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ECOLOGY AND PREVALENCE OF TICKS AND TICK-BORNE BACTERIAL PATHOGENS IN SOUTHEAST KANSAS

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ECOLOGY AND PREVALENCE OF TICKS AND TICK-BORNE BACTERIAL PATHOGENS IN SOUTHEAST KANSAS

An Abstract of the Thesis by
Abrar Alzahrani

Ticks are small arachnids which have the ability to acquire, maintain, and transfer pathogenic bacteria to human and animals by feeding on their blood. Diseases such as Anaplasmosis, Babesiosis, Colorado tick fever, Ehrlichiosis, Lyme disease, and Rocky Mountain spotted fever (RMSF), are becoming more prevalent in populations, creating global health and economic problems.

This research aimed to determine the frequency of three species of ticks, the Lone Star tick (*Amblyomma americanum),* the Dog tic*k (Dermacentor variabilis),* and Black-legged tick (*Ixodes scapularis)* in southeast Kansas and adjacent areas. Another objective of the study was to determine the infection prevalence of bacterial pathogens among the identified ticks using molecular techniques. Ticks collected from June to August (2016 and 2017) were identified at the species level using taxonomic keys. The type of land such as woodland or pasture used by the ticks will also be analysed. In order to detect the presence of pathogenic bacteria, DNA from individuals or groups of adult ticks or nymphs was extracted and quantified. Using bacterial species-specific primers, infection prevalence was assessed by polymerase chain reaction (PCR). Tick populations in southeast Kansas have been surveyed to a limited extent. The current research represents interesting findings in terms of changing climatic conditions. To reduce the incidence of tick-borne illnesses in this region and to find an effective means of treatment, our data will be shared among healthcare providers.

Keywords: *Southeast Kansas; Ticks; Tick-borne disease; Amblyomma; Dermacentor; Ixodes; woodland; pasture; Francisella tularensis; Rickettsia rickettsii*

TABLE OF CONTENTS

LIST OF TABLES

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

CHAPTER I

INTRODUCTION

Biology of ticks

Classification:

There are two families of ticks, the Ixodidae (hard ticks) and Argasidae (soft ticks). Family Ixodidae has been classified into three subfamilies which are Ixodidae, Argasidae, and Nuttalliellidae. The Lone Star tick, (*Amblyomma americanum*), the Dog tick, (*Dermacentor variabilis*), and the Black-legged tick (*Ixodes scapularis*) are three hard ticks in the family Ixodidae. The most commonly encountered ticks in the southeastern U.S. are American Dog tick, Lone Star tick, Black-legged or "Deer" tick, and Brown Dog tick (Fig. I.1) (Clemson Cooperative Extension, 2008). The taxonomic classification of ticks is shown below (Estrada-Peña 2015).

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Chelicerata
Class: Arachnida
Subclass: Acari
Superorder: Parasitiformes
Order: Ixodida
Superfamily: Ixodoidea
Family: Ixodidae

Genus: *Ixodes*	**Genus:** *Amblyomma*	**Genus:** *Dermacentor*
Species: *scapularis*	**Species:** *americanum*	**Species:** *variabilis*

Fig. I.1. Three most common ticks depicting their various life stages.

[Source: University of Rhode Island Tick Encounter Resource Center]

Morphology:

Hard ticks have three visible components of mouthparts which are the paired palps, the chelicerae, and hypostome (Fig. I.2). Palp pairs move while ticks are sucking host blood. Chelicerae protect the center rod-shaped structure. The hypostome has

many sharp beaks which stick into the host's skin while feeding and it prevents easy removal of the attached tick. In addition, most hard ticks secrete a mixture of substances through their salivary glands [which are also called ‘feeding cavity’] to help ticks attach to the host pathogens. Transmission starts about 24 hours after a tick begins to feed. After their blood meal, ticks drop to the ground to molt to the next stage. Female ticks become engorged and lay thousands of eggs at in sites with a high relative humidity to ensure their survival (Estrada-Peña 2015).

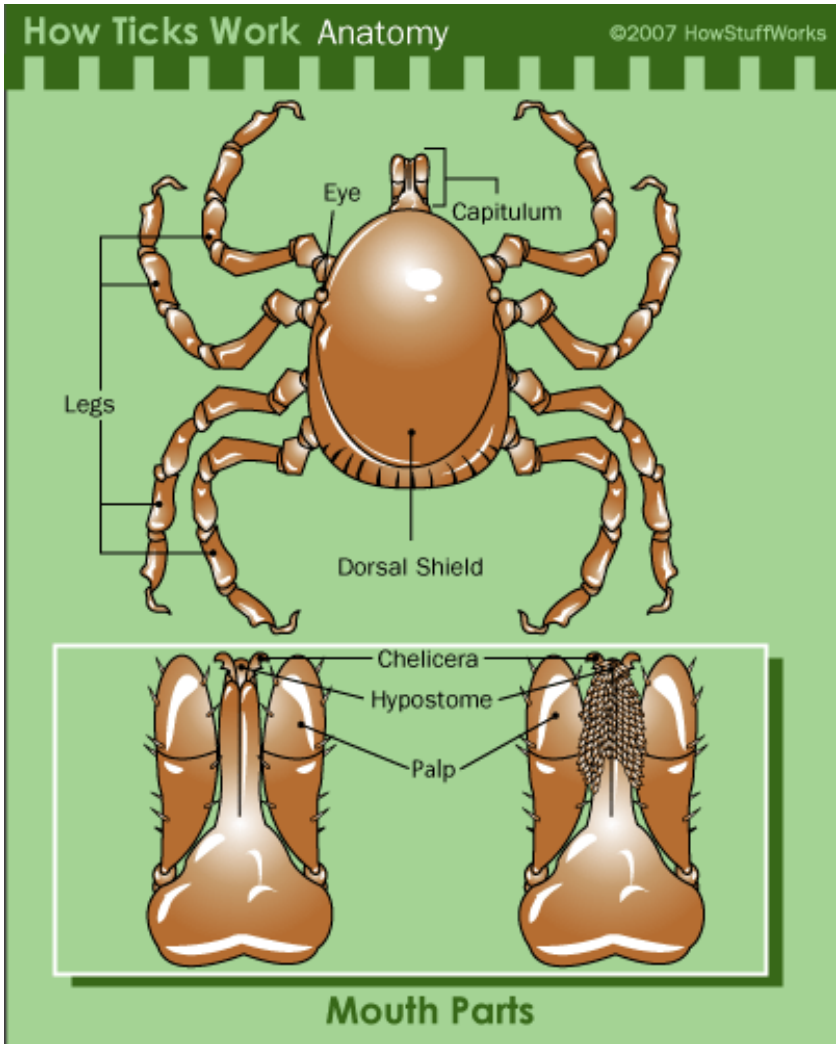


Fig. I.2. Tick anatomy, including the piercing mouthparts.

[Source: https://animals.howstuffworks.com/arachnids/tick1.htm]

Behavior:

"***Questing***" - an interesting behavior of hard ticks for host seeking. Ticks sneak up the stems or perch on the edges of leaves on the ground in a typical posture with the front legs extended as a response to a host passing by (Fig. I.3). They climb on to the host using their extended front legs whenever there is an opportunity. Carbon dioxide, heat, and movement are incentives to such questing behavior.

Fig. I.3. Adult Ixodes tick questing. Photo by Anna Perez, courtesy of CDC [source: https://www.bayarealyme.org/blog/beware-the-questing-tick/]

Life cycle:

Ticks have a complex life cycle that takes about two years to be completed. It includes four life stages: the egg, the 6-legged larvae, the 8-legged nymph, and the adult (Fig. I.4). Development from one stage to another requires two to three different hosts. In the fall and early spring, adult ticks feed and mate on large animals such as deer. The eggs are dropped off on the ground by female ticks. During summer to fall, eggs hatch into larvae that feed on mice and/or rabbits and then remain inactive until the next spring. In the late spring, they molt to nymphs, and then they feed on small rodents and other small mammals such as dogs. In the fall, they molt to adults, which completes the life cycle (Fig. I.4). Larvae and nymphs become infected with pathogenic bacteria when they feed on infected animals (CDC 2015).

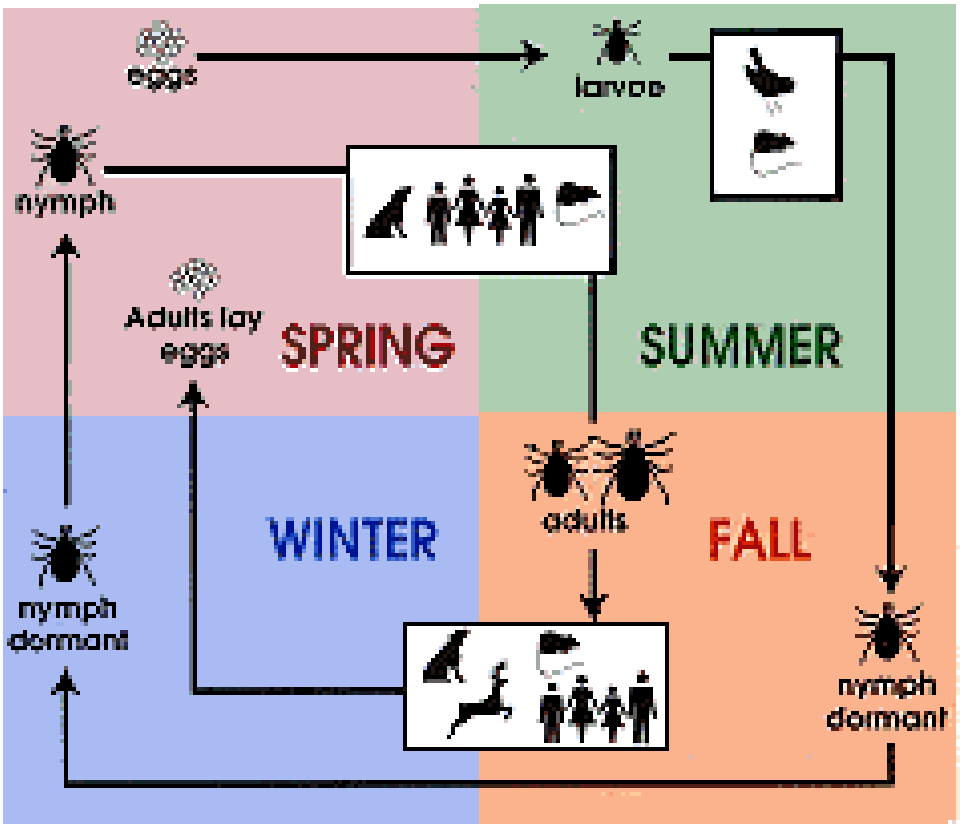


Fig. I.4. Two-year life cycle of deer ticks.

[Source: https://www.cdc.gov/lyme/transmission/blacklegged.html]

Physiology and habitat:

Ticks come out as small ectoparasites living outside the host's body and feeding on blood. They belong to Parasitiformes order, and they have evolved over the Cretaceous era to fossilization form. The *Ixodes*, *Dermacentor*, and *Amblyomma* stand out as the main tick variety in the Peri-urban landscape. These ticks depend greatly on their physiology and ecological adaptation (Cohen et al. 2010). The salivary glands are the important organ for the tick as it fosters its survival on and off the host. The physiology of the gland promotes the acquisition of blood meal from the host as well as maintains the hydration of the tick outside the feeding season.

The multifunctional salivary organs help the ticks in feeding, as well as promoting the transmission of the pathogens to the hosts. In the saliva, the ticks produce chemicals such as catecholamines and dopamine that regulate the homeostasis of the

host to allow feeding (Kazimírová and Štibrániová 2013). Similarly, the salivary gland nurtures the multiplication of the pathogens that in turn plays a critical role in infection. Over time, the ticks have coevolved with pathogens to ensure a symbiotic relationship. Here, the saliva provides the pathogens with an environment for growth promoting their subsequent infections. Moreover, the saliva facilitates the multiplication of pathogens that increases their infection rate (Rynkiewicz and Clay 2014).

Although ticks cause disease to animals and human, there is still not enough information on pathogen-tick interaction (Liu & Bonnet 2014). It has been shown that ticks' developmental stages facilitate their ability to transmit diseases to different hosts. For instance, *Ixodes* transmit Lyme disease from infected birds to people at its nymphal stage. Since nymphs are too small, it is difficult to notice their bite. Therefore, they take a long time siphoning blood and allowing the infection of the pathogen to the people. The peri-urban areas in the Midwestern US have gone through a successive environmental change. The agrarian revolution (1860 – 1910) led to the extensive deforestation and increase of agricultural land. This caused a significant reduction of natural forests leaving bushes and thickets (Hamer et al. 2012). The small bushes are good habitats for the deer and small mammals that host various ticks. Therefore, this region exhibits the presence of the *Ixodes*, *Dermacentor*, and *Amblyomma* ticks that remain crucial vectors of the major diseases such as Lyme in humans. In this case, the habitat plays an important role in the availability of ticks responsible for pathogen transmission. The physiology and environmental factors stand out as fundamental components in understanding the infection and transmission of diseases by ticks.

Tick-borne diseases

Ticks, as a group, are second only to mosquitoes as vectors of pathogens to humans and are the primary vector for pathogens of livestock, companion animals, and wildlife (Mansfield et al. 2017). Recent report by CDC shows a remarkable increase in the occurrence of vector-borne diseases. Over 13 years (2004 - 2016), illnesses from mosquito, tick, and flea bites have tripled in the U.S., with more than 640,000 cases reported (Fig. I.5). Nine new germs spread by mosquitoes and ticks were discovered or introduced into the United States during this time (CDC 2018).

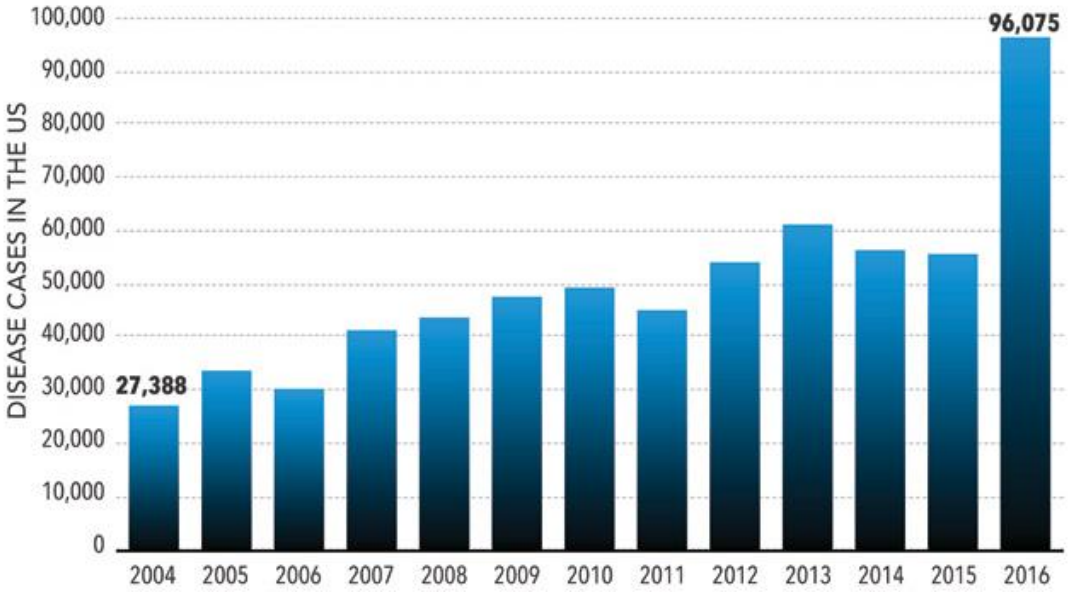


Fig. I.5. Disease cases from infected mosquitoes, ticks, and fleas have tripled in 13 years. [Source: Source: CDC Vital Signs, May, 2018]

*The number of disease cases from infected mosquitoes, ticks, and fleas has increased from 27,388 in 2004 to 96,075 in 2016.

CDC also has published data on reported cases of tick-borne disease between 2004 and 2016 (Fig. I.6). Total cases reported from Kansas during this period was 1,164 (Rosenberg et al. 2018).

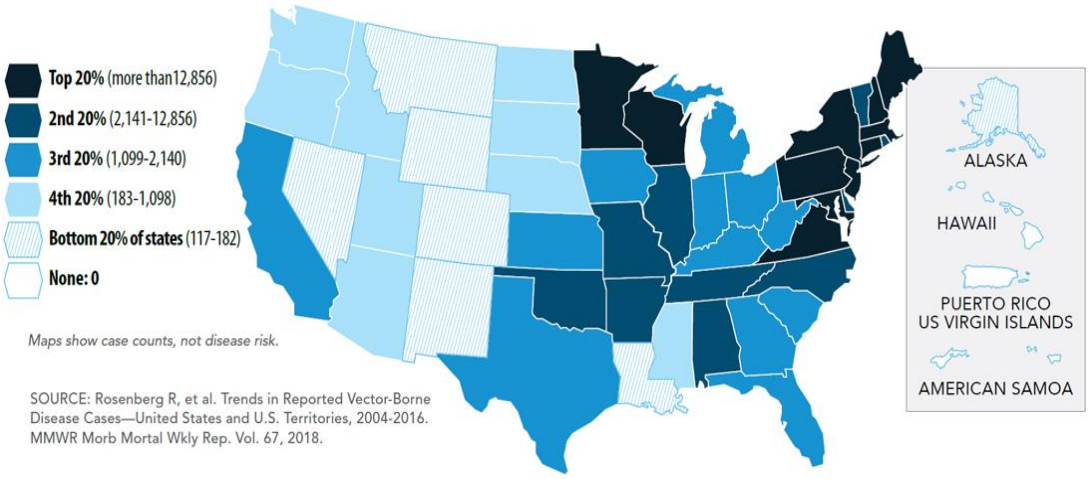


Fig. I.6. Map showing reported disease cases from ticks during 2004-2016.

