this pathogen cause Lyme disease. (Little S. et al., 2010)

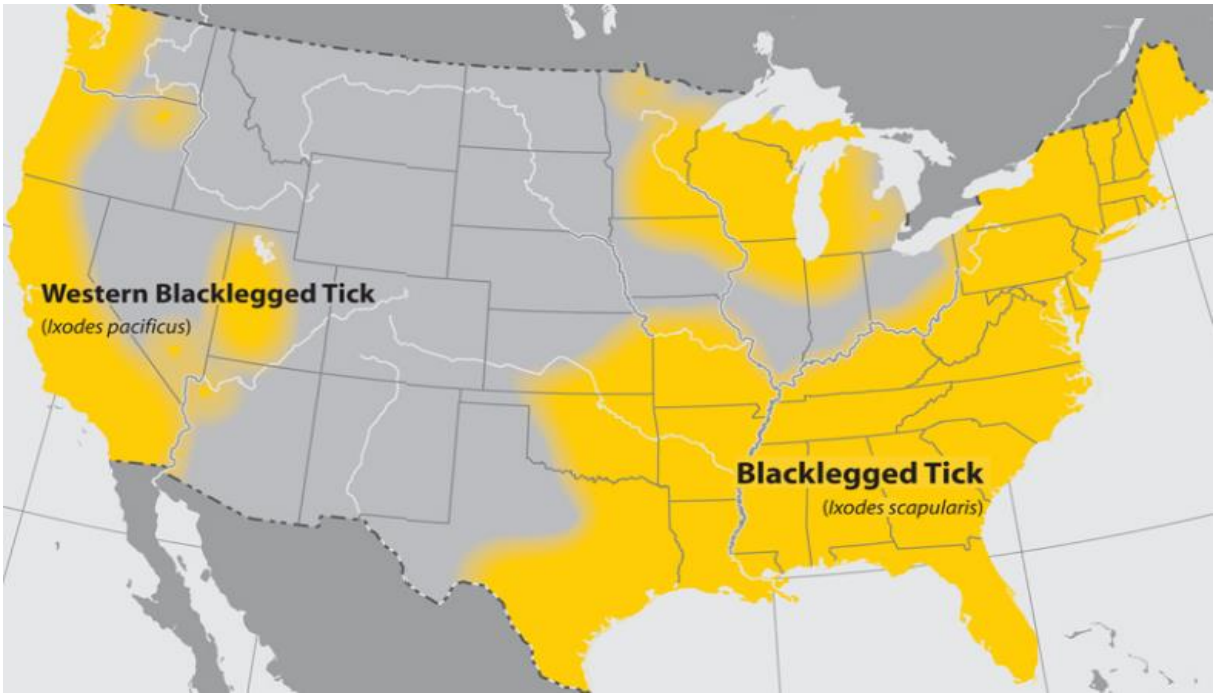
This study focuses on Ixodes ticks as a vector for Lyme disease, the tick-borne disease most commonly prevalent in humans and dogs in the USA (Little S. et al., 2010). A total of 17 *B. burgdorferi* isolates are associated with the disease. Altogether these 17 isolates are generally referred to as the *B. burgdorferi* sensu lato complex. In the eastern United States *B. burgdorferi* sensu stricto is currently the only species considered pathogenic in dogs and humans. (Bacon R.M. et al., 2008) (Krupka I. et al., 2010)

Eight species of *Ixodes* ticks are known to be involved in the transmission of the bacterium in the USA: *Ixodes angustus, I. dentatus, I. jellisoni Cooleyi , I. minor, I. pacificus (I. pacificus), I. scapularis (I. scapularis), I. spinipalpis, and I. uriae*. (Poucher K.L. et al., 1999)

I. scapularis (commonly known as the deer tick) is the most common vector of *B. burgdorferi* sensu stricto for the Eastern and Midwest regions of the USA, while *I. pacificus* (commonly known as the Western black-legged tick) is the most common vector for the Pacific Coast region, see map 1. (Poucher K.L. et al., 1999) (Bacon R.M. et al., 2008)

These two most common vectors for the pathogen both have three-host life cycles (Diuk-Wasser et al., 2006). This means that after each completed life stage, the tick typically molts on the soil before attaching to another host.

Map 1: Area's in the USA were *I. pacificus* and *I. scapularis* are most prevalent (CDC[2]., 2015)



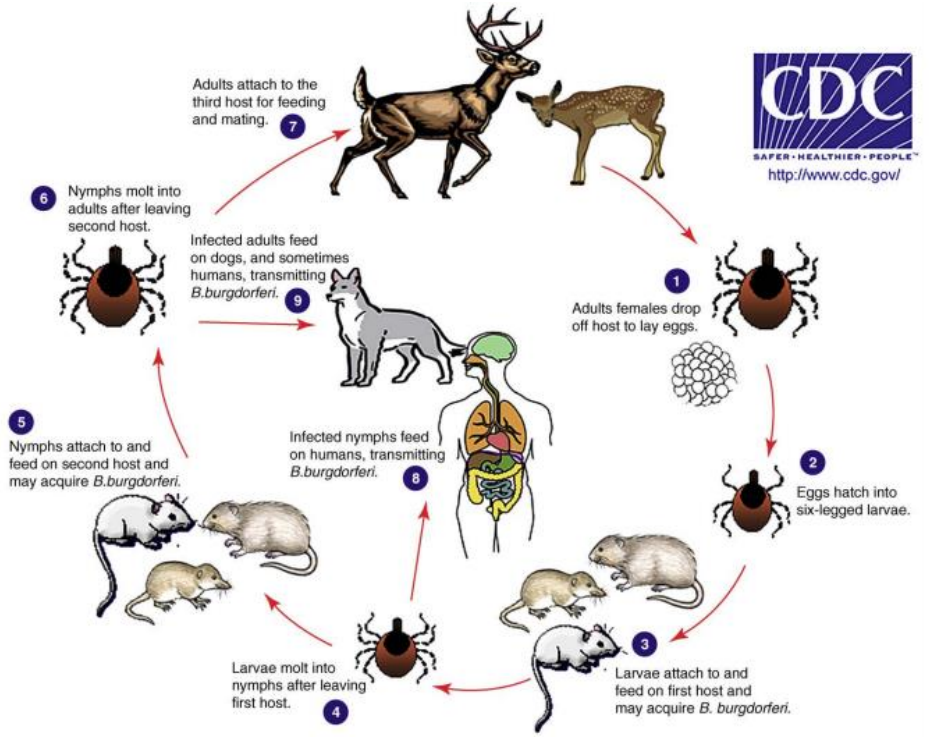
Depending on host availability, hosts can be a variety of animal species. When host encounters are limited, ticks can complete all three life stages utilizing a single host species in order to survive. (CDC[1]., 2013)

The main reservoir host for juvenile ticks is the white footed mouse (*Peromyscus leucopus*) and the eastern chipmunk (*Tamias striatus*). It is known that *I. scapularis* feeds on at least 125 species of North American vertebrates, 14 lizard species, 54 mammalian species and 57 avian species. (Hamer S.A. et al., 2011) ([2]Keirans J.E. et al., 1996)

Ixodes ticks stay attached to their host up to several days while feeding. The life cycle may often span three years while the tick ages from the larval stage to nymph, to adult; however, the life cycle can be completed in less than three years depending on environmental conditions. (CDC[1]., 2013)

Most human infection occurs through the bite of an infected nymph. Humans may serve as a first, second or third host, see figure 2. (CDC[1]., 2013) It is thought dogs are primarily infected by adult ticks (Little S. et al., 2010).