The transmission of infectious disease through ticks is higher than other external parasites, making them very critical vectors. Ticks can transmit protozoan, viral, bacterial, and fungal pathogens, posing a threat to human, livestock, and wildlife health. In addition to transmitting diseases, ticks can also diminish the value of livestock by damaging the hide (leather quality) (Lysyk 2013).

In the United States, there are currently 14 vector-borne diseases that are of national public health concern. These diseases account for a significant number of human illnesses and deaths each year and are required to be reported to the National Notifiable Diseases Surveillance System at the Centers for Disease Control and Prevention (CDC). In 2013, state and local health departments reported 51,258 vector-borne disease cases to the CDC.

Table I.1: Summary of reported case counts of notifiable tick-borne diseases in the United States.

Tick-Borne Diseases	2013 Reported Cases	Median (range) 2004–2013[a]
Lyme disease	36,307	30,495 (19,804–38,468)
Spotted Fever Rickettsia	3,359	2,255 (1,713–4,470)
Anaplasmosis/Ehrlichiosis	4,551	2,187 (875–4,551)
Babesiosis[b]	1,792	1,128 (940–1,792)
Tularemia	203	136 (93–203)
Powassan	15	7 (1–16)

[a] State Health Departments are required by law to report regular, frequent, and timely information about individual cases to the CDC in order to assist in the prevention and control of diseases. Case counts are summarized based on annual reports of nationally notifiable infectious diseases.

[b] Babesiosis and dengue were added to the list of nationally notifiable diseases in 2011 and 2009, respectively. Median and range values encompass cases reported from 2011 to 2013 for babesiosis and from 2010 to 2013 for dengue.

[Source: https://health2016.globalchange.gov/vectorborne-diseases]

The highest risk of being bitten is by *D. variabilis* occurs during spring and summer in the southern, southeastern, central and northeastern parts of the U.S. Adult females, normally found on dogs, are most likely to bite humans and will readily attack larger animals, such as cattle and horses. The 8-legged adult female is a vector for the pathogens causing Rocky Mountain spotted fever (RMSF) and tularemia and can cause canine tick paralysis (Chan and Kaufman 2013). Much like the *D. variabilis*, the *A. americanum*, has also migrated from its original location. This tick species is found mainly in Texas and the Ozark mountains of Missouri, with scattered loci in other parts of southern USA. It is a vector for RMSF (Alderdice and Burgess 1998), human monocytic ehrlichiosis, human ewingii ehrlichiosis, tularemia, southern tick-associated rash illness, and feline cytauxzoonosis (Raghavan et al. 2016). Table I.2 illustrates bacterial pathogens that are associated with various tick-borne disease (CDC 2018). Phenomenological model indicated climate change has affected the distribution of *Ixodes* in the Midwestern region (Ostfeld and Brunner 2015). Since in phenomenological models the important predictor variables can be chosen arbitrarily, it is often hard to interpret biologically.

Table I.2: Diseases caused by tick

Disease	Tick species	Pathogen(s)
Tularemia	*Dermacentor variabilis*	*Francisella tularensis*
Lyme disease	*Ixodes scapularis* *I. pacificus*	*Borrelia burgdorferi*
Rocky mountain spotted fever	*Amblyomma americanum* *D. variabilis*	*Rickettsia rickettsi*
Ehrlichiosis	*A. americanum* *I. scapularis*	*Ehrlichia chaffeensis* *Ehrlichia ewingii*
Anaplasmosis	*A. americanum* *I. scapularis*	*Anaplasma phagocytophilium* *Rickettsial parasite*

Besides carrying bacterial pathogens, there are a few viral strains reportedly carried by ticks caused fatal diseases as listed in table I.3. According to CDC, approximately 100 cases of Powassan virus (POWV) neuroinvasive disease cases were reported in the upper Midwest and northeast of United States in the past 10 years and about 10% of those cases were fatal. Bourbon virus (BRBV) was isolated in 2014 from a resident of Bourbon County, Kansas. Heartland virus disease cases were identified in the Midwestern and southern United States.

Table I.3. Emerging tick-borne viruses in North America.

Virus	Classification (family, genus)	Distribution	Primary tick vector
Severe Fever with Thrombocytopenia syndrome virus	Bunyaviridae, *Phlebovirus*	East Asia, North America	*Haemaphysalis longicornis*
Heartland virus	Bunyaviridae, *Phlebovirus*	North America	*Amblyomma americanum*
Powassan virus	Flaviviridae, *Flavivirus*	North America, Russian Federation	*Ixodes scapularis, Ixodes cookei*
Deer tick virus	Flaviviridae, *Flavivirus*	North America	*Ixodes scapularis*

Based on the connection and interests of the researcher to Middle-east, it is worth to note that ticks were found to cause relapsing fever, encephalitis, Boutonneuse fever and Q fever in various parts on that region (Regional Disease Vector Ecology Profile, 1999). Table I.4 illustrates cases of tick-borne diseases that were reported in counties of southeast Kansas, and this emphasizes the need to study ticks in this region.

Table I.4. Tick-borne disease MMWR (Morbidity & Mortality Weekly Report) prepared by CDC for 2013-2015 years; counties include Allen, Bourbon, Cherokee, Crawford, Labette & Neosho.

Disease data provided by Kansas Dept. of Health and Environment (KDHE)	**Total count (2013-2015)**
Ehrlichiosis (*Ehrlichia chaffeensis*)	12
Ehrlichiosis (*Ehrlichia ewingii*)	2
Lyme Disease (*Borrelia burgdorferi*)	4
Tularemia (*Francisella tularensis*)	4
Grand Total	22

Dahlgren et al. (2015) created models based available ecological, clinical, epidemiological, and laboratory data and indicated that increasing incidences of tick-borne rickettsial diseases was associated with expansion of geographic distribution of *A. americanum* in the United States. The maps below depict how incidences of Lyme disease expanded over 15 years (Fig. I.7).

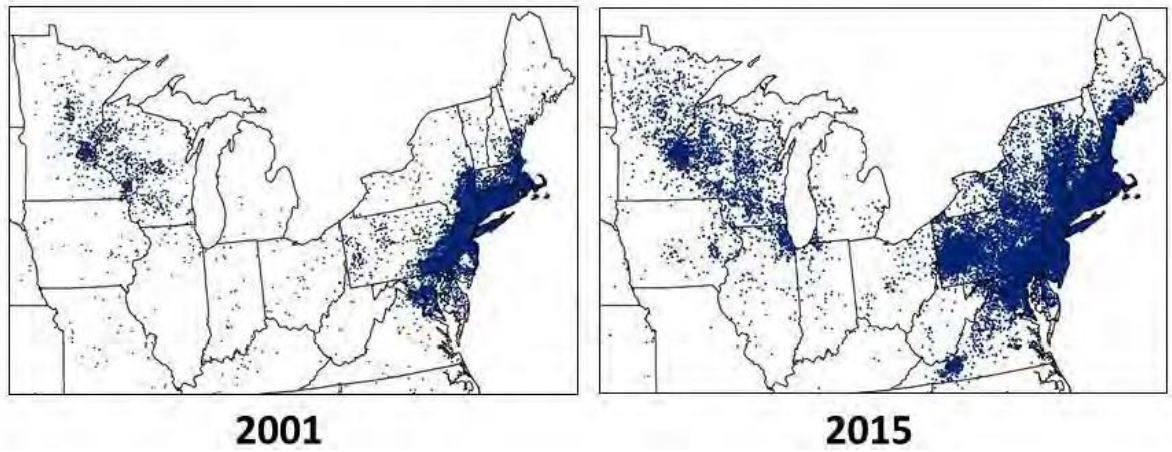


Fig. I.7. Expanding numbers and geographic distribution of Lyme disease cases mirrors other tickborne diseases
[Source: cdc.gov/lyme/stats/index.html]

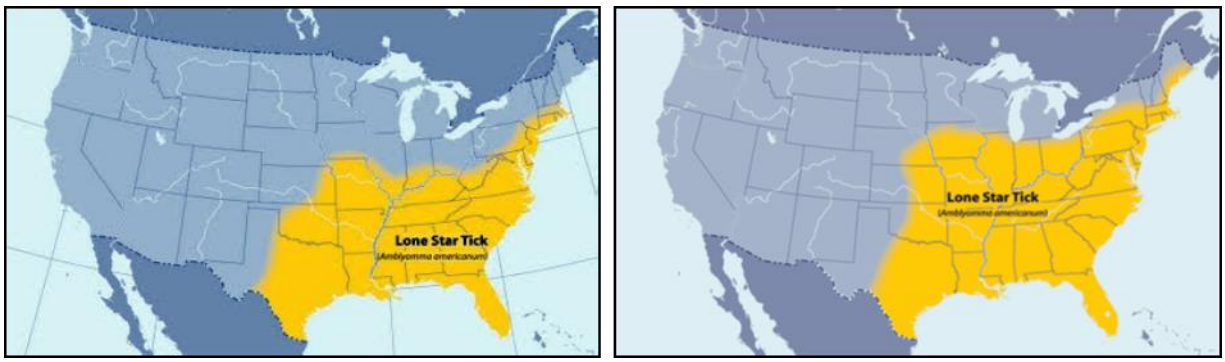


Fig. I.8. These maps show the extent of established *Amblyomma americanum* tick populations (left panel: 2007, right panel: 2011), commonly known as lone star ticks. However, tick abundance within this area varies locally. The map does not represent the risk of contracting any specific tickborne illness.
[Source: https://www.cdc.gov/ticks/geographic_distribution.html]

Dantas-Torres (2015) anticipated that warmer winters and extended autumn and spring seasons will influence the distribution of some tick species to northern latitudes and to higher altitudes. Ecological processes such as trophic cascade as well as increased emission of greenhouse gases and increased rate of deforestation have multitude impact on environment. In a sense, human behavior is considered as a strong determinant of environmental health. The transmission risk for tick-borne diseases may be impacted by changes in human behavior. Avoidance of tick-infested areas and rapid removal of ticks after a person is bitten, will reduce the transmission risk. Due to the

complexity and dynamics of interacting factors, it is difficult to comprehend the tick-borne transmission system. Based on this background the following goals were set for the current study.

Research goals:

i) Determine the prevalence and distribution of three tick species *Amblyomma* spp., *Dermacentor* spp., and *Ixodes* spp. in southeast Kansas.

ii) Evaluate the type of land cover that is associated with the distribution of each tick species.

iii) Detect selected bacterial pathogens among the identified tick species using molecular technique.

CHAPTER II

MATERIALS AND METHODS

Materials required for tick collection and processing:

The material that was needed to collect ticks were 1 m^2 flannel cloth attached to a 0.5 inch-thick wooden flag pole, sterile plastic containers, sharp forceps, and an ice cooler. All collecting personnel were equipped with protective gear such as gloves, all body cover white suit, insect repellents (DEET/Deep Woods), insecticide (Permethrin), ducktape to seal the gaps between clothing and boot or gloves. Field assistants were also recommended to wear light colored full-sleeve shirts and long pants. In the laboratory, materials needed were dissecting microscope (stereoscope), liquid nitrogen, bead beater, microcentrifuge tube, DNA isolation kit, sterile plastic pestle, TAE buffer, agar powder, UV Transilluminator, pipettes and pipette tips, vortexer, Ethanol (70% and 100%), 0.5% sodium hypochlorite solution, sterile water, fine tip brush, liquid nitrogen container, and liquid nitrogen.

Sampling procedures:

Ticks were collected from a total of 36 locations in 2016 and 25 locations in 2017 during mid-April through mid-August ***(see Appendix A and B)***. The standard tick collection technique was called 'Flag and Drag' where the personnel hold the pole and drag it on the vegetation on either side over about 10 meters of walking steps. Ticks were carefully removed from the flannel cloth using forceps and were stored in plastic containers. At the end of collections of each day, the samples were stored in an ice

cooler until transportation to the laboratory. In the lab, ticks were identified by using stereoscope and the identification key to species, life-stages, and sexes, and the data were recorded.

Fig. II.1. Tick collection using 'Flag and Drag' technique.

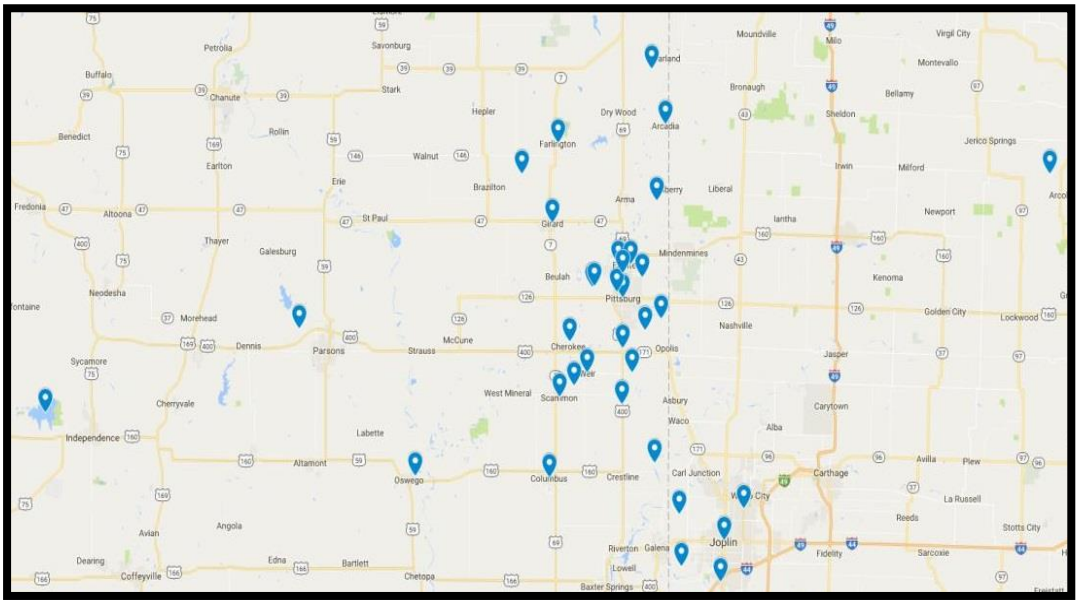


Fig. II.2a. Tick collection sites for 2016 (pinned as dark blue in google map).

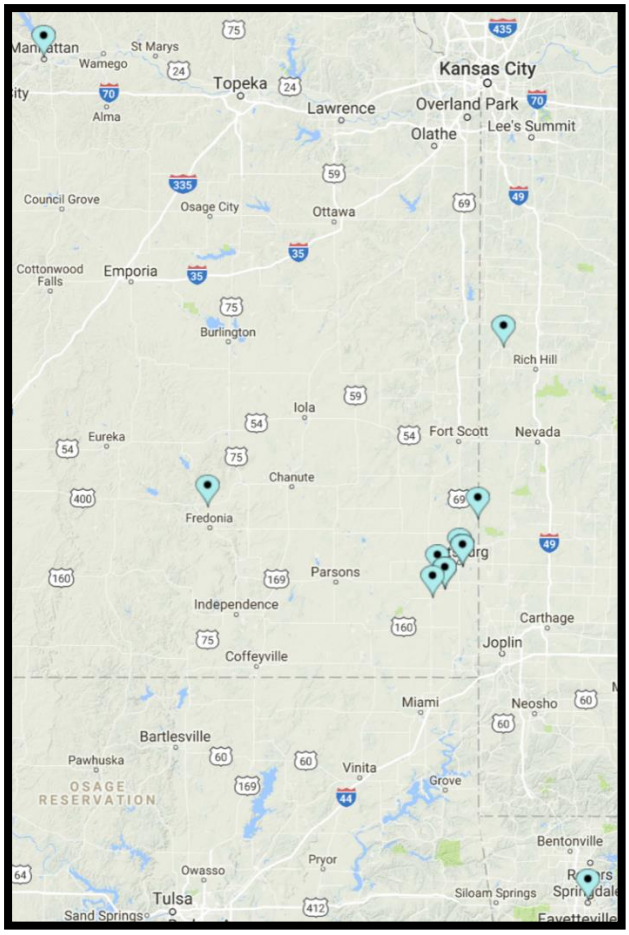


Fig. II.2b. Tick collection sites for 2017 (pinned as light blue in google map).

DNA isolations:

Extracting DNA involves multiple steps to gently break and open the cell, nuclear membrane, and separate the DNA from proteins and then cause it to precipitate out of a solution. This is achieved by using various chemicals, based on the membrane structure, size of whole genomic DNA, and its electronegativity.

Two to three adult ticks or five nymphs (from the same location and date of collection) were sterilized by washing them with 0.5% sodium hypochlorite solution, 70% ethanol, and sterile water for one minute respectively. Then they were transferred to a microcentrifuge tube and exposed to liquid nitrogen until frozen and then crushed by sterile plastic pestle. DNA was isolated using the Fast soil kit following manufacturer's protocol with minor modification ***(see Appendix E)***. Briefly, the steps

involved: 978 microliters of sodium phosphate buffer were added to a microcentrifuge tube. The solution was transferred to lysing matrix along with 122 µl of MT buffer. It was shaken by bead beater for one minute and then kept on ice for two minutes. The lysed sample was centrifuged at 14,000× g for five minutes. The supernatant was transferred to a clean two ml microcentrifuge tube and 250 µl of protein precipitation solution was added to solubilize and precipitate the proteins in order to get a pure DNA extract. It was mixed by flipping the tube by hand ten times and then incubated at room temperature for ten minutes. The suspension was centrifuged for five minutes and 750 µl of supernatant was transferred to a clean two ml microcentrifuge tube. Equal amount of Binding matrix was added to the suspension. Eight hundred µl of the solution was transferred to a spin filter. This step was repeated for the remaining suspension. The flow-through was discarded. Five hundred µl of prepared SEWS-M solution was added to the spin filter tube and mixed by flipping by hand. It was centrifuged for five minutes to remove the residual ethanol. The spin filter was transfer to a new catch tube and dried at room temperature for five minutes. A hundred µl of DES was added to the filter tube in two parts of 50 µl each time and incubated at room temperature for one minute before centrifuging at 14,000× g for one minute each time to elute bound DNA into the catch tube. The spin filter was discarded in the end.

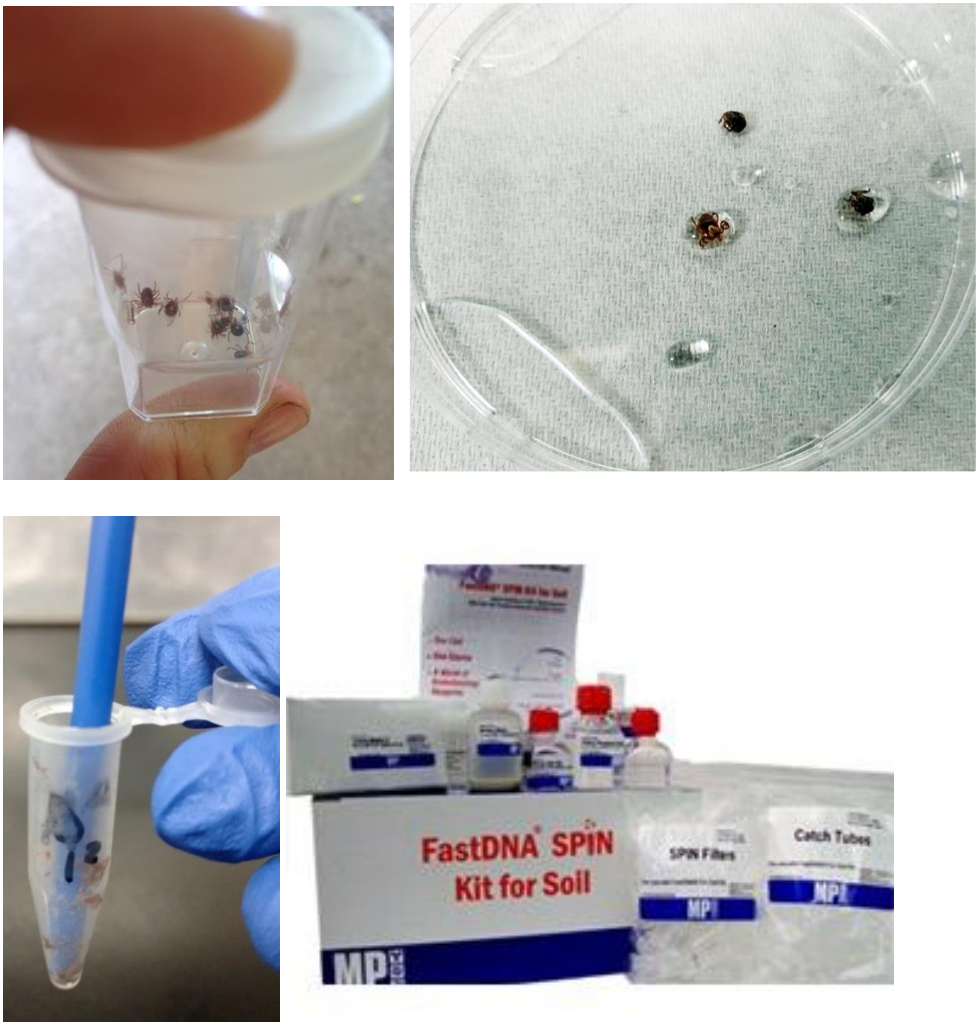


Fig. II.3. Ticks in plastic vial (upper left); surface sterilization on Petri plates (upper right), Freeze-thaw and grinding of ticks using sterile plastic pestle (lower left); DNA isolation kit (upper right)

DNA quality and concentration:

The DNA quality and the quantity were checked by nanodrop lite (Thermo Scientific). The upper and lower optical surfaces were cleaned by distilled water. Three microliters of water were added on the lower surface of the lever arm and was set as blank using DN application software. One microliter of sample was placed for measuring double-stranded DNA concentration. The upper and lower optical surfaces were wiped by water after each use. Extracted DNA was also visualized by running 0.7% agarose gel (see below).

Agarose gel electrophoresis:

Gel media was prepared for 0.7% agarose gel by mixing 0.45 g of agarose powder, 60 ml of 1X TAE buffer (Tris-acetate-EDTA) and 2.5 µl Red Safe dye (Intron Scientific) and then the suspension was brought to boiling in microwave for about 2-3 minutes to achieve a homogeneous mixture. Once it cooled down to around 60°C, Red Safe dye was added to the mixture and then the gel was poured into a Fisher Biotech Electrophoresis Unit until solidified (30-40 minutes). The solidified gel was submerged in gel tank with TAE buffer which is useful for separation of smaller DNA fragments (MW < 1000).

Loading samples were prepared by adding five µl, of genomic DNA/PCR reaction, three µl of sterile water, and two µl loading buffer (6x DNA loading buffer). Five µl of Lambda DNA/ 100 bp DNA ladder was used as DNA marker ***(see Appendix D)***. The samples were run at 75 volts for 40 minutes followed by visualization of the gel in Fluor Chem E transilluminator.

Polymerase chain reaction (PCR) amplification of selected genes:

In order to amplify eubacterial 16S rRNA gene, PCR was performed on each of the isolated DNA using universal primers as mentioned in Table II.1. Each PCR reaction contained 10µl of Promega 2X Master mix per sample, 0.5 µl of each of forward and reverse primers (20 pmole) and 7.5 µl of distilled water a centrifuge tube. Calculated amount (30-50 ng) of isolated genomic DNA was used as template in the range of 0.5-3.0 µl. The tubes were briefly centrifuged and then transferred to the PCR machine (BioRad C1000 Touch Cycler). The PCR protocol was composed of initial denaturation at 95°C for 3 minutes followed by 30 cycles of denaturation at 95°C for one minute, annealing at 50°C for one minute, and extension at 72°C for one minute.

For species level identification of ticks, *Dermacentor variabilis, Amblyomma americanum* and *Ixodes scapularis* specific primers were used, PCR reaction composition was as described above. However, the annealing temperature varied as 50°C, 53°C and 47°C, respectively. For detection of bacterial pathogens *Francisella tularensis, Ehrlichia chaffeensis* and *Rickettsia rickettsii*, species-specific primers were used. PCR reaction composition was as described above, however the annealing temperature varied between 45-55°C. To check all the PCR reactions usually 15 microliters of each sample was analyzed on 1.0% agarose gel.

Table II.1. Primers used in this study.

Target Gene	**Primer ID.**	**Sequence (5' -> 3')**
Eubacterial 16S rRNA	**27 (F) *** **1492 (R)**	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT
Tick spp.	tickLCO1490 (F) tickHCO2198 (R)	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAATCA
Amblyomma americanum	**AamITS2Sh (F) *** **AamITS2Sh (R)**	GCAGCAGTTCGGCTACACGTA ACGACGTAACGCGGGACGGC
Amblyomma americanum	AamITS2V (F) AamITS2V (R)	CCTCCTCGAACGGGCGCAAAGTCG TAACGCAGAGAGTTTCGAGCCC
Dermacentor variabilis	**DermITS2-(F) *** **DermITS2-(R)**	GTGCGTCCGTCGACTCGTT TCGCCCAACACGGCGCTACT
Ixodes scapularis	**IxoCOI907 (F) *** **IxoCOI907 (R)**	TTAGGGGCACCAGACATAGC TAGCAAAAACGGCTCCTATTG
Rickettsia sp.	R17-122 (F) R17-500 ((R)	CAG AGT GCT ATG AAC AAA CAAGG CTT GCC ATT GCC CAT CAG GTT G
Rickettsia rickettsii	**RRi6 (F) *** **RRi6 (R)**	AAA TCA ACG GAA GAG CAA AAC CCC TCC ACT ACC TGC ATC AT
Ehrlichia chaffeensis	**EHR01(F) *** **EHR02 (R)**	GCC TAA CAC ATG CAA GTCGAACG GCC CAA TAA TTC CGA ACAACG
Ehrlichia ewingii	EE72-159 (F) EE72-160 (R)	CAATTCCTAAATAGTCTCTGACTATT TATAGGTACCGTCATTATCTTCCCTAT
Francisella tularensis	**FTP1(F)*** **FTP2 (R)**	TGG CGA GTG ATA CTG CTT G TAG GAT CCC ATT AGC TGT CCA CTT ACC

Anaplasma sp.	AnaGe9 (F) AnaGe10 (R)	AACGGATTATTCTTTATAGCTTGCT TTCCGTTAAGAAGGATCTAATCTCC
Borrelia burgdorferi	BB126 (F) BB127 (R)	TGCGAGTTCGCGGGAG TCCTAGGCATTCACCATAGACTCTT

*The primer sets that yielded useful information in this study are in **bold** and the results obtained from the PCR reactions using those primers are included in result section below.

Each PCR reaction consisted of positive and no template DNA negative controls. For detection of bacterial pathogen, isolated genomic DNA from respective bacterial species was obtained from our collaborator in University of Nebraska (Kearney). Based on positive controls and relevance of pathogens in southeast Kansas, two species of ticks were tested for *E.chaffeensis*, *R.rickettsii*, and *Francisella tularensis.*

Statistical analyses:

Statistical analysis was performed using R-Statistical Software, Version 3.2.2 (R-Core Team 2015). Differences between tick species prevalence in woodland versus pasture land cover types were analyzed. The %land cover type for a 0.5 km diameter was extracted from the Arc-GIS map for each collection site. Presence or absence of each species of tick was calculated.

CHAPTER III

RESULTS AND DISCUSSION

Ticks collected in 2016 and 2017 were counted and identified at the species level. In addition to species, the ticks in the collection were classified based on gender and life stages such as adults and nymphs. Tables III.1, III.2 and Figs. III.1 and III.2 provide details of the collection.

In 2016, out of a total of 1301 ticks collected, the majority of ticks were identified as *Dermacentor* 49.7% (n = 647) and *Amblyomma* 48% (n = 626); very few (2%) *Ixodes* females and nymphs were also identified (Table III.1 and Fig. III.1). For all the species, more females (F) were found than males (M): *Dermacentor* F (n = 339) > M (n = 267), *Amblyomma* F (n = 299) > M (n = 199), *Ixodes* F (n = 13) > M (n = 0). The number of nymphs varied: *Dermacentor* N (n = 41), *Amblyomma* F (n = 128), *Ixodes* F (n = 15) and they were found throughout the collection period.

Table III.1. Distribution of ticks for the year 2016.

Dermacentor			***Amblyomma***			***Ixodes***		
Male	**Female**	**Nymph**	**Male**	**Female**	**Nymph**	**Male**	**Female**	**Nymph**
87	100	32	42	71	40	0	0	0
53	45	4	33	30	10	0	1	2
59	57	3	34	61	20	0	3	1
12	15	0	31	56	2	0	0	1
2	6	0	24	25	37	0	0	7
14	29	0	5	15	1	0	0	2
12	56	0	14	17	1	0	4	2
28	31	2	16	24	17	0	5	0

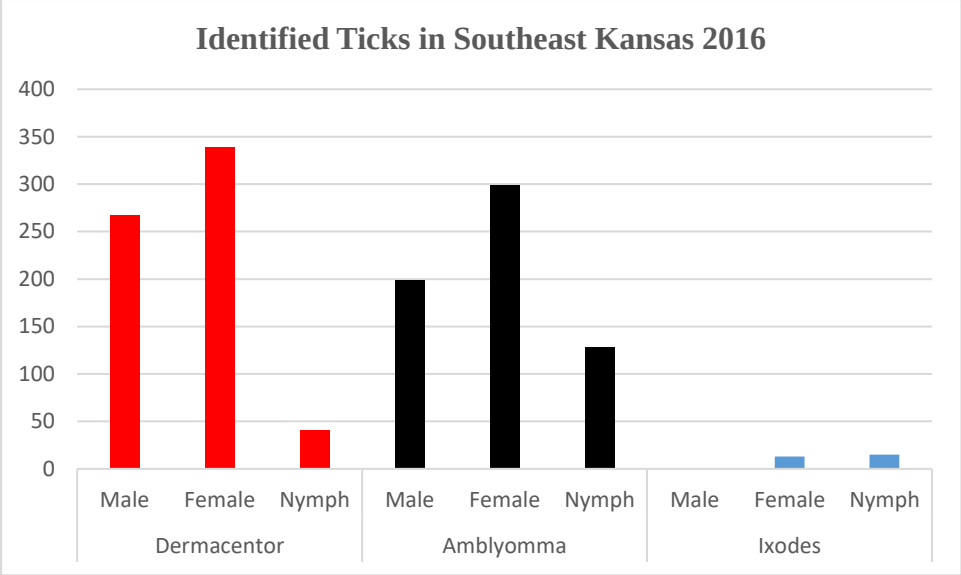


Fig. III.1. Distribution of ticks in the year 2016.

In 2017, out of a total of 377 ticks collected, the majority of ticks were identified as *Dermacentor* 52.3% (n = 197) and *Amblyomma* 44.5% (n = 168); very few (3.4%) *Ixodes* were also identified (Table III.2 and Fig. III.2). Likewise, in 2016, for all the species, the number of females were found to be more than males: *Dermacentor* F (n = 93) > M (n = 65), *Amblyomma* F (n = 51) > M (n = 47), *Ixodes* F (n = 2) > M (n = 1). Number of nymphs varied: *Dermacentor* N (n = 39), *Amblyomma* F (n = 70), *Ixodes* F (n = 9) and they were found throughout the collection period.

Table III.2. Distribution of ticks for the year 2017.

Dermacentor			***Amblyomma***			***Ixodes***		
Male	**Female**	**Nymph**	**Male**	**Female**	**Nymph**	**Male**	**Female**	**Nymph**
5	4	1	10	1	3	1	2	7
20	29	2	9	1	10	0	0	2
4	11	36	3	4	42			
13	12	0	2	4	2			
6	1	0	5	2	6			
1	2	0	2	4	3			
1	3	0	2	1	2			
3	2	0	4	1	2			
5	8	0	3	6	0			
5	8	0	2	6	0			
1	2	0	2	1	0			

1	9	0	0	1	0			
0	1	0	0	5	0			
0	1	0	3	3	0			
0	0	0	0	2	0			
0	0	0	0	2	0			
0	0	0	0	3	0			
0	0	0	0	4	0			

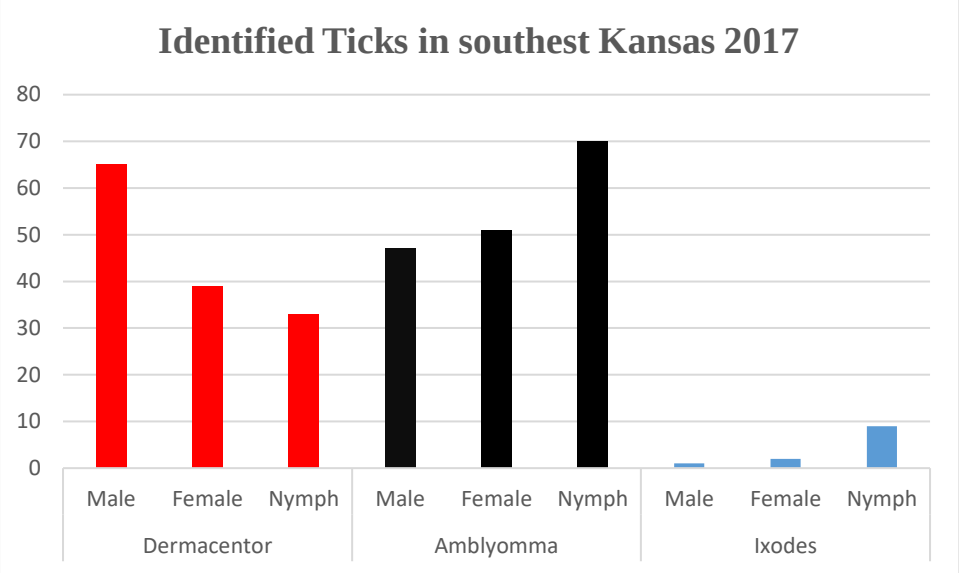


Fig. III.2. Distribution of ticks in the year 2017.

Study sites for this work were selected around the city of Pittsburg, Crawford County, in southeast Kansas. Most of the samples were collected within the city limits in areas where human interaction with the outdoors was expected to be higher. These included recreational areas and areas adjacent to low and medium-intensity residential zones. However, a few locations were in Bourbon County (KS) and in part of Missouri. In 2016, a group of entomologists from CDC were collecting ticks from Bourbon County (KS) to screen for the Heartland and Bourbon viruses (Savage et al. 2018). Our Pitt State team helped them in their collection and included few ticks that were collected along with the CDC group in this study for analysis. The land cover was later classified as woodland and pasture types (Fig. III.3). Locations of collection sites were recorded

using Global Positioning Systems. The land cover type variables were estimated from the NLCD (National Land Cover Database) in ArcGIS. The details of the location are provided in ***Appendix A and B***.