Fig 2: Lifecycle of *B. burgdorferi* in ticks and hosts



***Ixodes* ticks as a vector for *B. burgdorferi* sensu stricto**

B. burgdorferi sensu stricto relies on the host for metabolic products and body heat, 30-42°C, and cannot survive in the environment without a host (Krupka I. et al., 2010).

Ticks get infected with *B. burgdorferi* while feeding on infected hosts, since no transovarial transmission of the pathogen occurs (Kung F. et al., 2013) (Magnarelli L.A. et al., 2005).

When a non-infected tick is attached to an infected host, the bacterium migrates from the vertebrate host dermis into the gut of the tick. This process takes place within the first 36 hours of tick-host attachment.

When *B. burgdorferi* arrives in the tick's gut, most of the bacteria colonize the gut lumen and remain there until the next blood meal. A fraction of the pathogens invade the salivary glands, causing *B. burgdorferi* to be transmitted with tick saliva. (Kung F. et al., 2013)

Once a tick acquires *B. burgdorferi*, the tick remains infected for life. *B. burgdorferi* continues to persist in the gut lumen during molting periods and throughout the remaining developmental stages. (Pal U. et al., 2001)

To establish transmission of *B. burgdorferi* to the host, the pathogen must recognize the interaction between tick and host. The transmission of the pathogen begins when the tick is taking its blood meal, *B. burgdorferi* rapidly multiplies in the gut's lumen. A population of phenotypically diverse bacteria is generated. A fraction of this population crosses the epithelial barrier of the tick's gut and travels to the hemocoelic space, invading the tick's salivary glands. The precise mechanism for *B. burgdorferi* crossing the epithelial tissues is still unknown. (Kung F. et al., 2013) *B. burgdorferi* is transferred to the host dermis trough the tick's saliva (Piesman J. et al., 1993) (Piesman J. et al., 2001). The pathogen disseminates out of the host dermis to several internal organs, heart, nervous system and joints (Kung F. et al., 2013).

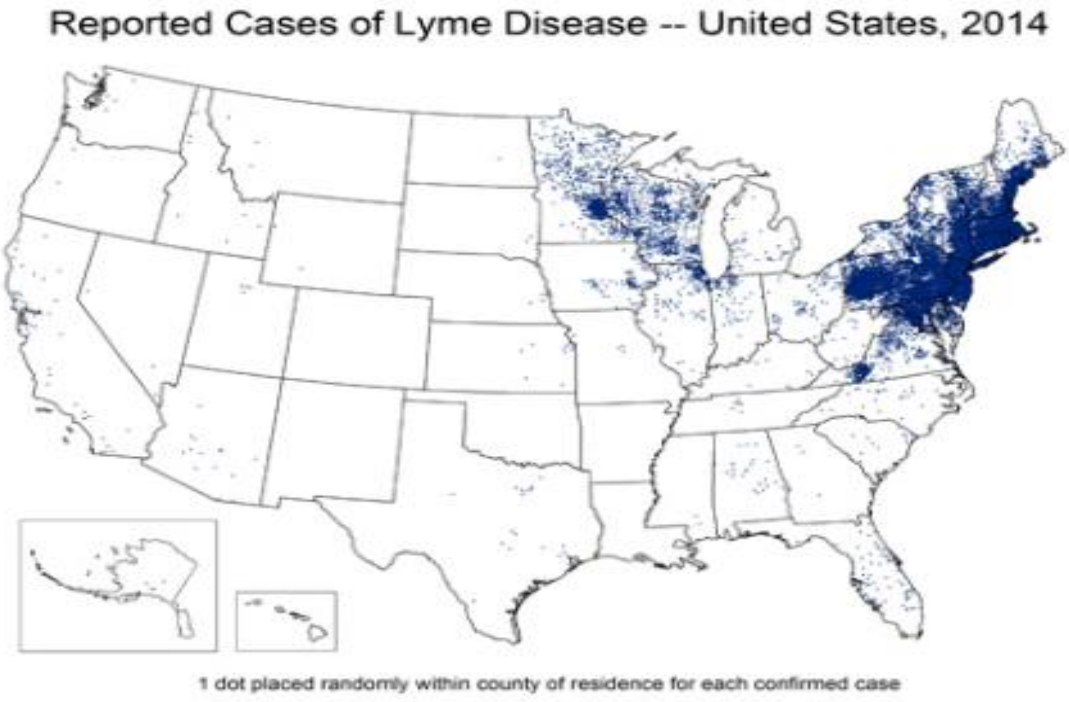
Recent studies in the Northeast and Midwest USA have shown prevalence rates of *B. burgdorferi* in *I. scapularis* adults ranging from 27-47 % (Scott J.D. et al., 2016) (Hutchinson M.L. et al., 2015) (Turtinen L.W. et al., 2016). Generally, the infection rate of *I. scapularis* increases with the life cycle of the vector (Krupka I. et al., 2010).

Lyme disease and its importance

Lyme disease is a multisystem, emerging disease and occurs in humans and animals in North America, Europe and Asia (Bacon R.M. et al., 2008). Since the endemic areas of *B. burgdorferi* sensu stricto are growing, incidences of the disease have increased (Marconi R.T. et al., 2010). This disease is one of the most commonly reported vector-borne illnesses in humans in the United States of America (USA). (Beard C. et al., 2014) (Poucher K.L. et al., 1999) In 2012 Lyme disease was the 7th most common reportable condition; there were over 30,000 reported human cases at the Centers for Disease Control and Prevention. (Beard C. et al., 2014)
However this disease does not occur nationwide and is concentrated heavily in the northeast and upper Midwest of the US, see map 2. (CDC[3]., 2015)

In 2014, a total of 96% of the confirmed cases of Lyme disease were reported from 14 states: Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, Virginia and Wisconsin. (CDC[3]., 2015)

Map 2: The geographic origin of confirmed human Lyme disease cases in 2014 (CDC[3]., 2015)



This regional occurrence can be explained by the presence of lizards in the states where *B. burgdorferi* is not as endemic. Immature *I. pacificus* larvae and nymphs feed heavily on the Western Fence Lizard (*Scleroporus occidentalis*). This lizard is native to California, Oregon, Washingon, Nevada, Idaho and Arizona, and appears to be unable to support *B. burgdorferi* infections. (Lane R.S. et al., 1998)

The lizard's vector incompetence for *B. burgdorferi* can be explained by a study by Kuo et al., 2000. The alternative complement pathway of reptilian sera was observed. Sera of two lizard species, the Western fence lizard (*Sceloporus occidentalis*) and the southern alligator lizard (*Elgaria multicarinata*), were inoculated in vivo with cultured *B. burgdorferi* bacteria. Complement-mediated killing was observed in the sera of these lizard species. The same experiment was performed on mouse and human's sera, where no complement-mediated killing was observed. (Kuo et al., 2000)

This lizard reservoir incompetence for *B. burgdorferi* impedes the vectorial capacity of *I. pacificus* and therefore reduces the risk of transmitting the pathogen to people located in western North America. (Lane R.S. et al., 1998)

Concerning the geographic variation and prevalence of *I. scapularis* and *B. burgdorferi,* immature *I. scapularis* larvae feed heavily on lizards in the southeast of the USA. The prevalence of *B. burgdorferi* is much lower in these areas. (Maupin G.O. et al., 1991)

Lyme disease symptoms

Clinical symptoms in the human

Lyme disease is known for its lack of a clear clinical case definition. Human clinical manifestation of Lyme disease can be associated with cardiac, dermatologic, neurologic or musculoskeletal abnormalities. (Bacon R.M. et al., 2008)

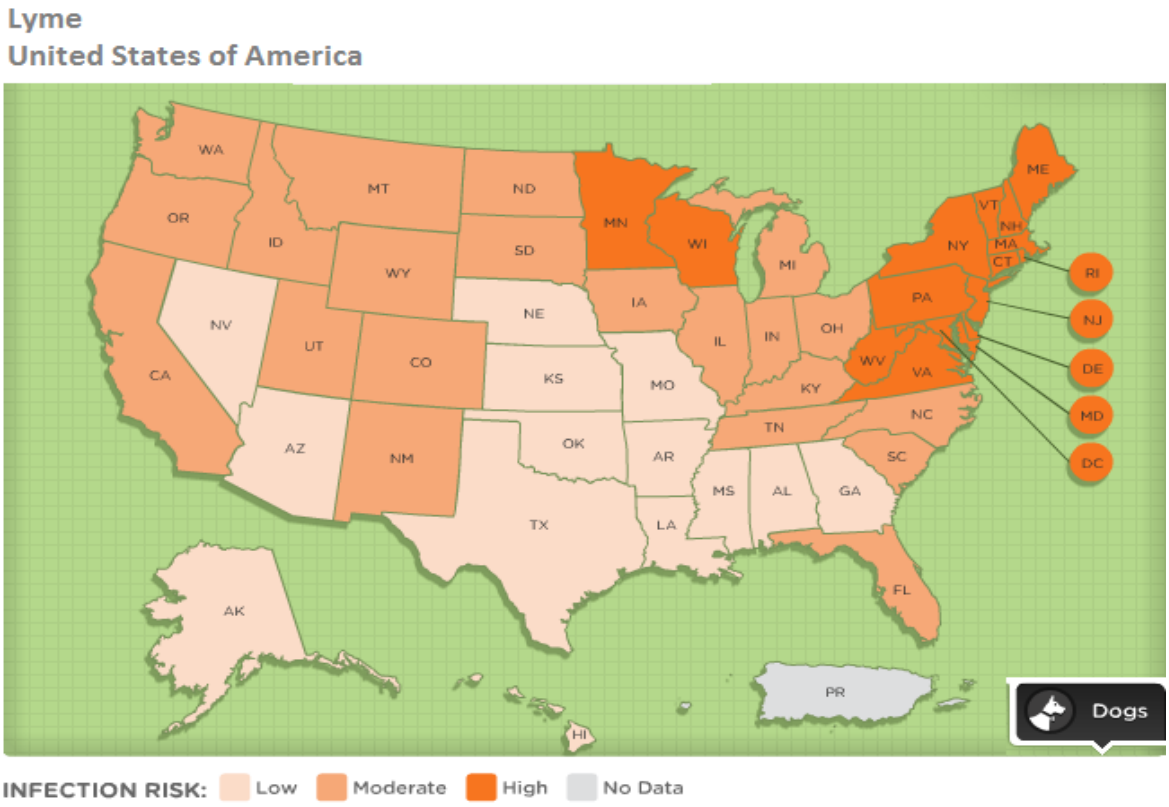
Approximately 70% to 80% of reported patient cases develop erythema migrans (EM) within 30 days of infection with *B. burgdorferi*. This characteristic rash is red and expands radially, forming a clearing at its center. This rash is often accompanied by arthralgia, symptoms of fatigue, fever, headache, mild stiff neck of myalgia. Without treatment, the infection can spread to other parts of the body within days to weeks. Neurological conditions (facial palsy, radiculopathy and meningitis symptoms) and cardiac abnormalities (carditis with an atrioventriculair heart block) can occur. If the infection remains untreated, mono- or aligoarticular arthritis can develop. (Bacon R.M. et al., 2008) Clinical manifestations within individual patients vary, due to variations in immune response among patients. (Marconi R.T. et al., 2010) Because the lack of a clear clinical case definition of this disease may cause misdiagnoses, many experts believe that the true number of cases is much higher(Bacon R.M. et al., 2008). The use of antibiotic treatment is available but not always successful (Marconi R.T. et al., 2010).

Clinical case signs in animals

Lyme disease in domestic animals is difficult to diagnose due to the lack of a clear clinical case definition. Signs associated with the disease can include anorexia, fever and malaise. (Magnarelli L.A. et al., 2005)

The majority of 95% of the dogs testing positive for Lyme disease, remain clinically normal. No characteristic erythema migrans rash, as seen in human infections, develops in dogs. Often illness is not apparent until after the bacteria is disseminated through the body. When the disease develops a clinical perspective, arthritis, fever, anorexia and lymphadenopathy are the most common signs observed. In some cases, glomerulonephritis develops. (Little S. et al., 2010) In 2015, 1 of every 16 dogs tested is found to be positive for Lyme disease (250,880 positive cases out of 4,062,155 dogs tested). The infection risk of Lyme disease for dogs differs in each state, see Map 3. (CAPC., 2015)

Map 3: The geographic risk of infection for Lyme disease in dogs, 2015 (CAPC., 2015)



Lyme disease in cats

There are no natural clinical cases of Lyme disease described or reported in the literature so far in cats. It is known that cats do suffer from *Ixodes* species tick bites. Experimental infections with the pathogen resulted in short-lived bacteremia. (Krupka I. et al., 2010)
The following studies discussed provide a short review of *B. burgdorferi* infections in cats.

In a study in the UK by Shaw S.E. et al., 2005, the prevalence of *B. burgdorferi* sensu lato is determined in the blood samples of 60 cats, using Polymerase Chain Reaction (PCR) targeting. One or more of the following criteria was recorded in each cat: anaemia and/or thrombocytopenia, splenomegaly/lymphadenopathy, acute or recurrent pyrexia, intraocular inflammation with systemic signs or polyarthritis/muscle pain.
B. burgdorferi sensu lato was detected in two cats, which provides molecular evidence of naturally occurring *B. burgdorferi* sensu lato infections in cats. However, no statistically significant associations were found between the infection and the clinical signs. (Shaw S.E. et al., 2005)

There have been cats infected experimentally. A study by Lappin M.R. et al., 2015, demonstrated an antibody response of cats against *B. burgdorferi*, no clinical signs of illness were observed. In this study, wild caught *I. scapularis* ticks (n=25) were fed experimentally to 4 cats. *B. burgdorferi* DNA was present in 50% of the ticks. All 4 cats exhibited an antibody response against the pathogen. However, no significant clinical signs developed. (Lappin M.R. et al., 2015)

In another study,by Burgess E.C. et al., 1992, 15 cats were experimentally inoculated with *B. burgdorferi*. The majority of the inoculated cats developed an IgG antibody response to the pathogen. No histological abnormalities or clinical signs were observed in any of the inoculated cats. (Burgess E.C. et al., 1992)

These results along with lack of documentation of clinical disease may suggest *B. burgdorferi* sensu lato is not a primary pathogen in cats, explaining a lack of clinical appearance.