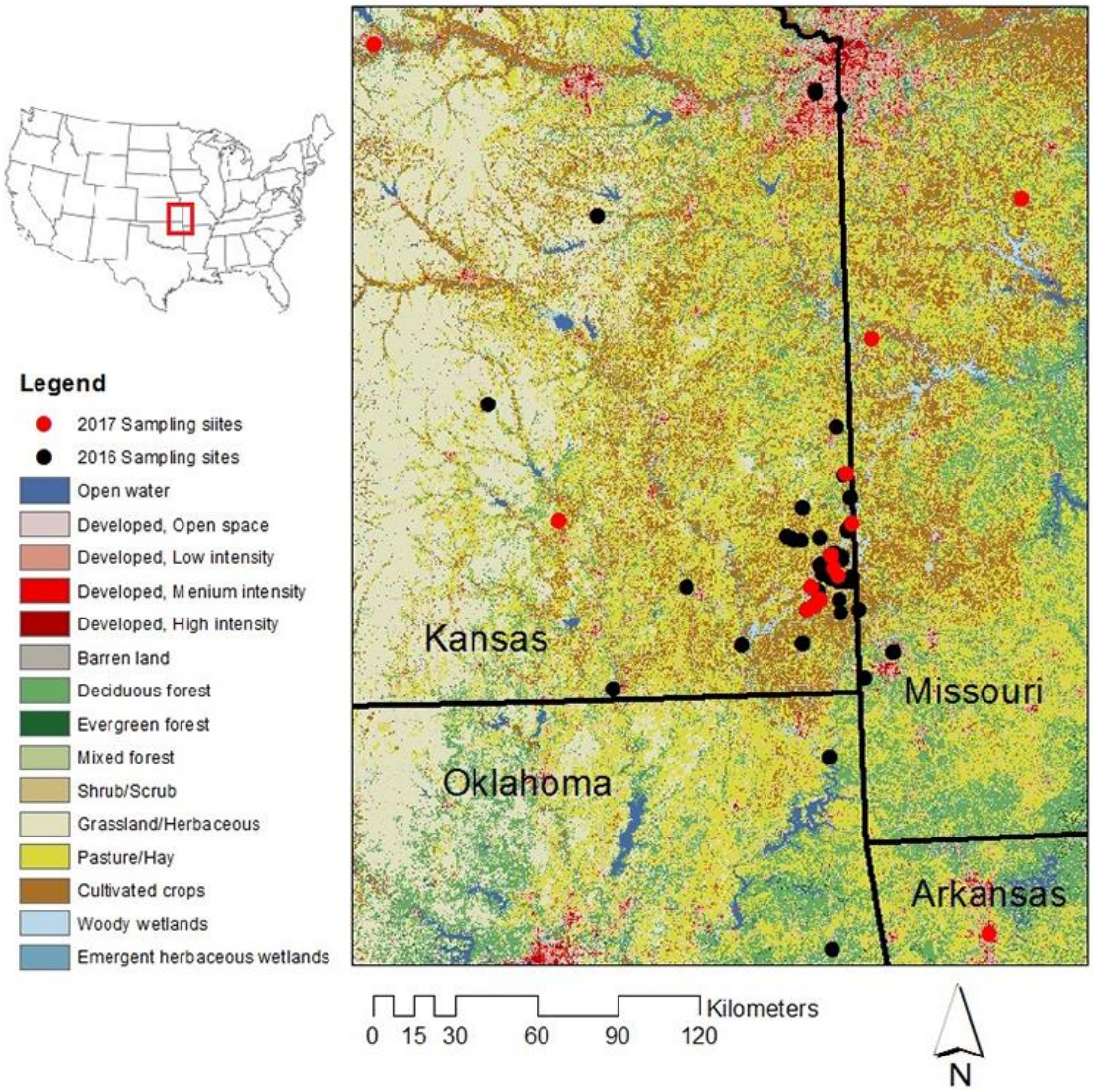


Fig. III.3. Map of peri-urban Pittsburg, Kansas, and collection sites where ticks were sampled for prevalence estimation of three tick-borne bacterial pathogens.

Table III.3a shows which type of land cover is used by three different types of ticks collected in the year 2016. While *A. americanum* were more frequently found in pasture (42.6%), *D. variabilis* and *I. scapularis* were found in woodland (68.6%) and (80.9%), respectively.

Table III.3a. Distribution of tick species in woodland and pasture in the year 2016.

Type of land cover	**n (%)**
Woodland	
A. americanum	173 (47.39)
D. variabilis	48 (68.57)
I. scapularis	17 (80.95)
Pasture	
A. americanum	192 (52.60)
D. variabilis	22 (31.42)
I. scapularis	4 (19.04)

Table III.3b. represents the multivariate logistic model constructed based on different variables to show which land use land cover parameters predict the distribution of different species in 2016. Significant factors have a $P < 0.05$. Association coefficient for *Amblyomma* and %pasture/hay is high (0.661) and this is in agreement with the data in table III.3a. Association coefficient for *Dermacentor* and %mixed forest is high (0.892) and this is in agreement with the data in table III.3a. Association coefficient for *Ixodes* and %mixed forest is high (0.320) and this is in agreement with the data in table III.3a as well.

Table III.3b. Association of land cover/land use indices with the presence of tick species in the year 2016.

A. americanum	**Coefficient**	**Std. E**	**Exp**	**P**
TECI	0.401	0.021	1.55	0.01
Edge density	0.122	0.082	3.11	0.68
% pasture/hay	0.661	0.087	2.33	0.00

D. variabilis	**Coefficient**	**Std. E**	**Exp**	**P**
TECI	0.322	0.018	1.88	0.00
% mixed forest	0.892	0.109	2.41	0.02
Patch cohesion	0.054	0.005	1.73	0.08
% Shrub/scrub	0.104	0.001	1.31	0.05

I. scapularis	**Coefficient**	**Std. E**	**Exp**	**P**
TECI	0.211	0.083	2.11	0.04
% mixed forest	0.320	0.117	3.21	0.00
Edge contrast	0.021	0.010	1.01	0.01

TECI = Total edge contrast index

Table III.4a shows which type of land cover is used by three different types of ticks collected in the year 2017. In contrast to 2016 data, *A. americanum* were collected in high number from woodland (52.4%) compared to pasture (47.6%). In congruence with 2016 data, *D. variabilis* and *I. scapularis* were found more frequently in woodland (61.4%) and (91.6%), respectively.

Table III. 4a. Distribution of tick species in woodland and pasture in the year 2017.

Type of land cover	**n (%)**
Woodland	
A. americanum	88 (52.38)
D. variabilis	121 (61.42)
I. scapularis	11 (91.66)
Pasture	
A. americanum	80 (47.61)
D. variabilis	76 (38.57)
I. scapularis	1 (8.33)

Table III.4b. represents the multivariate logistic model constructed based on different variables to show which land use land cover parameters predict the distribution of different species in 2017. Significant factors have a $P < 0.05$. Association coefficient for *Amblyomma* and patch cohesion is high (0.781) and this is in agreement with the data in table III.4a. Association coefficient for *Dermacentor* and %mixed forest is high (1.762) and this is in agreement with the data in table III.4a. Association coefficient for *Ixodes* and %mixed forest is high (0.399) and this is in agreement with the data in table III.4a as well.

Table III. 4b. Association of land cover/land use indices with the presence of tick species in the year 2017.

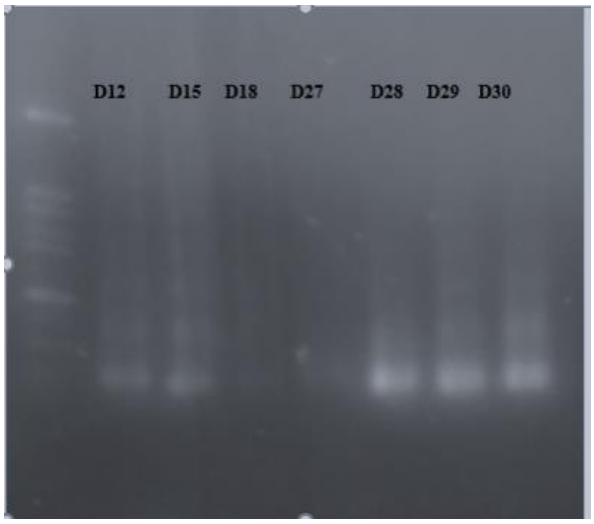
A. americanum	**Coefficient**	**Std. E**	**Exp**	**P**
TECI	0.283	0.071	1.55	0.02
Edge density	0.278	0.282	1.11	1.78
% pasture/hay	0.177	0.087	1.89	0.00
Patch cohesion	0.781	0.651	0.14	2.19

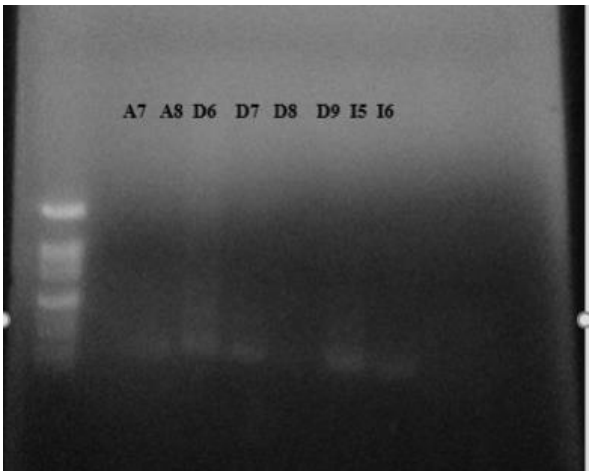
D. variabilis	**Coefficient**	**Std. E**	**Exp**	**P**
TECI	0.459	0.098	2.17	0.00
% mixed forest	1.762	0.119	1.81	0.01
Patch cohesion	0.031	0.081	1.21	0.09
% Shrub/scrub	0.081	0.011	1.71	0.06

I. scapularis	**Coefficient**	**Std. E**	**Exp**	**P**
TECI	0.284	0.120	1.87	0.02
% mixed forest	0.399	0.184	2.12	0.00
Edge contrast	0.019	0.001	1.10	0.04

TECI = Total edge contrast index

In order to detect bacterial pathogens carried by the identified ticks in 2016, total genomic DNA was isolated from adults and nymphs. A total of 89 DNA samples were extracted after pooling 2-3 adults and up to 5 nymphs from same locations/dates. Fig. III.4a, b, and c are representative 0.7% agarose gels that were run to check the quality of extracted DNA. The extracted DNA showed smear on gel but most of them had larger fragment lengths. The concentration was checked with nanodrop in nanogram per microliter. DNA with a concentration of >10ng per µl were appropriate for further analysis. The A260/280 ratio was noted and the range between 1.7-1.9 was considered good quality DNA ***(see Appendix C)***.





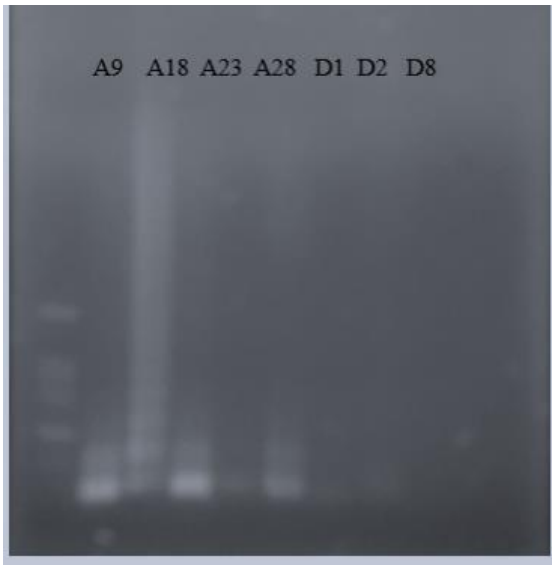


Fig. III.4a-c. Total extracted DNA from ticks (adults and nymph) on 0.7% agarose gels. Either Lambda DNA or 100 bp DNA ladder was used as marker on gels.
A = *Amblyomma*, D = *Dermacentor*, I = *Ixodes*, C = Control

The 16S rRNA gene (~1500 bp) is present in all eubacteria and could be amplified using universal primers. The universal primers target the most conserved sequences of the gene that are present across the domain. All the 89 extracted DNA samples were subjected to PCR amplification for 16S rRNA gene. Out of 89, 77 samples (86.5%) were found to be positive for PCR reaction on 1% agarose gel showing an amplicon length of 1500 bp. Fig. III.a-e. represent the gels showing expected 1.5 kb PCR amplicon from various samples. The total extracted DNA samples that could not be amplified using these universal primers were not pursued for further analysis.

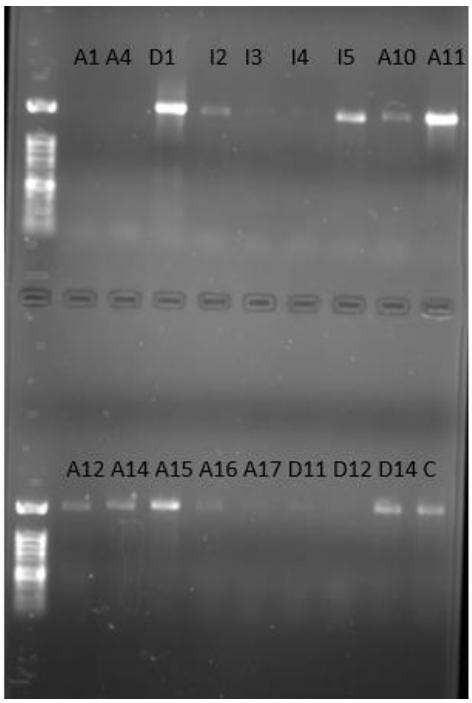


Fig. III.5a. PCR amplification of 16S rRNA gene using total extracted tick DNA as template. Each PCR reaction used positive control of DNA from cultured bacteria. 100 bp DNA ladder was used as marker on each gel.

A = *Amblyomma*, D = *Dermacentor*, I = *Ixodes*, C = Control

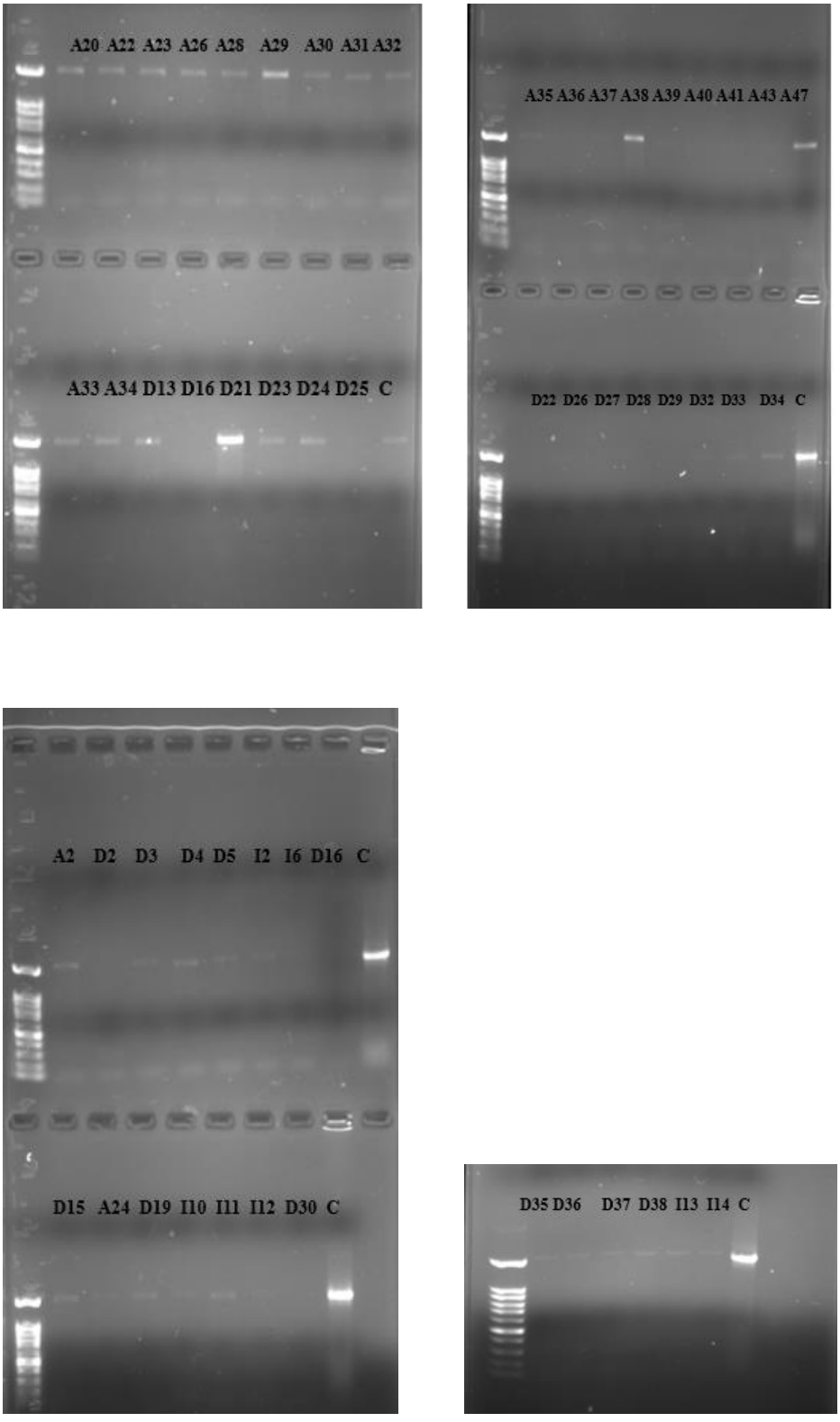


Fig. III.5b-e. PCR amplification of 16S rRNA gene using total extracted tick DNA as template. Each PCR reaction used positive control of DNA from cultured bacteria. 100 bp DNA ladder was used as marker on each gel.

A = *Amblyomma*, D = *Dermacentor*, I = *Ixodes*, C = Control

Further to confirm the tick identification, a subset of total extracted tick DNA was subjected to PCR amplification of tick species specific gene. Out of 39 *Amblyomma* DNA, 13 samples (33.3%) were tested and expected an amplicon length of ~200 bp; out of 28 *Dermacentor* samples, 9 (32.1%) were tested and expected an amplicon length of ~900 bp and all of *Ixodes* samples (100%) were tested and expected an amplicon length of ~1000 bp. Fig. III.6a shows 1% agarose gel with *Amblyomma* and *Dermacentor* samples as positive. While 100% *Amblyomma* were positive for *A. americanum*, 7 out of 9 (77.7%) *Dermacentor* were positive for *D. variabilis*. Only one out of 11 *Ixodes* samples (9.0%) tested were positive for *I. scapularis* (Fig. III.6b).

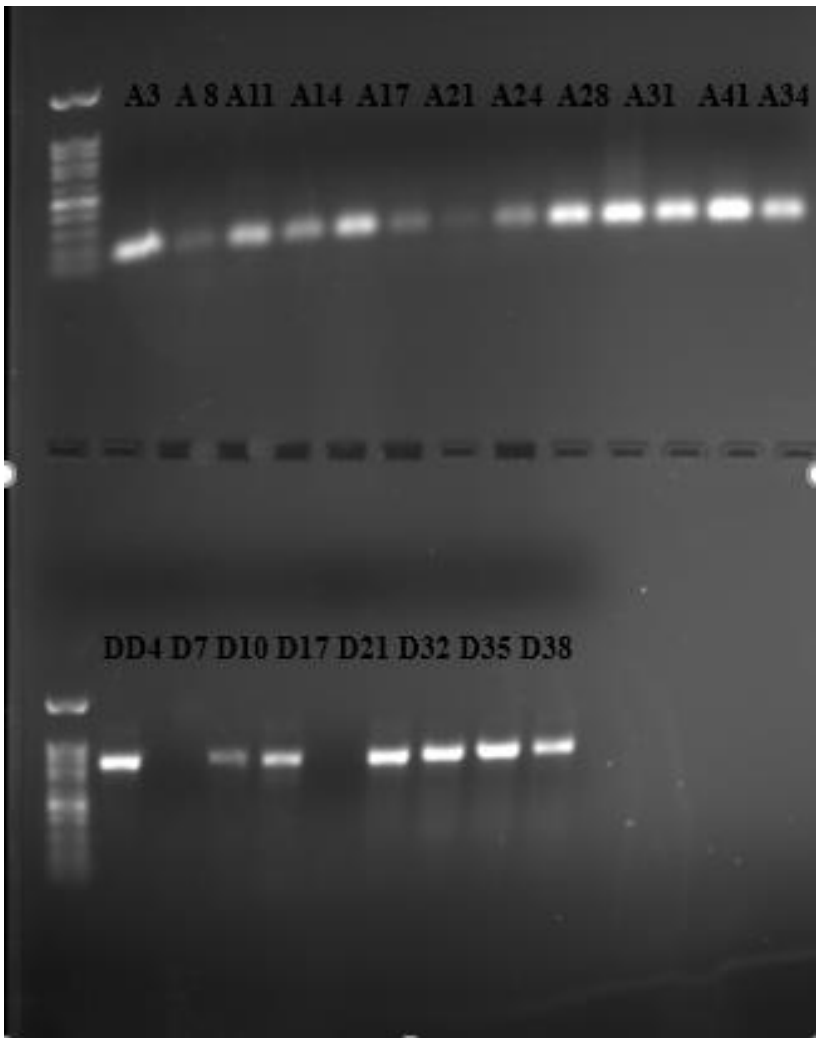


Fig. III.6a. PCR amplification of *A. americanum* and *D. variabilis* using species-specific primer sets. 100 bp DNA ladder was used as marker on each gel.
A = *Amblyomma*, D = *Dermacentor*, C = Control

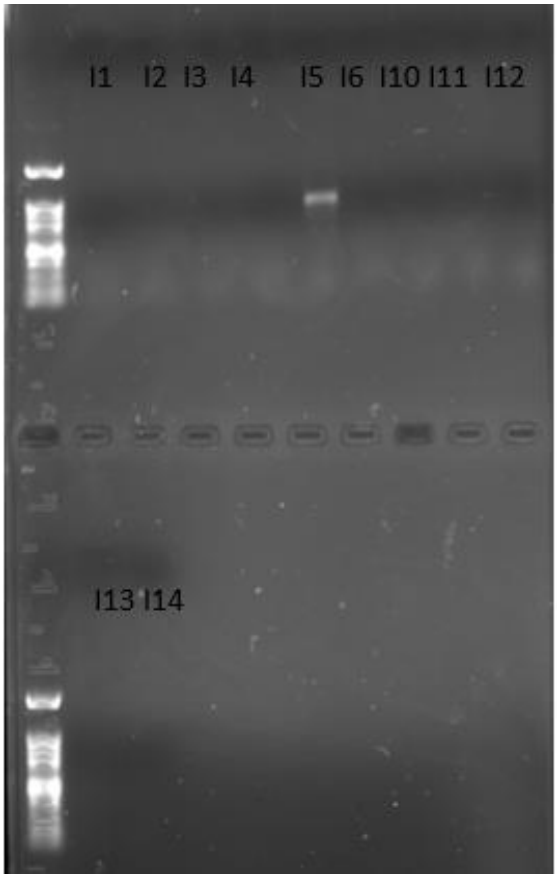


Fig. III.6b. PCR amplification of *I. scapularis* using species-specific primer sets. 100 bp DNA ladder was used as marker on each gel.
I = *Ixodes*, C = Control

To detect the prevalence of bacterial pathogens, PCR amplification was performed using species-specific primers and genomic DNA from control bacterial strains. Based on the positive results *E. chafeensis*, *F. tularensis* and *R. rickettsii* were selected for further analysis.

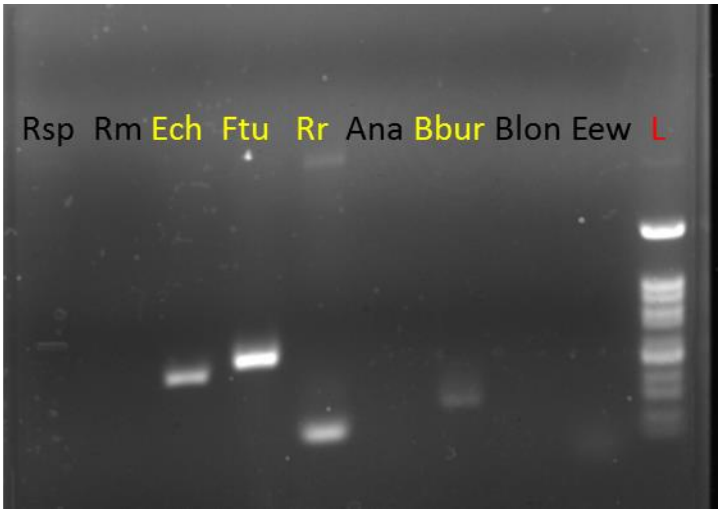
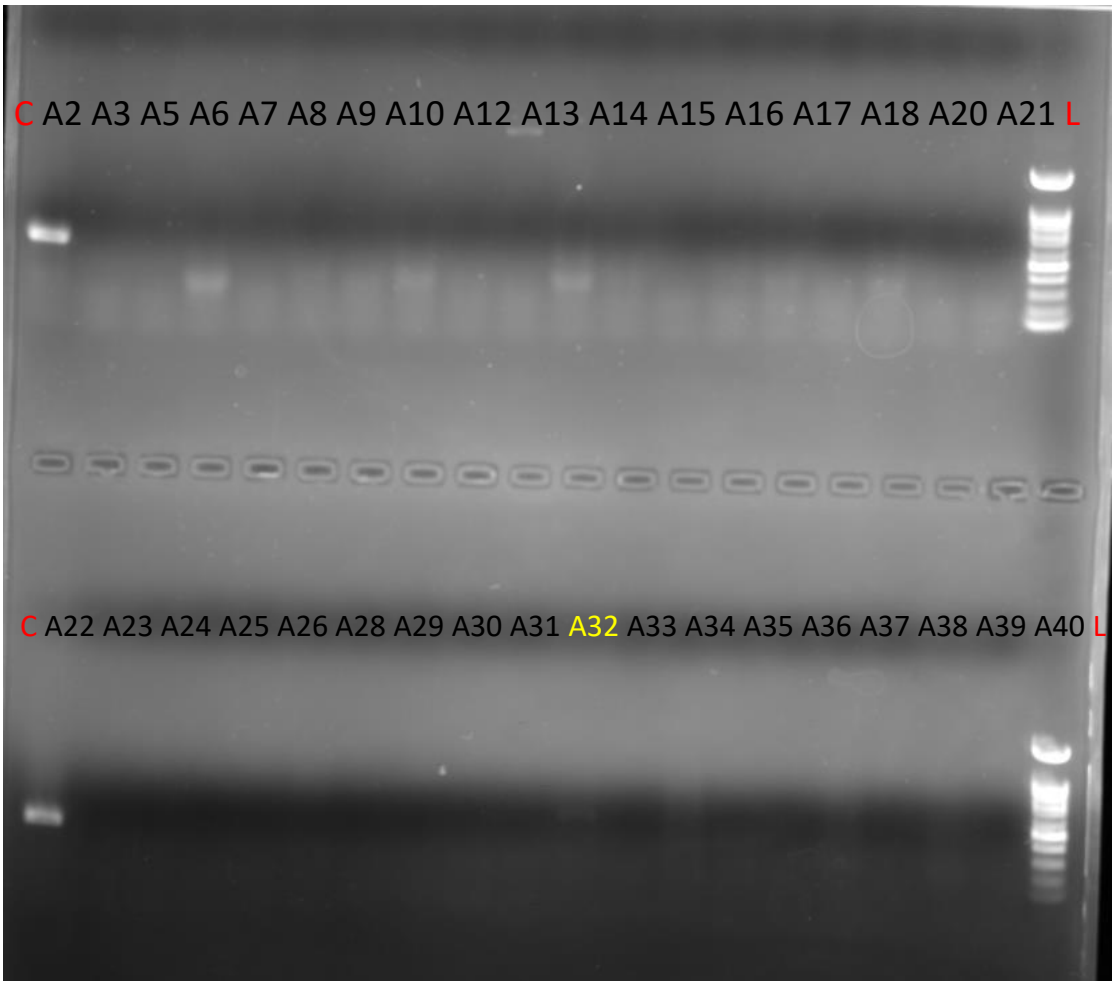


Fig. III.7. PCR amplification of selected species-specific genes from control bacterial strain on 1% agarose gel. 100 bp DNA ladder was used as marker on this gel. Rsp = *Rickettsia* sp., Rm = *R. montanensis*, Ech = *E. chaffeensis*, Ftu = *F. tularensis*, Rr = *R. rickettsii*, Ana = *Anaplasma* sp., Bbur = *B. berghotferi*, Blon = *B. lonstarii*, Eew = *E. ewingii*

PCR amplification was carried out using *Amblyomma* and *Dermacentor* DNA as templates. Although one of the *Ixodes* was positive using molecular method, *Burkholderia* sp. or *Anaplasma* sp. was not tested.



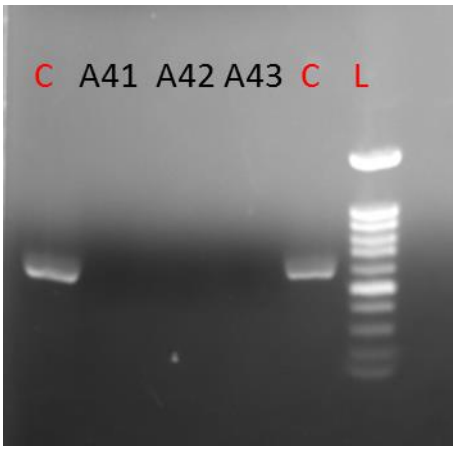


Fig. III.8a-b. Detection of *Francisella tularensis* in *A. americanum* by species-specific PCR on 1% gel. Positive samples are highlighted in yellow.
A = *Amblyomma*, C = Control, L = 100 bp DNA ladder

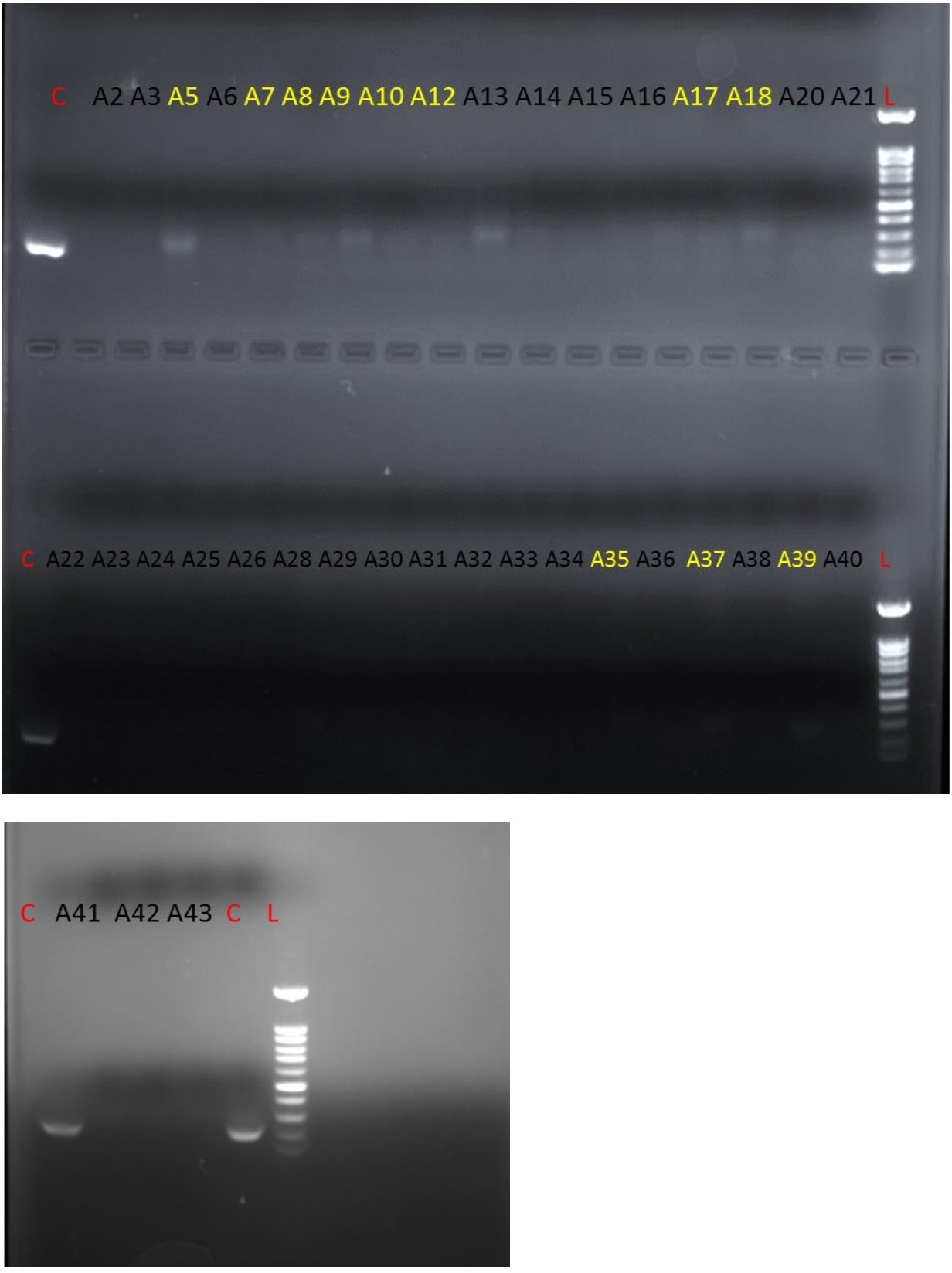
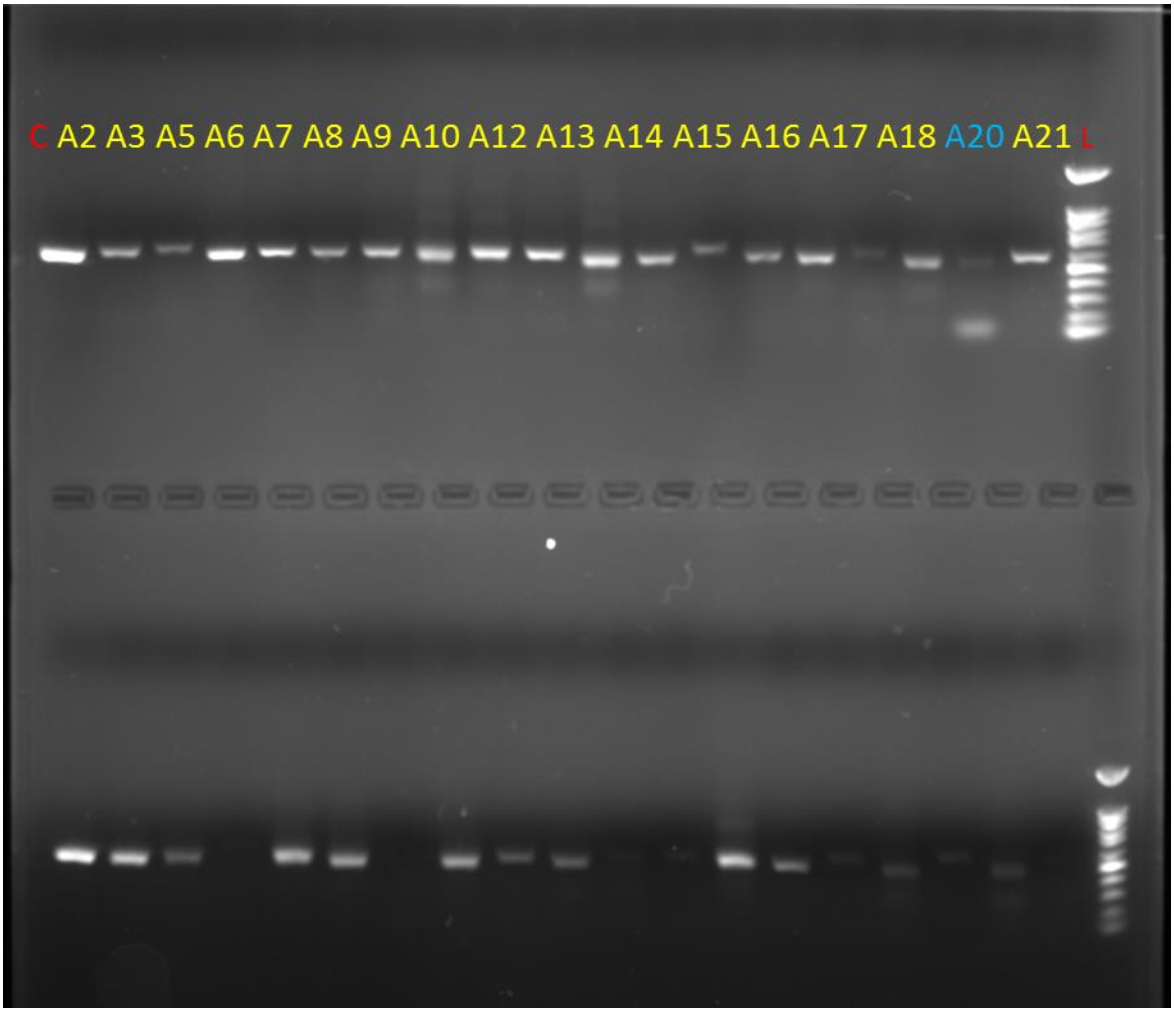