Study objectives

The aim of this study is to identify ticks found on domestic cats located in the Eastern and Midwest regions and Southeastern parts of the USA.

Morphological determination, genetic analyses and sequencing will be performed. With these results, it can be assessed if the two most common vectors for *B. burgdorferi* sensu stricto in the US, *I. scapularis* and *I. pacificus,* are naturally occurring on cats. If so, this will eliminate the question if cats are not (regularly) bitten by the main two vectors for *B. burgdorferi* sensu stricto, and therefore not having infections and clinical signs.

The research question during this research project is as follows: Do the two most common vectors for *B. burgdorferi* sensu stricto in the US, *I. scapularis* and *I. pacificus*, naturally feed on domestic cats located in the Eastern and Midwest regions and Southeastern parts of the USA?

The results can and will be used in further studies to determine the correlation between the ticks used in this study and whether they are infected with *B. burgdorferi* sensu stricto.

The ticks used in this study will be tested in the near future for containing *B. burgdorferi* DNA.

Materials and Methods

Tick specimens collection

A total of 54 intact ticks recovered of domestic housecats were collected in the North-East, Southeast and Midwest regions of the United States (see Map 4). The ticks were removed by the cat's owners and sent to the parasitology laboratory at Cornell University.

A completed information sheet was submitted with each tick (see Attachment 1). On this information sheet it was noted if the tick was walking or feeding on the cat when recovered. In addition, acquisition date and the location where the tick was found was noted.

Upon arrival at the laboratory, each tick was given a number, FHC1-FHC54, and data capture was maintained in an excel file. Ticks were stored dry at -20°C until examined and processed.

Morphology

Each tick was identified through morphological examination, using an Olympus dissection microscope with magnification 40x. Ticks were manipulated with insect forceps to reduce damaging appendages such as legs and mouth parts. A tick was identified as a member of the genus *Ixodes* by observing the characteristic anal groove extending anteriorly around the anus. Dorsal and ventral pictures of each individual tick were taken using a SPOT 14.2 camera by Diagnostic Instruments Inc. Sex or age determination was not performed.

Map 4: Geographic origin of ticks submitted

DNA extraction

Each individual tick was placed on a clean slide and cut in half with a new razor blade, and transferred to a 1.5 ml Eppendorf tube. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen), with slight modifications to the manufacturer's instructions as per personal communication with Lorenza Beati (Curator U.S. National Tick Collection, Georgia Southern University, Statesboro Georgia), see attachment 2. Extracted DNA was stored at −20°C, until further processing.

PCR, amplification of the ITS-2 Region

To identify each tick genetically, the polymerase chain reaction (PCR) was performed initially using the reagents and thermal cycling conditions for amplification of the Internal Transcribed Region(ITS) region as described by West D.F. et al., 1997, along with conserved forward primers 5.8s (5'-YTG CGA RAC TTG GTG TGA AT-3') and reverse primers 28s (5'-TAT GCT TAA RTT YAG SGG GT-3') with (Y= C/T, R=A/G, S=C/G), produced by INVITROGEN (as described by Poucher K.L. et al., 1999). These primers amplify a ~900 bp fragment of the ITS region that extends from the 3' half of the 5.8S gene to the end of the 28s gene.

For confirmation of test validity, one positive and one negative sample were used in each PCR reaction. The template DNA of a known *I. scapularis* tick (source: National Tick Research and Education Resource, Oklahoma State University) DNA extraction date 10-29-'08, served as the positive control sample. The negative control contained all reaction materials except template DNA.

To test the amplification and Restriction enzyme [RE] digest, a test run was performed using the positive control *Ixodes scapularis* and a morphologically identified species of *Ixodes pacificus* (source: National Tick Research and Education Resource, Oklahoma State University). Both RE digest patterns matched the genetic key.

A total of 1 µL extracted tick DNA was transferred to 49 µL PCR reaction mixture, using sterile aerosol barrier pipet tips. The PCR reaction contained the following reagents concentrations: 1 µM primer 5.8s F (5'-YTG CGA RAC TTG GTG TGA AT-3'), 1µM primer 28s R (5'-TAT GCT TAA RTT YAG SGG GT-3'), 1 U Taq polymerase, 1X PCR buffer 1- containing 15mM MgCl2, 200 µM dNTP and molecular grade H20.

Initially, amplification was performed during a program consisting of 10 cycles [denaturation step at 92 °C for 1 min, annealing at 50 °C for 1 min and an elongation step at 72 °C for 2 min] directly followed by 30 cycles: 30 cycles [denaturation step at 92 °C for 1 min, annealing at 48 °C for 35 sec and an elongation step at 72 °C for 2 min] with a final extension step at 72°C for 7 minutes.

PCR optimizations were performed to improve the samples amplification. PCR buffer 1 was replaced by 1X 5Prime MasterMix-2.5X (#2900177), containing the following reagents and concentrations: 2.5 mM self-adjustable MgCl2, 200 µM dNTP, 45 mM KCL and 1.0 U Taq DNA polymerase, 0.5 µM Primer F, 0.5 µM primer R. Optimal primer concentrations were determined during tests, using [1.0], [0.5] and [0.1] µM concentrations. The test showed that the 0.5 µM primer concentration gave the best amplification results, seen as a strong positive band with no visible nonspecific bands on the 2% agar gel.

Samples with weak PCR amplification products were amplified using twice the amount of template DNA. PCR cycling conditions were optimized; those with the best amplification result were used throughout the project which consisted of a pre-dwell, 1 cycle [94 C 2min] followed by 30 cycles [denaturation step at 94 °C for 30 sec, annealing step at 44 °C for 30 sec min and an elongation step at 72 °C for 30 sec], and a final extension step at 72°C for 7 minutes.

For tick specimens which were unable to amplify the 900-bp fragment and therefore too weak to be useful for RE analysis, a 2 tube nested PCR procedure was performed. The entire ITS region, consisting of 1600 bp, was amplified in a primary PCR using the reagents and conditions used in the original 900 bp PCR amplification with the use of different primers: 18s rDNA Gene 5' –GTA AGC TTC CTT TGT ACA- 3' and of the 28s gene 28SA 5' –GTG AAT TCT ATG CTT AA- 3'. Following this first amplification step, a second amplification was performed. A total of 1 µl of primary PCR product was used as template DNA using the internal primers, 5.8S and 28s to amplify the 900-bp region as previously described.
All PCR products were stored at -20 °C until further use.

Visualization PCR products

PCR products were analyzed by electrophoresis on a 2% agarose gel for 35 minutes with ethidium bromide staining. A GIBCO BRIL Horizon 58 (Form No 14294B) gel Electrophoresis System box was used. An amount of 5 µl PCR product was mixed with 1 µl of 6 × orange loading dye and loaded onto the gel. Band sizes were determined using a molecular weight marker of 25 bp ladder and 50bp. PCR products were visualized by trans illumination under UV-light. Products with amplification, shown as a visible band approximately 900~ bp long on the 2% agar gel, were selected for restriction digest.