Fig. III.9a-b. Detection of *Rickettsia rickettsii* in *A. americanum* by species-specific PCR on 1% gel. Positive samples are highlighted in yellow.
A = *Amblyomma*, C = Control, L = 100 bp DNA ladder



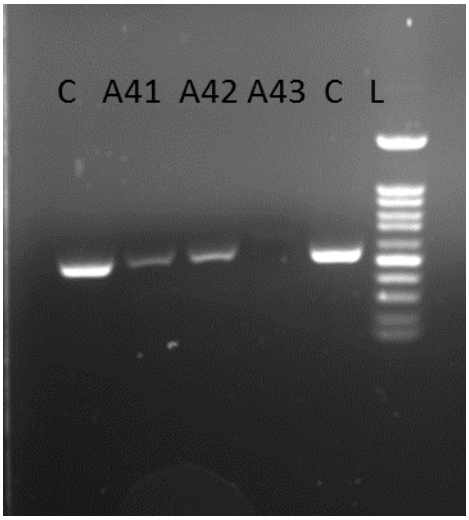


Fig. III.10a-b. Detection of *Ehrlichia chaffeensis* in *A. americanum* by species-specific PCR on 1% gel. Positive samples are highlighted in yellow.
A = *Amblyomma*, C = Control, L = 100 bp DNA ladder

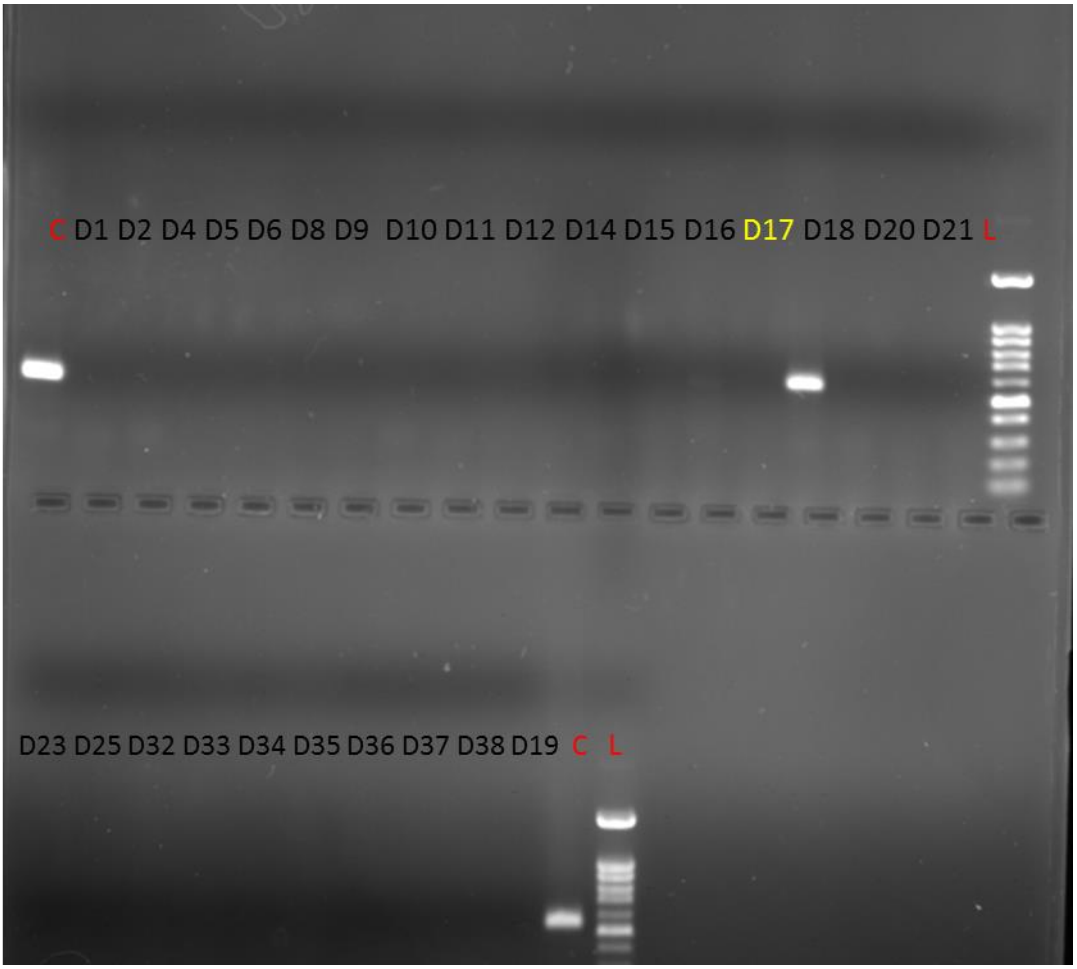


Fig. III.11. Detection of *Francisella tularensis* in *D. variabilis* by species-specific PCR on 1% gel. Positive samples are highlighted in yellow.
D = *Dermacentor*, C = Control, L = 100 bp DNA ladder

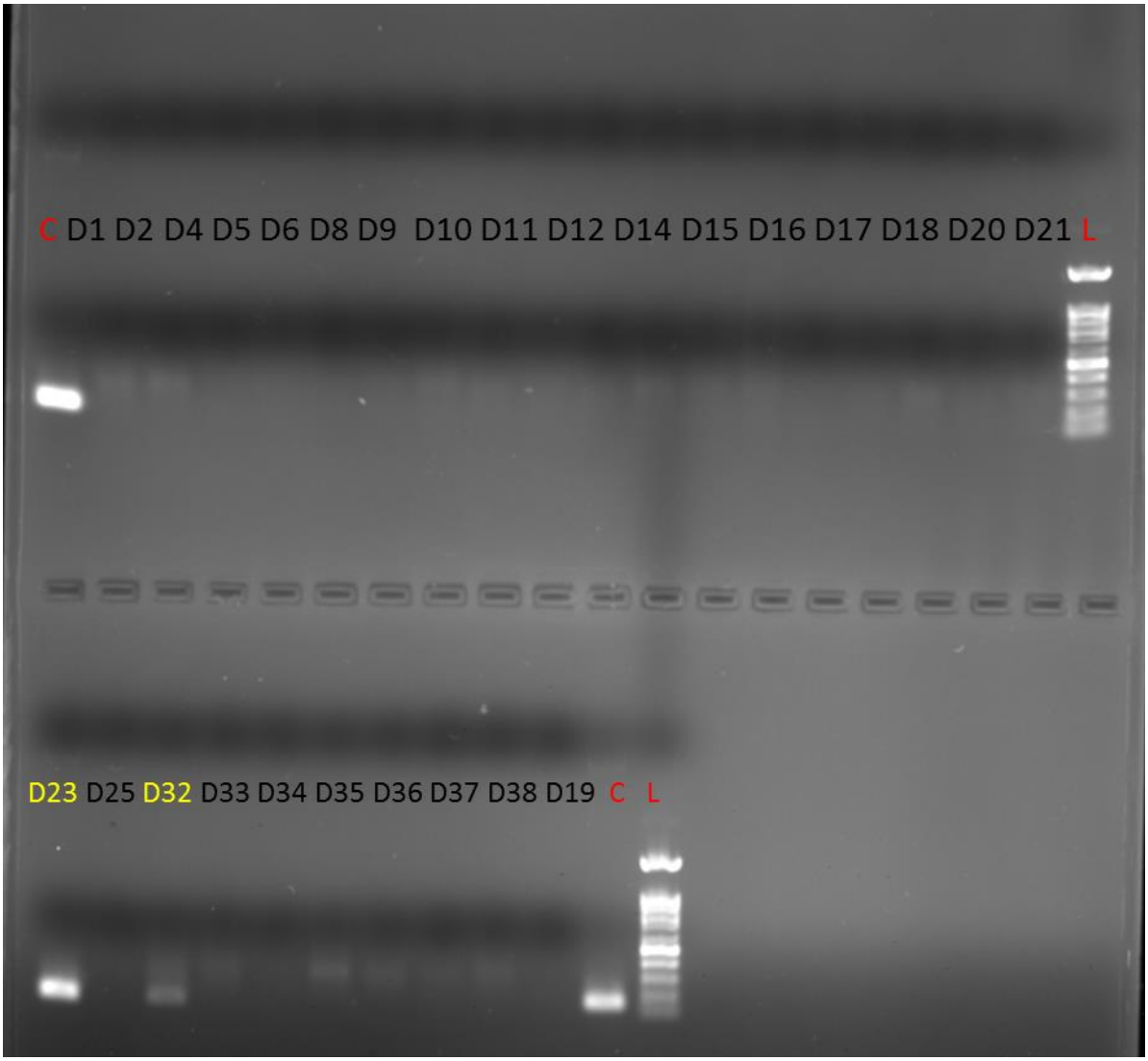


Fig. III.12. Detection of *Rickettsia rickettsii* in *D. variabilis* by species-specific PCR on 1% gel. Positive samples are highlighted in yellow.
D = *Dermacentor*, C = Control, L = 100 bp DNA ladder

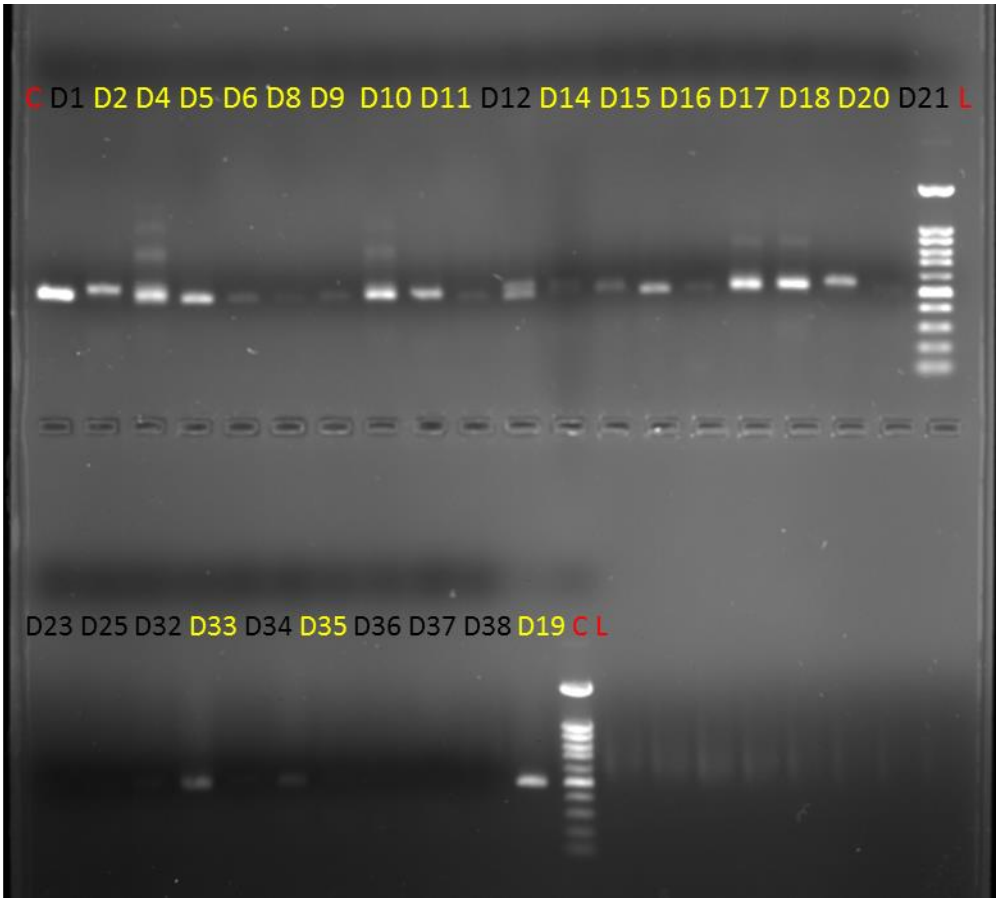


Fig. III.13. Detection of *Ehrlichia chaffeensis* in *D. variabilis* by species-specific PCR on 1% gel. Positive samples are highlighted in yellow.
D = *Dermacentor*, C = Control, L = 100 bp DNA ladder

The rate of detection in *Amblyomma* for *F. tularensis* was 2.56% (Fig. III.8a-b, total pooled isolation = 39), for *R. rickettsii* was 28.2% (Fig. III.9a-b, total pooled isolation = 28). The rate of detection in *Dermacentor* for *F. tularensis* was 3.57% (Fig. III.11), for *R. rickettsii* was 7.14% (Fig. III.12). Detection rate for *E. chaaffensis* in *Amblyomma* and *Dermacentor* was 100%, much higher than expected (Table III.5). Therefore, the primer sets were tested *in silico* once again and were found to be non-specific to *E. chaffeensis*. Tables III.6a and b illustrates the collection sites of the ticks that were positive for selected bacterial pathogens.

Table III.5. Rate of detection of bacterial pathogens among identified pooled ticks.

Rate of detection	***F. tularensis***	***R. rickettsii***	***E. chaffeensis***
Amblyomma	2.56%	28.2%	ND
Dermacentor	3.57%	7.14%	ND

Table III.6a. Details of samples showing presence of *Francisella tularensis* and *Rickettsia rickettsii* in *Amblyomma americanum.*

Bacterial pathogen	*Amblyomma* ID.	life stage & number	Date of collection	Location name	GIS coordinates
Francisella tularensis	A32	3 Adults	6-12-16	Wilderness park	(37.454882, -94.713836)
Rickettsia rickettsii	A5	3 Adults	6-2-16	Weir KS	(37.310055, -94.771904)
	A7	3 Adults	7-22-16	Madison Pet Clinic Webb City	(37.128007, -94.474864)
	A8	3 Adults	5-8-16	Pittsburg/KS	(37.410884, -94.70496)
	A9	3 Adults	6-6-16	231 N 255th St Mulberry KS	(37.537039, -94.639714)
	A10	3 Adults	6-12-16	Wilderness park	(37.454882, -94.713836)
	A12	3 Adults	7-13-16	wilderness park	(37.454882, -94.713836)
	A17	3 Adults	6-3-16	Joplin newton county	(37.128007, -94.474864)
	A18	3 Adults	5-25-16	1022 S 210th Pittsburg KS	(37.378913, -94.722207)
	A35	3 Adults	6-10-16	Wilderness Park	(37.454882, -94.713836)
	A37	3 Adults	5-25-16	Country Side Vet Pittsburg KS	(37.428056, -94.71589)
	A38	3 Adults	5-23-16	Dr Beezley Vet Clinic	(37.428056, -94.71589)

Table III.6b. Details of samples showing presence of *Francisella tularensis* and *Rickettsia rickettsii* in *Dermacentor variabilis.*

Bacterial pathogen	*Dermacentor* ID.	Life stage & number	Date of collection	Location name	GIS coordinates
Francisella tularensis	D17	3 Adults	5-25-16	Country Side Vet Pittsburg KS	(37.428056, -94.71589)
Rickettsia rickettsii	D23	3 Adults	07-10-2016	7792 NE 20th Wier KS	(37.266027, -94.686593)
	D32	3 Adults	8-1-16	Osage Rd Fort Scott	(38.600900, -95.645795)

Discussion

Prevalence of tick species

According to our two-year data on tick collection, *A. americanum* was identified in the majority of the population followed by *D. variabilis*. Zurek et al. (2004) reported that these two species of ticks are predominant in Kansas. These two species are particularly abundant in southeast Kansas (CDC 2016, Raghavan et al. 2016b).

Furthermore, detection of *Ixodes* spp. In this region is of great importance. In our study we show that this species is not just obtainable in this area but also their prevalence increased from 2% in 2016 to 3.1% in 2017. However, this increase in *Ixodes* proportion could be year-to-year variability or due to sampling error. We aim to continue our annual sampling for 5-years in order to obtain convincing data. White and Mock (1991) noticed presence of this species not only in southeast but also northeast Kansas. Based on ecological niche modeling, it was shown that this species ranges continuously across eastern North America; this distributional pattern is supported by independent occurrence data from the eastern Great Plains, in Kansas (Petersen and Raghavan 2017).