Restriction Digest

A double digest was performed (RE Digest), using MspI and CfoI enzymes (Promega Biotech), as described by Poucher K.L. et al., 1999. Each digestion was performed with of a total reaction volume of 20 µl, consisted of 8 µl of PCR product, 0.5 µl of each digest enzyme (10 U/ µl) , 2 µl RE 10X Buffer (Multi-Core: 6mM tris-HCL [PH 7.5], 6mM MgCl2, 50 mm NaCl2, 1mM dithiothreitol), 0.2 µl Acetylated BSA (10mg/ml) and sterile H20. DNA was digested for 3 hours at 37 °C. To inactivate the enzymes, 1 cycle at 65 °C was run/performed for 15 minutes.

The amplified ITS-2 region of *I. scapularis* contains 5 restriction sites located at positions 240, 360, 479, 651 and 768 of the ITS-2 genomic. Using the restriction enzymes, 5 fragments arise of 56, 117, 119, 120, 172 and 240 bp long. Run on a 2% agar gel the bands fractionated in banding patterns. Samples with an RE digest pattern not as discernible as preferred were digested again with twice the amount of PCR product. RE Digest products were stored at -20 °C until further use.

Visualization RE digest products

RE digest products were analyzed by electrophoresis on a 2% agarose gel as previously described. To make the RE digest analyses easier, the gel was run for 70 minutes therefore separating the fragments further. RE digest products consisting of twice the concentration of PCR product as template DNA were run in a gel electrophoresis using a gel deep enough to hold a loading volume of 8 µl digest product. Products were visualized by trans illumination under UV-light.

RE Digest Identification

The RE digest banding patterns were analyzed using a molecular genetic key by (Poucher K.L. et al., 1999), which distinguishes between 17 species of *Ixodes* ticks found in the USA, see attachment 3. The following 17 species are included in the molecular key: *I.scapularis, I. spinipalpis, I. affinis, I. dentatus, I. muris, I. brunneus, I. pacificus, I. texanus, I. uriae, I. angustus, I. jellisoni, I. minor, I. sculptus, I. woodi, I. baergi, I. cookei, I. kingi)*

DNA Sanger sequencing

For samples with faint or indistinct RE digest banding patterns, DNA Sanger sequencing was performed to determine *Ixodes* species. PCR products were prepared for sequencing by removing excess primer using ExoSAP-IT (Affymetrix) following the manufacturer's instructions (see attachment 4).

Each prepared PCR product was sequenced in the forward and reversed directions using the primers (5'-YTG CGA RAC TTG GTG TGA AT-3') and (5'-TAT GCT TAA RTT YAG SGG GT-3') (Y= C/T, R=A/G, S=C/G). Prepared PCR products are sequenced at the Cornell University Institute of Biotechnology Genomics Core Facility using Applied Biosystems Automated 3730xl DNA Analyzer and Big Dye Terminator chemistry.

The resulting sequences, sequences, one forward and one reverse for each sample, were analyzed using Finch TV and CLC sequence viewer 7 to generate a consensus DNA sequence. This resulting DNA sequences was compared to reference sequences in GenBank using the standard nucleotide Basic Local Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (NCBI).

Results

Morphology and notes made at tick intake

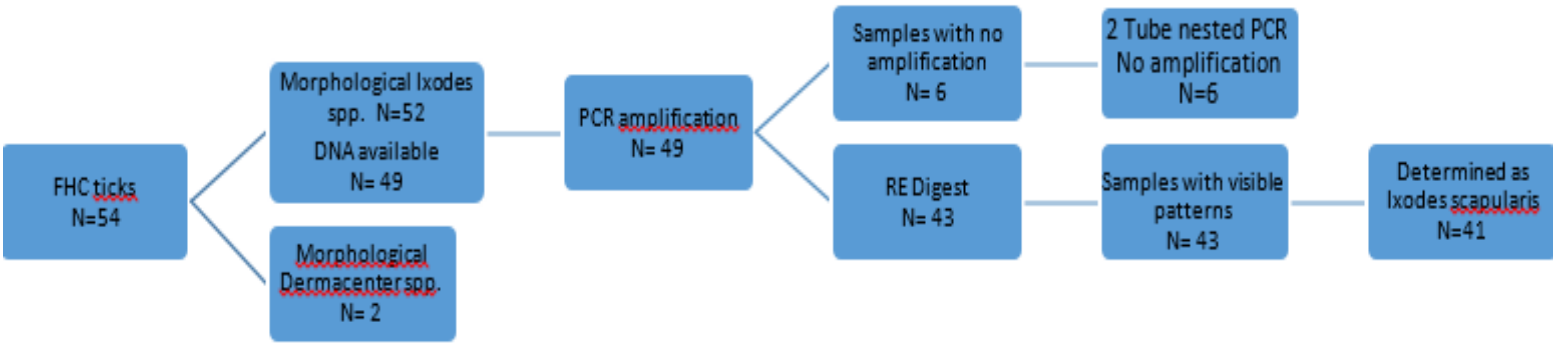
From the total of 54 ticks submitted, 52 were morphologically identified as a species of *Ixodes* (96,3%). The 2 remaining non *Ixodes* ticks were identified as species of *Dermacentor*.

A total of 4 ticks were walking on the cat when removed. The other 50 ticks were embedded and required forcible removal for collection. Out of the 4 unattached ticks, 3 were identified as an *Ixodes* spp, and one 1 as a *Dermacentor* spp. DNA extraction took place on the ticks determined as species of *Ixodes*, N=52. The extracted DNA of 49 ticks was available.

PCR, amplification of the ITS-2 Region

Samples were considered positive when matching the ~900 bp bright band of the positive control. Out of the 49 amplified samples, 43 samples gave an amplification of the expected molecular base pair length. The amplified region ranged in size from 900bp up to 1000bp. The 43 samples with amplification were used for RE digest. A 2 tube nested PCR was performed on the 6 samples that did not amplify in the PCR. After analyzing the 2 tube nested PCR products, none of the 6 samples amplified (12,2%), see graphic 1

Graphic 1: morphological tick determination, PCR and RE digest results



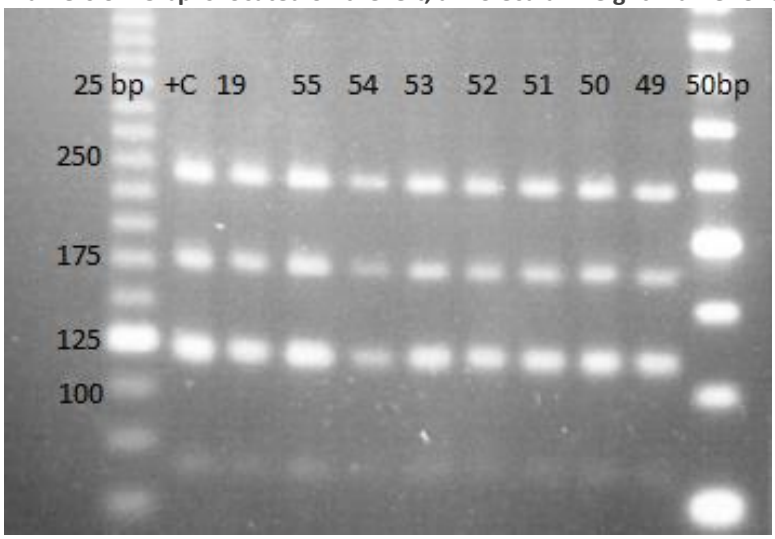
Restriction Digest

The double digest by restriction enzymes Mspi and CfoI of the ITS-2 region yielded distinct banding patterns for 43 out of 43 samples. Digested fragments ranged in size between 100 and 250 bp, with 3 bands per sample as shown in figure 3.