Ecological distribution of ticks

The type of land use by *A. americanum* differed in two subsequent years. This may be due to small sampling size in 2017. Our analyzed data shows 47-52% use for both woodland and pasture. Raghavan et al. (2016a) based on a larger sample size reported higher rate of recovery of *A. americanum* from woodland (n=2720) compared to pasture (n=1637).

It has been shown that *I. scapularis* nymphal tick exposure is greater in woodland than grassland (CDPH 2018, Lane et sl. 2007, Hickling et al. 2018). Although

the number of *Ixodes* sp. Collected in this study was very low, they were predominantly collected from woodland as well. Species of Dermacentor were located more frequently in woodland compared to pasture in both years. Similar distribution was reported by Eisen et al. (2006).

Molecular detection of bacterial pathogens

We were able to cover a broader region for our sampling in 2016; therefore, the molecular analysis was performed only on ticks collected in 2016. There were a total of 89 DNA extraction samples obtained from pooled male, female and nymphs (n=1031 from the year 2016) in this study. Out of 89, 77 extracted DNA samples were further analyzed. The rest 12 samples were not usable due to poor extraction efficiency.

Subset of ticks were also attempted to be identified at the molecular level to confirm our visual identification using taxonomic key. It was revealed that *A. americanum* was the predominant identified tick species in our sample, while there could be other species that are present among *Dermacentor* and *Ixodes*. *Ixodes brunneus* was detected in Missouri (Brown et al. 2011). In the western U.S. *I. pacificus* was the major vector for Lyme disease while *I. ricinus* in Europe *and I. persulcatus* in Asia (Walker 2014). Therefore, the ticks identified as *Ixodes* based on morphological keys in this study could belong to either *I. brunneus* or *I. scapularis*. *Dermacentor andersoni*, also called Rocky Mountain wood tick, is predominantly reported in the western part of the U.S. (CDC 2016). It will be worth to re-identify the *Dermacentor* species that were negative using the species-specific primers in this study.

Nymphal *D. variabilis* and *A. americanum* have been implicated as vectors for *F. tularensis* in previous studies by Mani et al. (2015) and Reese et al. (2010), respectively. Brown et al. (2011) provided an updated distribution map for *A.*

americanum and their association with county level reported tularemia cases in Missouri. According to the data obtained from the CDC, the incidence rate (reported cases per 100,000 residents) of tularemia in Kansas over a 10-year period of time (2006-2016) was 0.86 (Fig. III.1).

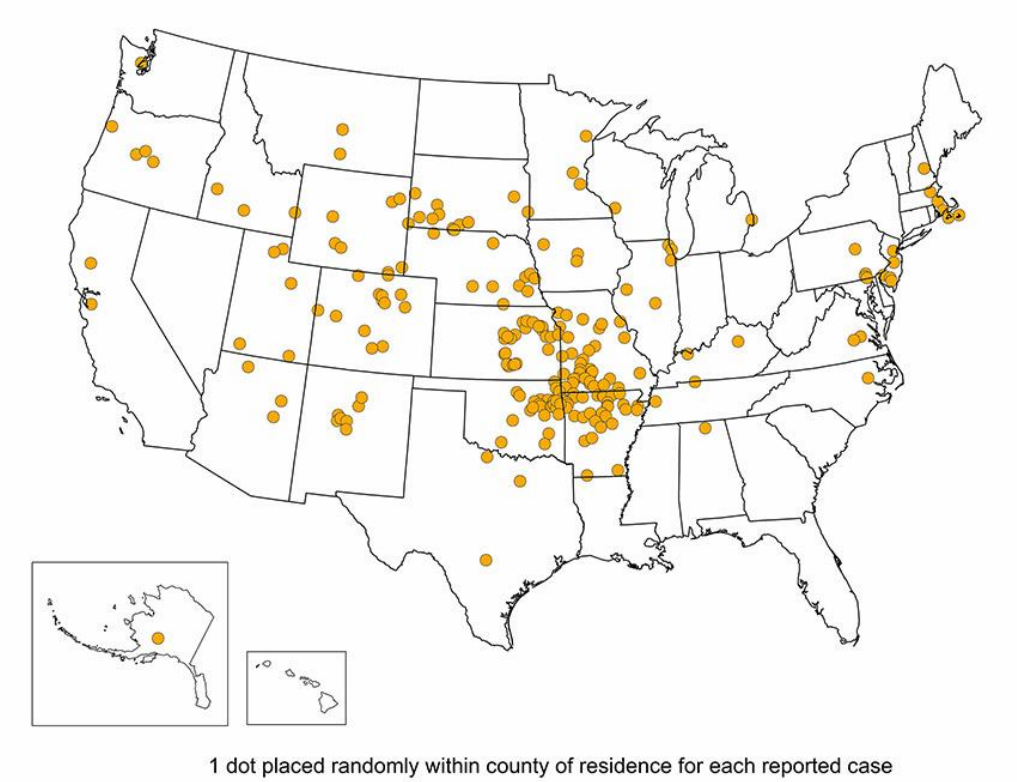


Fig. III.1. Map showing distribution of reported cases of Tularemia from all states except Hawaii, but is most common in the south central United States, the Pacific Northwest, and parts of Massachusetts.

[Source: https://www.cdc.gov/tularemia/statistics/index.html]

A study by Paquette (2016) over a period of 4 years (2012-2015) showed that there was not much fluctuation in the annual disease incidence (0.83-1.16 per 100,000 persons-year) (Fig. III.2). Detection of *F. tularensis* in *Amblyomma* and *Dermacentor* in this this study is a significant finding since this increases the risk of acquiring the pathogen in this area.

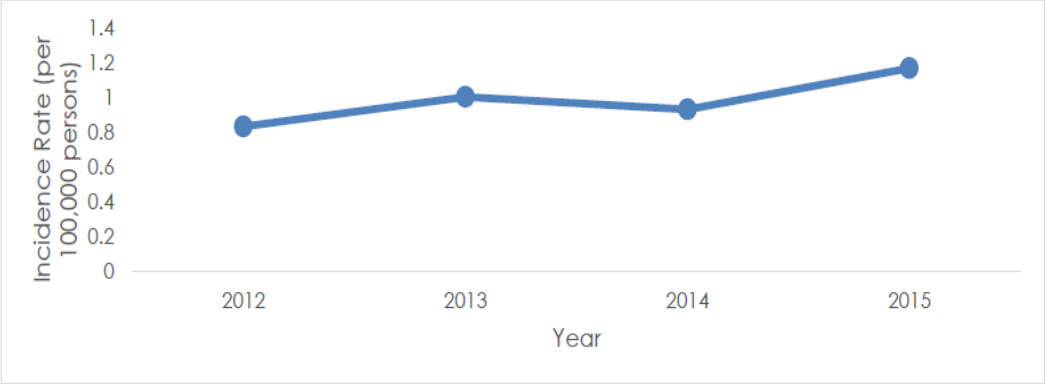


Fig. III.2. Incidence rate of tularemia per 100,000 persons – Kansas 2012-2015.

It is important to note that there are at least three recognized tick genera, *Dermacentor*, *Amblyomma*, and *Rhipicephalus* that are capable of transmitting Rocky Mountain spotted fever (RMSF) in the eastern and south-central U.S. (Breischwerdt et al. 2011).They found that *A. americanum* can transmit *Rickettsia amblyommii*, *R. parkeri*, and *R. rickettsii* to persons in the U.S. Paddock and Yabsley (2007) described that since white-tailed deer serve as a keystome host for all stages of *A. americanum*, rapid expansion of deer population in the eastern U.S. during the twentieth century influenced *A. americanum* borne zoonoses.

Since 2010, RMSF cases are reported under Spotted Fever Rickettsiosis (SFR). The number of SFR cases for every million persons has increased from <2 cases per million persons in 2000 to over 11 cases per million in 2014 (Fig. III.3, CDC 2017). In 2016, around 230 cases of tick-borne diseases were reported to the Kansas Department of Health and Environment, and the highest number of those cases were RMSF. Moreover, an eight-year (2000-2008) period data depicted a sharp rise of incidence rate (1.7 to 9.4 cases per million persons) of RMSF (Openshaw et al. 2010).

According to our detection method, *R. rickettsii* was found to be associated with *Amblyomma* more frequently than *Dermacentor*. In Missouri, Satanello et al. (2018)

noticed *R. amblyommatis* as the predominant spotted fever group in *A. americanum, D. variabilis,* and *I. scapularis*. It is predicted that high risk of exposure to this rickettsial species may provide some cross-protective immunity to *R. rickettsii*.

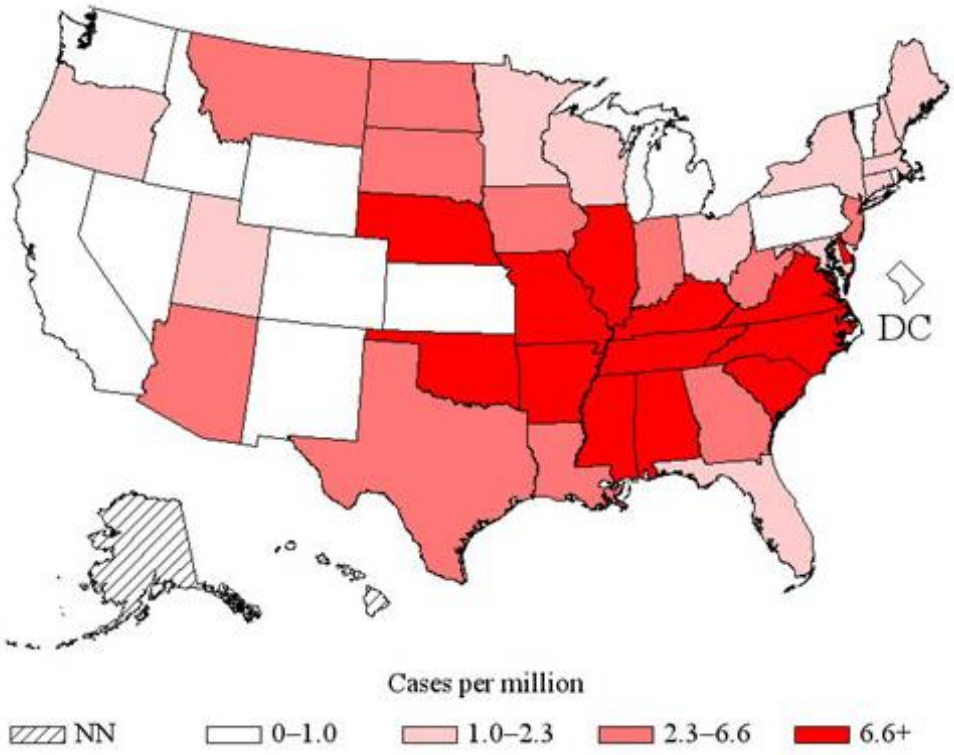


Fig. III.3. Map showing geographic distribution of Spotted fever Rickettsiosis (SFR). Reported incidence (per million population) of SFR cases by states in the United States for 2014. (NN= Not notifiable)

[Source: https://www.cdc.gov/otherspottedfever/stats/index.html]

Figure III.4 shows state-wide occurrence of ehrlichiosis with highest rate in the southeastern and south-central United States, from the Eastern Coast extending westward to Texas. Interesting, these areas overlap significantly with the geographic distribution of *A. americanum,* which is the primary vector of *E. chaffeensis* and *E. ewingii.* In 2016, 50% of all reported cases of ehrlichiosis were reported in Missouri, Arkansas, New York, and Virginia (CDC 2016). The incidence in Kansas was also very high > 6.4 cases per million persons (Fig. III.4). Enzoonotic cycle of *E. chaffeensis* is

maintained in white-tailed deer and thereby increasing incidence of Ehrlichiosis in the U.S. could be partially explained by expansion of deer population which is naturally infected with *E. chaffeensis* (Nichols et al. 2016).

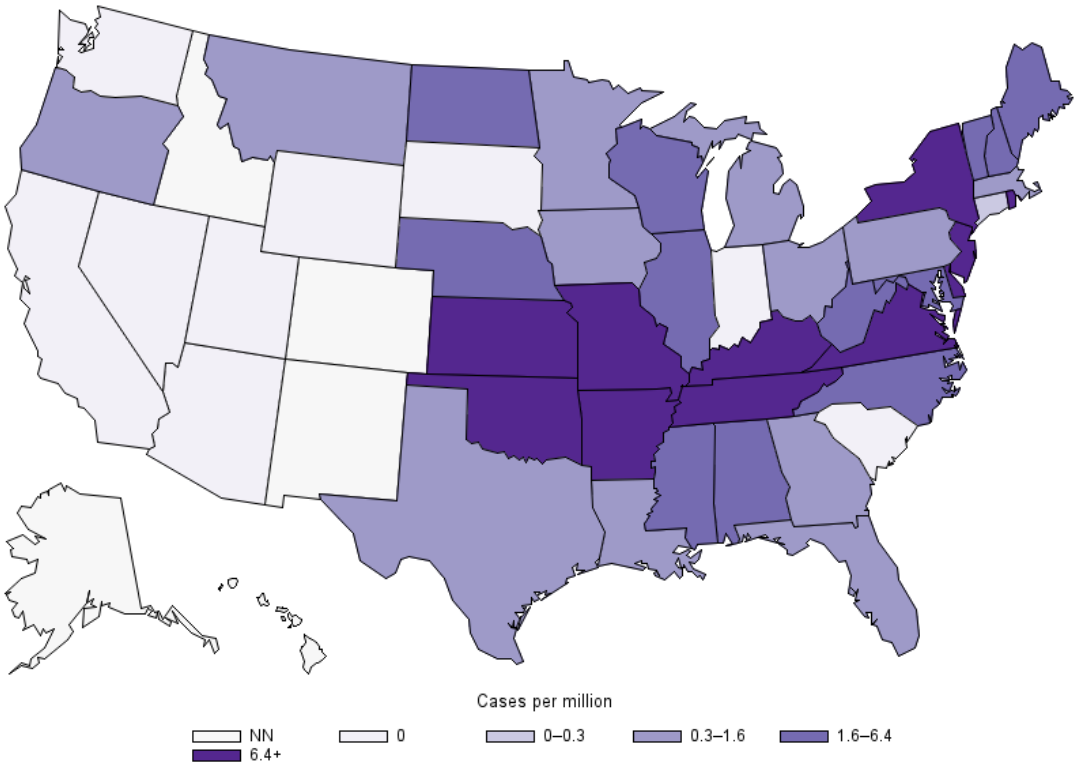


Fig. III.4. Map showing incidence of ehrlichiosis cases caused by *Ehrlichia chaffeensis* by state in 2016 per million persons in the United States in 2016. (NN= Not notifiable)

[Source: https://www.cdc.gov/ehrlichiosis/stats/index.html]

In the present study, detection of *E. chaffeensis* was attempted using PCR primers; however, due to error in primer design the observed results were not accounted for. Newer set of primers would be used in future to amplify *E. chaffeensis* specific DNA from all *Amblyomma* sp. and *Dermacentor* sp. isolated in this study.

Conclusions and future research

In conclusion, almost equal prevalence of *Amblyomma* and *Dermacentor* found in this study indicates an increased probability of rickettsial infections in the southeast Kansas region. Isolation of *Ixodes* ticks is of great importance in this region reflecting their expansion over a large geographic area in recent years because of changing conditions of temperature and humidity. As per the ecological distribution, *Amblyomma* were found in pasture while *Dermacentor* and *Ixodes* were frequently isolated from woodlands which corroborate with previous studies. Detection of bacterial agents such as *Rickettsia* and *Fransicella* although in low prevalence confirms effectiveness of our detection method. The data obtained in this study would help in implementing comprehensive surveillance and management programs for ticks and tick-borne disease risk for humans and animals in this region.

In future research attempts would be made to sequence PCR amplicons to confirm identify of bacterial pathogens. It will be interesting to find out the species of *Ixodes* identified in this study and PCR will be performed to detect *Borrelia burgdorferi* (the Lyme disease causing pathogen). In addition, our findings would be shared among local veterinary clinics and human hospitals (Community Health Centre) as well as efforts would be made to obtain information on reported tick-borne diseases in this region.