RFLP patterns were species-specific. The RE digest banding patterns with 3 single equidistant fragments between sizes 201-154, 298-220 and 134-75 were identified as *Ixodes scapularis.*

Out of the 43 RFLP band patterns, 41 were diagnostic for *I. scapularis*. The other 2 samples, FHC 32a and FHC32b showed an extra digest band of ~280-300bp. When analyzed using the molecular genetic key, these samples did not match any tick's RFLP banding pattern.

Figure 3: RE digest results of sample 19, 55, 54, 53, 52, 51, 50, 49. +C= positive control. Molecular weight markers of 25 bp is located on the left, a molecular weight marker of 50 bp is located on the right



DNA Sanger sequencing

A total of 13 samples were sequenced. These 13 samples were selected based on vague agar gel bands.

Out of the 13 samples sequenced, 2 samples gave Sanger sequencing results and a usable consensus, see table 1. The other 11 samples did not gave usable consensus, as the sequencing results came up as absent, homopolymeric or deteriorated/noisy. The tick used as the positive control sample, did not sequence properly and the result appeared to be homopolymeric. The 2 ticks with usable sequencing result were confirmed to be genetically identical to *I. scapularis.*

Table 1: Sanger sequencing results for the 2 FHC samples

Sample	Consensus	1st Match in BLAST
FHC 3	ATCATATATCAAGAGAGGAGAATTTGTTTTCTACCTCGTTTTGACTGTGT CGGATCGTGGGCAGCACGCAGTTTTATGCTTTCTTGCGTTGCGTTTTCTT TGAGCAAATGCACGAGTGGTGCGATTGCACGCGTGCGCTTAACCAGTCC TCCTCCTCCTACGAGTTTTTATCGAACACTGCATGGGAAAACGAAACTCG ATGGATACCGTTTGTGGAAAATCCCGTACCAAAAAAATCTTTCGCACGTT GAACGGCGCTGTGACGTCGGTGCGTTAGAAACGGAGATTTGAAACGGT TTCTTTTCGATCGATTCTGTTTTCTTTGGCGTGGATGTTGTTCGAGTGGAA AAACCGTGGGACGGAGTTGGCGTTGGCGCGTTGAAACGCGGCGTCGGC AAATGTAAATCTCGTGGCGTTGATTTGCACAAAAAAAAAGAAAAACGCT TCTGGGAGGAAAGTTTTTTGCGTCGTAGCCTTCCGTCAGTCTAAGACCTT CGCGTCCCCGATGAATACTGGAGCCATCCAGTAGGGGAATGCCGTTGGA TTGCGCGCTTTCTTTTTTGTCGAATCGAAAGCGATGCAAAGCGTGTGTAC AACAATCTGTTAGTTCGCCATCCTTTTGTGTTTTTG	*Ixodes scapularis* isolate D05 5.8S ribosomal RNA gene and internal transcribed spacer 2, partial sequence
FHC 4	GANTCNTCGNCTGTGATGAGGGTCGCATCATATATCAAGAGAGGAGAAT TTGTTTTCTACCTCGTGTTTGACTGTGTCGGATCGNGNGCAGCACGCAGT TTGATGCTTTCTTGCGCTTGCGTTTTCTTTGAGCAAATGCACGAGTGGTGC GATTGCACGCGTGCGCTTAACCAGTCCTCCNCCTCCTACGAGTTTTTATCG AACACTGCATGGGAAAACGAAACTCGATGGATNCCGTTTGTGGAAAATC CCGTACCAAAAAAATCTTTCGCACGTTGAACGGCGCTGTGACGTCGGTGC GTTAGAAACGGAGATTTGAAACGGTTTCTTTTCGATCGATTCTGTTTTCTT TGGCGTGGATGTTGTTCGAGTGGAAAAACCGTGGGACGGAGTTGGCGTT GGCGCGTTGAAACGCGGCGTCGGCAAATGTAAATCTCGTGGCGTTGATT TGCACAAAAAAAAAGAAAAACGCTTCTGGGAGGAAAGTTTTTTGCGTCG TAGCCTTCCGTCAGTCTAAGACCTTCGCGTCCCCGATGAATACTGGAGCC ATCCAGTAGGGGAATGCCGTTGGATTGCGCCTTTCTTTTTTGTCGAATCG AAAGCGATGCAAAGCGTGTGTACAACACCTGTTAGTTCGCCATCCTTTGG TGTTTTTGACCATGTGGTAAGGAGGCGAAG	*Ixodes scapularis* strain 07-038 5.8S ribosomal RNA gene and internal transcribed spacer 2, partial sequence

Discussion

As a result of the completed information sheet for each individual tick, we can determine which ticks were feeding on cats and confirmed to be an *Ixodes* species by RE digest. Out of the 54 ticks available, 52 were morphological determined to be ticks in the genus *Ixodes*. Of those 52 species of *Ixodes*, 43 were identified as *I. scapularis* by RE digest. Of these 43 ticks, 2 ticks were walking on the cats. The other 41 ticks were attached in such a way they had to be forcibly removed from the cat. Therefor we can definitely state that *Ixodes* ticks were embedded in the cat on the moment of collecting by the cat's owner.

The high rate of *I. scapularis* ticks (43/49) found in our study, corresponds with the prevalence data by CDC[2] of *I. scapularis* in the USA. Looking at the map which indicates *I. scapularis* prevalence, see map on page 5, we see most of the ticks in our study are located in the areas where *I. scapularis* is most prevalent (North-east and Midwest of the USA). This explains why most of the ticks in our study (43/49 by genetic analysis) are identified as *I. scapularis*.

Our tick identification results conclude *I. scapularis* naturally attaches to cats. It is known *I. scapularis* is a vector for *B. burgdorferi* with prevalences ranging from 27% to 47 % in *I. scapularis* adults. It is likely *I. scapularis* ticks can transfer *B. burgdorferi* to cats based on reported antibody responses against the pathogen. This supports the hypothesis that *B. burgdorferi* is not a primary acute pathogen in cats, explaining the lack of clinical disease appearance.

Among the 49 ticks morphologically identified as *Ixodes*, a total of 6 ticks (12.2%) did not give any amplification of the ITS-2 region using the PCR and the 2 tube nested PCR.
The method we have used has shown a 10% of samples with no amplification. Therefor our 12.2% can be explained with the 10% non-amplifying samples described in the method we have used. (Poucher K.L. et al., 1999)
Because these samples could not be sequenced since the lack of PCR amplification, it was not possible to genetically determined the *Ixodes* represented by the collected tick.
Because these ticks were identified morphologically as *Ixodes*, it seems likely that the lack of amplification was caused by DNA deterioration or inhibitory contamination (The gene pool, 2009).

Out of the 13 sequenced samples we were able to get a usable consensus from 2 samples. These two ticks sequenced matched parts of the genome of *I. scapularis* .

The lack of sequencing results in the 11 samples can be caused by DNA degradation or inhibitory contaminant in the samples (The gene pool, 2009). DNA contaminated or low DNA concentrations can cause sequencing to be absent.

For the two samples with extra RE digest fragment, sample 32a and 32b, a visible band consisted of approximately 280-300 bp. This digest pattern, consisting of 4 fragments between sizes 300-280, 298-229, 201-154 did not match any of the ticks included in the molecular genetic key.

Morphological examination have shown these ticks are *Ixodes* species. Given the lack of matching to any described tick banding pattern in the genetic key and the morphological identification results, it is likely that these extra bands were caused by DNA deterioration or inhibitory contamination. (The gene pool, 2009).

Studies by Wesson D. M. et al., 1993, and Rich S.M. et al. ,1997, have shown intraspecific and intraindividual variation in the restriction sites of the ITS region of *I. scapularis*. An intraindividual variation in ~300bp of the ITS region is reported by Rich S.M. et al. ,1997, in a set of 8 clones from 2 individual *I. scapularis* specimens. These studies examined single cloned copies of the ITS region.

Our RE digest analysis did not show any intraspecific variation, correlating to the study by Poucher K.L. et al., 1999, where no intraspecific variation was found. The absence of intraspecific variation seems to contradict earlier studies (Wesson D. M. et al., 1993, and Rich S.M. et al., 1997), where intraspecific and individual variation were found in the ITS region of *I. scapularis* ticks.

However our method is based on the RE digest analyses of many copies of the ITS region amplified directly. The RE digest will only determine intraspecific variation between the 5 restriction sites. Insertions, deletions and small-scale substitutions could be present in the rDNA copies, without affecting the overall banding pattern of the ITS region as observed in our study. The earlier studies by Rich S.M. et al. ,1997, and Wesson D. M. et al., 1993, examined the variation between the entire ITS sequence, explaining the documented ITS variation within and among individual *I. scapularis* ticks.

Since our RE digest patterns have not shown intraspecific variation, it is likely no intraspecific variation between the ITS regions existed in the ticks tested for this study.

Future work

In order to make sure that all *Ixodes* species included in the molecular genetic key, Poucher K.L. et al., 1999, are being identified by our RE digest protocol, all species should be tested. This makes a total of 15 species that need to be amplified, RE digested and analyzed using the genetic molecular key.

We were not able to amplify 6 samples by (2 tube nested) PCR. It would be optimal to amplify these remaining 6 samples. Optimizing the 2 tube nested PCR might give usable amplification of the ITS-2 region of *Ixodes* species, making this a future research project.

One of the future goals is to sequence all of the 49 samples. In order to do this, DNA needs to be purified. An method proposed for DNA purification is gel purification, isolating DNA fragments ~900 bp and eliminating non-visual amplified products and small fragments.

Most of the identified ticks were attached to the cat when collected. However, we did not test the ticks for cat blood in their gut. This is one of our future objectives, so that we can state the ticks were definitely feeding on the cats when collected.

Conclusion

A total of 96,3% ticks (52/54) were found morphologically to be members of the genus *Ixodes*. Of the morphologically identified *Ixodes* ticks, 41 were found to be *I. scapularis* by RE Digest. This study has shown that cats located in the eastern, midwestern, and southeastern USA, are natural hosts for *I. scapularis* ticks.

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Attachments

Attachment 1

Cornell Feline Health Center

Thank you for participating in the Feline Tick/Lyme Disease Surveillance Program! Please answer the following questions and include this completed form with your submitted ticks.

Place ticks in **two sealed zip-lock bags, one inside the other** via overnight or priority carrier.

Name:_____

1. County and State where the tick(s) on the cat was collected?

   County: _____

   State: _____

2. Was the tick(s) walking on the cat when collected? **Yes** or **No**

   OR

   Was the tick(s) attached in such a way that it had to be forcibly removed from the cat?

   Yes or **No**

3. What email should we use to send you the final results beginning in early 2016?

   Email: _____

www.vet.cornell.edu/fhc

Mail this form and ticks to:

FHC TICKs
Cornell University
C4-114 VMC
930 Campus Road
Ithaca, NY 14853-6401
607.253 .3394 –for shipping purposes only

Questions?

Email us at: fhcticks@cornell.edu

Attachment 2

Tick DNA extraction protocol

Day 1:

1. On slide, with a scalpel blade (or razor), cut a small fragment of the body of the tick in a way that will leave more than half of the tick intact.
2. Place all contents of the tick inside the Eppendorf vials and add 180 microliters of ATL lysis buffer (check for precipitate).
    a. If the tick is engorged, add 360 microliters ATP lysis buffer to cover the entire tick.
3. Add 40 microliters of the PK (proteinase K) stock solution to each tick.
    a. If the tick is engorged, add 80 microliters ATP lysis buffer.
    b. Vortex and place at 56 degrees C overnight.
        i. If possible, vortex a few times during lysis.

Day 2

1. Add 220 µL of AL buffer to each vial, vortex, and place at 72 degrees (which deactivates PK) C for 10 min.
    a. Add 440 to engorged tick to ensure that it is covered.
2. Add 250 µL (500 µL for engorged tick) of ethanol to each vial, vortex rapidly after adding alcohol, and pipet all the resulting solution into a Qiagen column → 100% ethanol from shelf.
3. Follow subsequent extraction steps:
    a. Spin column at (6,000 x g) → which is setting 8 on our centrifuge, discard the collection vial, then place the column in a fresh collection vial.
        i. The DNA is now on column.
    b. To clean the DNA, add 500 µL AW1 (washing buffer 1), spin the column at setting 8 (1 minute), discard the collection vial, and place the column in a fresh collection vial.
        i. Remember to pipette from surface of bottle (do not let pipette touch the bottom of column).

c. Add 500 µL AW2 (washing buffer 2), spin the column at setting 13 (full speed) for 3 min, discard content of collection vial, replace the collection vial under the column.
    i. Remember to pipette from surface of bottle (do not let pipette touch the bottom of column).
d. Place the column in a clean Eppendorf vial (the one with pointy bottom)***, elute DNA by adding 50 µL of AE Buffer to the column and spinning it at setting 8 (1 minute).
    i. Repeat from ***

Attachment 3

MOLECULAR GENETIC KEY TO 17 IXODES SPECIES IN THE U.S.A. (Poucher et al. 1999)

Gel results after *Ix.* spp. identification PCR and RE digest for PCR *B. burgdorferi* in cat ticks project *I. scapularis*✓, *I. spinipalpis, I. affinis, I. dentatus*✓, *I. muris*✓, *I. brunneus*✓, *I. pacificus, I. texanus, I. uriae, I. angustus*✓, *I. jellisoni, I. minor, I. sculptus, I. woodi, I. baergi, I. cookei*✓, *I. kingi*

spp. found in NY as of 1996 (Durden & Keirans) denoted by ✓ -- all spp. with described *B. burgdorferi* infection in literature are included in PCR ID key (see end of doc for details)

1(0). Fragment present at or immediately above 298. Single fragments also present at or near 220 and between both 201 and 154 and 154 and 134 → *I. uriae*

No fragment at or immediately above 298 → 2

2(1). One or more fragments present between 201 and 154 → 3

No fragment between 201 and 154 → 13

3(2). Only 1 fragment present between 298 and 220 → 4

Two fragments present between 298 and 220. Single fragments present between 201 and 154 (that is, approximately equidistant) at 154, and 2 fragments present between 134 and 75 → *I. brunneus*✓

4(3). Only 1 fragment present between 201 and 154 → 5

Two fragments present between 201 and 154 → 12

5(4). Fragment present at 154 → 6

No fragment at 154 → 7

6(5). Single fragment present between 201 and 154 that is closer to 201. Position of fragment between 298 and 220 is approximately equidistant; fragments also present at 154 and between 134 and 75 → *I. texanus*

Single fragment present between 201 and 154 that is closer to 154. Fragments also present between 298 and 220 (that is approximately equidistant), at 154, and between 134 and 75 I → *I. angustus*✓

7(5). Fragment present at 201; single fragments also present between 298 and 220, between 201 and 154 (but closer to 154), and 3 fragments between 134 and 75, the uppermost of which is immediately below 134; the other 2 fragments of these are closer to 75 than 134 → *I. kingi*
No fragment at 201 → 8

8(7). Fragment present immediately above 201. Two fragments also present between 201 and 154; fragments also between 298 and 220, at or near 134 and between 134 and 75 (that is closer to 75) → *I. jellisoni*
No fragment between 220 and 201 → 9

9(8). Fragment present at 134. Fragments also between 298 and 220, immediately below 201, and between 134 and 75 → *I. pacificus*
No fragment at 134 → 10

10(9). Single fragment between 201 and 154 that is immediately below 201 → 11
Single fragment between 201 and 154 that is approximately equidistant. A fragment also present between 298 and 220 (that is approximately equidistant), and between 134 and 75 → *I. scapularis* ✓

11(10). Only 1 thick fragment present between 134 and 75. A single fragment also present between 201 and 154 that is immediately below 201; a fragment is also present between 298 and 220 → *I. muris*✓
Two fragments present between 134 and 75. Fragments also present between 298 and 220 and immediately below 201 → *I. spinipalpis*

12(4). Two fragments present between 134 and 75; the uppermost of which is immediately below 134. Two fragments also present between 201 and 154; fragments also between 298 and 220 → *I. minor*
Three fragments present between 134 and 75, or without the above combination of characters. Two fragments also present between 201 and 154 and a fragment between 298 and 220 → *I. dentatus*✓

13(2). Fragment present at 220 → 14

No fragment at 220 → 15

14(13). Fragment present at 201. Fragments also present at or near 220 and between 134 and 75 → *I. affinis*

No fragment at 201 but fragment present at 220. Fragment also present between 298 and 220 (that is approximately equidistant); additional fragments present at or near 134 and between 134 and 75 → *I. woodi*

15(13). Fragment present at or immediately below 154. Position of fragment between 298 and 220 is immediately above 220; fragments also present at or near 154, 134, between 134 and 75, and at or near 75 → *I. cookei*✓

No fragment at 154 → 16

16(15). Fragment present between 154 and 134 that is closer to 134; a fragment also present that is nearly equidistant between 298 and 220, and 2 fragments between 134 and 75 → *I. baergi*

No fragment between 154 and 134, but a thick fragment present at 134. A fragment also present between 298 and 220 → *I. sculptus*

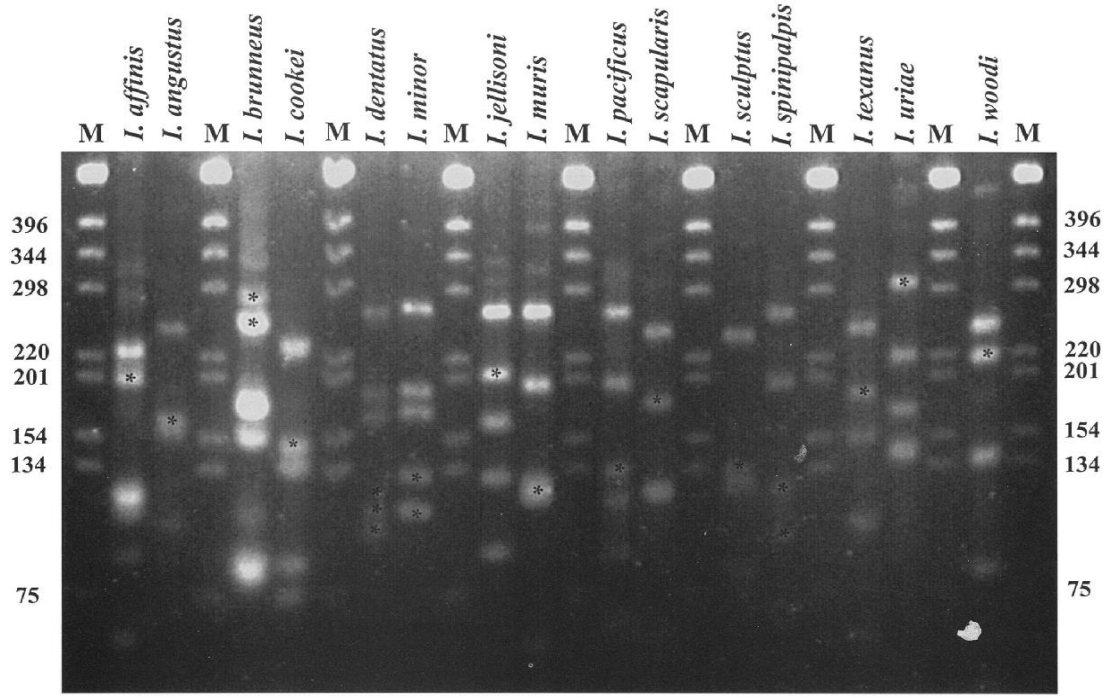

FIGURE 1. High-resolution agarose gel showing CfoI and MspI digest banding pattern of the ITS-2 region for 15 species of Ixodes ticks found in the contiguous U.S.; M = molecular markers;

asterisks indicate fragments in terminal couplets. Not pictured but included in the key are I.baergi and I. kingi.

Ixodes spp. In NY as of 1996 (Durden & Keirans)

1. ✓*angustus- rodents, predators
2. banksi - humans etc
3. ✓brunneus- exclusive birds
4. ✓*cookei - mammals, humans
5. ✓*dentatus - rabbits, birds
6. marxi - squirrels, occasionally chipmunks/foxes/raccoons
7. ✓muris- rodents
8. ✓*scapularis-mammals, birds lizards humans domestic animals etc
9. ✓texanus - raccoons, carnivores, rodents, domestic animals, humans etc

*=*B. burgdorferi* described as found in sp., ✓=included in PCR ID key

Attachment 4

ExoSAP-IT PCR Product Clean-Up Recording Sheet

- Place a clean diaper on the bench.
- Use DNA pipettes and barrier tips.
- Label tubes appropriately, sample ID, date, PCR ExS
- Warm up Thermocycler 30 mins before running.
- Place 5 ul PCR product in PCR tube.
- Add 2 ul ExoSAP-IT to each tube.
- Run in Thermocycler/ heat block :
- Incubate 37 °C, 15 mins (activates enzyme)
- Incubate 80 °C, 15 mins (deactivates enzyme)
- Samples now ready for use in sequencing protocol

Project _____ Date performed_____

Sample #	**Sample ID**	**Sample date**	**2° PCR Date**