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APPENDIX

APPENDIX A

Tick collection sites for the year 2016. (D= *Dermacentor*, A= *Amblyomma*, I= *Ixodes*)

Date	location	GPS	Genus	n
6-15-16	1195 E 530th Pittsburg	(37.381041, -94.632486)	D	3
6-27-16	7792 NE. 20th Weir KS	(37.291396, -94.792435)	I	2
7-13-16	N. Freeking Hwy	(37.438122, -94.668069)	D	1
6-27-16	NE. Hwy 400 Weir KS	(37.339078, -94.771317)	A	1
6-15-16	Wick KS	(37.989471, -96.122496)	D	2
6-21-16	4 Woodmore, Oswego KS	(37.171555, -95.097735)	A	1
6-29-16	NE. Weir Rd & NE 80th	(37.309678, -94.687045)	D	1
6-14-16	Deer Rd & 250th St Bourbon County	(37.716882, -94.649507)	D	2
6-15-16	Webb City Pet Clinic	(37.128007, -94.474864)	I	1
5-31-16	Joplin Newton County	(37.128007, -94.474864	D	1
06/05/2016	Joplin Newton County	(37.128007, -94.474864	D	3
05/09/2016	1280 26000 Rd Parson KS	(37.369156, -95.316831)	D	1
06/16/16	4033 Parkview Dr Frontenac	(37.444740, -94.70597)	D	2
5-13-16	127 N 130th St	(37.517336, -94.870416)	D	1
5-22-16	Crawford County Fairground	(41.638298, -80.149112)	I	1
5-25-16	Rural Weir KS	(37.343390, -94.777818)	D	1
5-25-16	Peterson Vet Clinic Pittsburg	(37.406506, -94.704504)	A	4
5-25-16	Peterson Vet Clinic Pittsburg	(37.406506, -94.704504)	A	14
05/08/2016	7792 NE 20th Wier KS	(37.291396, -94.792435)	A	21
05/18/2016	Scammon KS	(37.277557, -94.82496)	I	1
05/01/2016	Henry Schein Animal Health	(38.984997, -94.707819)	D	26
05/02/2016	Columbus KS	(37.169228, -94.844124)	D	1
05/01/2016	1520 N Main St Pittsburg	(37.413594, -94.726915)	D	1
06/10/2016	Wilderness Park	(37.454882, -94.713836)	A	17
4-25-16	530-540th Pittsburg KS	(37.396658, -94.754448)	D	1
06/10/2016	Wilderness Park	(37.454882, -94.713836)	A	37
06/10/2016	Arcadia KS	(37.454882, -94.713836)	A	29
06/03/2016	1195 E 53 Ave Pittsburg KS	(37.381041, -94.632486)	D	2
05/08/2016	Pittsburg KS	(37.381041, -94.632486)	A	19
07/08/2016	Spring River	(36.792014, -94.752729)	D	6
6-30-16	Weir KS	(37.310055, -94.771904)	D	7
06/11/2016	Deer Rd & 250th St Bourbon County	(37.716882, -94.649507)	A	4
05/10/2016	Farligton KS	(37.617824, -94.827744)	A	3
06/02/2016	Weir KS	(37.310055, -94.771904)	D	3
7-16-16	Frontenac KS	(37.455605, -94.689127)	D	2

06/10/2016	Wilderness Park	(37.454882, -94.713836)	D	2
5-27-16	Weir KS	(37.310055, -94.771904)	A	12
05/11/2016	Weir KS	(37.310055, -94.771904)	A	5
5-29-16	Weir KS	(37.310055, -94.771904)	D	6
6-13-16	Frontenac KS	(37.455605, -94.689127)	A	1
06/11/2016	Deer Rd & 250th St Bourbon County	(37.716882, -94.649507)	D	10
05/11/2016	Weir KS	(37.310055, -94.771904)	D	4
9-19-16	Beezley Bypass	(38.993792, -94.705427)	I	1
05/10/2016	Farligton KS	(37.617824, -94.827744)	D	1
7-17-16	LP Pittsburg	(37.511741, -94.862866)	D	1
05/05/2016	1195 E 53 Ave Pittsburg KS	(37.637341, -97.250068)	A	1
7-21-16	John Duffy Drive	(37.049638, -94.59316)	A	1
05/05/2016	1195 E 53 Ave Pittsburg KS	(37.637341, -97.250068)	D	1
6-21-16	Arcadia KS	(37.641989, -94.623848)	A	1
6-29-16	Girard KS	(37.511160, -94.838021)	D	6
05/11/2016	Monahan Site	(54.234170, -6.99737)	A	28
4-21-16	7792 NE 20th Wier KS	(37.291396, -94.792435)	A	9
5-25-16	1101 Deer creek Land	(37.369034,-94.690036)	D	4
05/09/2016	S 19th pittsburg ks	(37.424776, -94.707141)	D	10
5-25-16	Free kiny Hwy 4108 N	(35.303997, 134.907993)	D	1
4-28-16	7792 NE 20th Wier KS	(37.291396, -94.792435)	D	9
05/11/2016	Monahan Site	(54.234170, -6.99737)	D	4
4-21-16	7792 NE 20th Wier KS	(37.291396, -94.792435)	D	5
5-25-16	4108 N Freeking Hwy	(37.446324, -94.666193)	A	1
05/12/2016	Broadway Vet Clinic	(37.406506, -94.704504)	I	1
05/11/2016	Monahan Site	(54.234170, -6.99737)	A	28
05/12/2016	Broadway Vet Clinic	(37.406506, -94.704504)	A	3
05/12/2016	Broadway Vet Clinic	(37.406506, -94.704504)	D	3
5-25-16	Broadway Vet Clinic	(37.406506, -94.704504)	A	1
05/09/2016	S 190th Pittsburg KS	(37.429313, -94.759914)	A	4
05/12/2016	Frontenac KS	(37.455605, -94.689127)	D	1
5-25-16	406 Utah Ave Pittsburg KS	(37.385037, -94.699202)	D	3
5-25-16	1022 S 210th Pittsburg KS	(37.378913, -94.722207)	D	2
5-25-16	Broadway Vet Clinic	(37.406506, -94.704504)	D	1
05/10/2016	Osage Rd Fort Scott	(37.877716, -94.67417)	I	1
05/10/2016	Osage Rd Fort Scott	(37.877716, -94.67417)	A	20
6-16-16	231 N 255th St Mulberry KS	(37.537039, -94.639714)	A	7
07/10/2016	7792 NE 20th Wier KS	(37.291396, -94.792435)	D	2
7-18-16	1195 E 520th Pittsburg KS	(37.367327, -94.631447)	D	1
7-21-16	4033 Parkview Dr Frontenac	(37.444740, -94.70597)	I	1
6-19-16	1195 E 530th Pittsburg	(37.381041, -94.632486)	D	19
6-16-16	1195 E 530th Pittsburg	(37.381041, -94.632486)	D	5
6-16-16	281 N 255th Mulberry KS	(37.539916, -94.639754)	D	4
07/10/2016	7792 NE 20th Wier KS	(37.291396, -94.792435)	A	3

07/01/2016	Schlanger Park	(37.4134031-94.6940803)	I	1
7-21-16	4033 Parkview Dr Frontenac	(37.444740, -94.70597)	D	1
08/01/2016	Osage KS	(38.600900, -95.645795)	D	3
07/01/2016	Schlanger Park	(37.381041, -94.632486)	A	5
06/06/2016	SEK HS(Humane Society)	(37.424920, -94.762116)	A	13
6-26-16	S1	37.525731, -94.895695	D	12
6-26-16	S2	37.525731, -94.895695	A	3
06/06/2016	SEK HS	(37.037048, -95.632596)	I	2
06/06/2016	SEK HS	(37.037048, -95.632596)	D	7
6-13-16	Columbus KS	(37.169228, -94.844124)	A	2
6-13-16	Columbus KS	(37.169228, -94.844124)	I	2
05/10/2016	Osage KS	(38.600900, -95.645795)	D	7
06/10/2016	Wilderness park	(37.454882, -94.713836)	D	6
6-22-16	Wilderness park	(37.454882, -94.713836)	A	1
06/07/2016	1034E.520th Ave pittsburg	(37.367337, -94.662368)	A	1
6-15-16	Johnson Broadway	(37.406506, -94.704504)	D	1
6-15-16	1101 Deer creek Land	(37.369034,94.690036)	D	1
6-15-16	Georgia St	(33.753068, -84.385282)	D	3
6-15-16	Georgia St	(33.753068, -84.385282)	A	1
6-15-16	Farris KS	(38.936710, -94.603249)	A	5
6-15-16	Cherokee Adair	(36.155869, -94.773133)	A	1
6-15-16	pittsburg KS	(37.381041, -94.632486)	A	1
6-15-16	108 W 24(?) Pittsburg KS	(37.430068, -94.706393)	A	1
6-15-16	Beezley Bypass	(38.993792, -94.705427)	D	4
06/04/2016	Beezley Bypass	(38.993792, -94.705427)	A	3
6-14-16	Beezley Bypass	(38.993792, -94.705427)	D	1
6-15-16	414 Field Crest Pittsburg KS	(37.385711, -94.699201)	A	1
06/02/2016	pairter 69	(37.461483, -94.704477)	A	2
06/02/2016	pairter 69	(37.461483, -94.704477)	D	1
6-15-16	Gordon KS	(37.588630, -96.990592)	D	1
06/06/2016	Frontenac KS	(37.455605, -94.689127)	D	1
06/02/2016	pairter 69	(37.461483, -94.704477)	A	1
06/02/2016	Pittsburg KS	(37.410884, -94.70496)	D	1
06/02/2016	pairter 69	(37.461483, -94.704477)	A	1
06/02/2016	Weir KS	(37.310055, -94.771904)	I	1
07/02/2016	John Duffy Drive	(37.049638, -94.59316)	D	6
7-22-16	Madison Pet Clinic Webb City	(37.128007, -94.474864)	D	9
5-30-16	1034 E 520th Ave Pittsburg KS	(37.367337, -94.662368)	D	3
6-17-16	1034 E 520th Ave Pittsburg KS	(37.367337, -94.662368)	D	6
07/12/2016	Spring River	(36.792014, -94.752729)	A	3
6-22-16	Wilderness Park	(37.454882, -94.713836)	D	3
5-31-16	Joplin Newton County	(37.128007, -94.474864)	I	14
06/05/2016	Joplin Newton County	(37.128007, -94.474864)	I	7
06/06/2016	Bourbon County	(38.217075, -84.22788)	I	15

06/10/2016	Wilderness Park	(37.454882, -94.713836)	I	14
6-16-16	Monahan Site	(53.2734,-7.7783203126)	I	1
06/10/2016	Wilderness Park	(37.454882, -94.713836)	I	27
5-13-16	Dr Beezley Vet Clinic	(37.428056, -94.71589)	D	1
5-18-16	Dr Beezley Vet Clinic	(37.428056, -94.71589)	D	1
05/12/2016	Dr Beezley Vet Clinic	(37.428056, -94.71589)	A	1
7-13-16	211 N Newcomb Pittsburg KS	(37.409671, -94.705041)	A	1
4-28-16	7792 NE 20th Wier KS	(37.291396, -94.792435)	I	7
5-25-16	S 220th Whitlock Pittsburg KS	(37.374729, -94.705147)	D	2
05/09/2016	Pittsburg KS	(37.410884, -94.70496)	A	1
5-25-16	Country Side Vet Pittsburg KS	(37.428056, -94.71589)	D	1
5-25-16	Coalfield Rd Weir KS	(37.266027, -94.686593)	A	3
5-25-16	W Rouse	(37.379152, -94.685651)	A	1
4-25-16	530-540th Pittsburg KS	(37.396658, -94.754448)	A	3
5-25-16	47th Girard KS	(37.514537, -94.756843)	D	33
4-28-16	7792 NE 20th Wier KS	(37.291396, -94.792435)	A	24
5-25-16	4108 N Freeking Hwy	(37.446324, -94.666193)	I	5
5-18-16	Arcadia KS	(37.641989, -94.623848)	A	2
5-17-16	Asbury MO	(37.274500, -94.60551)	A	2
5-25-16	Country Side Vet Pittsburg KS	(37.428056, -94.71589)	A	3
06/10/2016	Wilderness Park	(37.454882, -94.713836)	I	8
5-25-16	1101 Deer creek Land	(37.369034,94.690036)	A	3
05/09/2016	Frontenac KS	(37.455605, -94.689127)	A	1
05/12/2016	Frontenac KS	(37.455605, -94.689127)	D	1
5-24-16	Pittsburg KS	(37.410884, -94.70496)	A	2

APPENDIX B

Tick collection sites for the year 2017.

Date	location	GPS	*Ixodes*	*Amblyomma*	*Dermacentor*
07/08/2017	Stockton, KS	(39.4380656,-99.2650967)	10	14	9
7/18/17	prairie, KS	(37.6010334,-95.8353836)	0	10	50
6/29/17	manahan, KS	(39.18360819,-96.5716694)	0	0	15
07/10/2017	monahan, KS	(37.353599, -94.803012)	0	7	25
7/14/17	wilderness park	(37.454882, -94.713836)	0	4	3
06/07/2017	wilderness park	(37.454882, -94.713836)	0	5	8
5/28/17	mulberry, KS	(37.5569911,-94.6219032)	0	6	0
07/10/2017	weir,ks	(37.3100548,-94.7719043)	0	1	0
6/26/17	springdale, AR	(36.1867442,-94.1288141)	0	1	0
6/22/17	S12	(37.291622-94.793962)	0	21	4
06/09/2017	wildreness park, KS	(37.454882, -94.713836)	0	8	0
06/03/2017	foster, MO	(38.166138,-94.507453)	0	3	3
____	1356 sw county, mo,64752	(38.6064889,-93.8521660)	0	1	12
07/07/2017	form Alex kng		0	0	13
07/11/2017	nature reach, KS	(37.3902857,-94.6915708)	2	42	36
6/22/17	crawling	(37.291622-94.793962)	0	11	0
06/02/2017	crawling	(37.72429-94.639130)	0	12	0
5/25/17	crawling		0	2	0
5/20/17	crawling		0	4	0
6/29/17	Scammon, KS	(37.2775569,-94.8249601)	0	5	2
6/29/17	bultte david Jones Pittsburg		0	1	0
6/22/17	crawling		0	6	14
7/31/17	weir, KS	(37.3100548,-94.7719043)	0	2	1
___	nature reach, KS	(37.3902857,-94.6915708)	0	2	0
08/01/2017	Pittsburg, KS	(37.410884,-94.7049600)	0	0	1
08/04/2017	Pittsburg, KS	(37.410884,-94.70496000)	0	0	2

APPENDIX C

DNA quality and quantity determined by using nanodrop.

Sample no.	Sample ID.	Concentration (ng per µl)	Quality (260/280)
1	A1	19.7	2.05
2	A10	21.3	1.9
3	A11	16.4	1.8
4	A12	85	1.8
5	A13	26.7	1.85
6	A14	7.8	1.5
7	A15	24.3	1.73
8	A16	34.5	1.78
9	A17	19.7	1.72
10	A18	49.8	1.8
11	A19	1.4	1.49
12	A2	9	2.19
13	A20	36.6	1.24
14	A21	24.7	1.05
15	A22	18	1.68
16	A23	51.2	1.63
17	A24	5.1	1.89
18	A25	26.7	1.75
19	A26	43.9	1.82
20	A27	1.2	1.4
21	A28	127.5	2.31
22	A29	21.9	1.8
23	A3	13.3	1.97
24	A30	10.7	1.8
25	A31	25	1.87
26	A32	32.9	1.77
27	A33	5.8	1.8
28	A34	137	1.83
29	A35	34.7	1.93
30	A36	1.2	5.11
31	A4	25	1.88
32	A5	101.7	1.85

33	A6	22.8	2.1
34	A7	23.6	1.73
35	A8	21.6	1.76
36	A9	81.7	1.72
37	D1	64.6	1.82
38	D10	25.9	
39	D11	38.3	1.9
40	D12	51.1	1.84
41	D13	28.8	1.8
42	D14	32.1	1.86
43	D15	98.4	1.84
44	D16	18.9	1.83
45	D17	22.9	1.84
46	D18	52.6	1.77
47	D19	7.6	1.74
48	D2	87.7	2.1
49	D20	28	2.35
50	D21	35.5	1.86
51	D22	21.8	1.57
52	D23	12.4	1.8
53	D23	13.8	1.79
54	D24	80	1.8
55	D24	66.4	1.83
56	D25	37.2	1.67
57	D25	19.1	1.7
58	D26	9.7	1.38
59	D26	8	1.51
60	D27	56.2	1.5
61	D27	27.3	1.31
62	D28	124	1.61
63	D28	64.3	1.37
64	D29	52.4	2.7
65	D29	14.5	1.57
66	D3	10.1	2.04
67	D30	209	1.69
68	D30	150.9	2.33
69	D31	3.8	1.74
70	D31	3	2.82

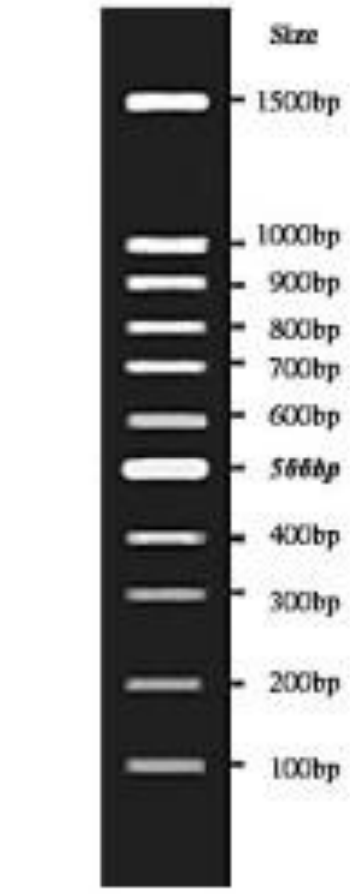
71	D4	13.5	2.08
72	D5	12.2	2.13
73	D6	10.7	1.47
74	D7	22.3	1.88
75	D8	55.7	1.91
76	D9	32.2	1.9
77	I 10	8.7	1.45
78	I 13	14.3	1.64
79	I1	28	1.6
80	I11	10.3	1.52
81	I12	8.7	1.4
82	I12	8.7	1.4
83	I14	8.9	2.34
84	I2	10.7	1.9
85	I3	13.3	1.79
86	I4	12.8	1.92
87	I5	16.1	1.7
88	I6	10.9	2.88

APPENDIX D

Information on 100 bp ladder

100BP DNA Ladder | Bullseye 100BP DNA Ladder | 100BP DNA Marker

Bullseye 100bp DNA Ladder



- Ready to use
- Contains 11 DNA bands: 100-1500bp.
- Clearly identifiable 500bp band as reference
- 500ng DNA/6µl/loading
- Easy to load
- Stable at room temperature
- Supplied with 6x sample loading buffer

Bullseye 100bp DNA Ladder consists of 11 DNA fragments ranging in size from 100-1500 base pairs (bp). 6µl will yield at least 30ng DNA in any single band. The intensity of the 500bp band has been increased to serve as a reference for easy identification.

Size: 1200µl
Storage: Store at -20°C.
Concentration: 500ng/6µl
Loading Buffer Composition:

10mM Tris-HCl
1mM EDTA (pH 8.0)
0.02% Bromophenol blue
0.02% Xylene cyanol
5% Glycerol

Usage: Add at least 6µl Bullseye 100bp DNA Ladder directly to wells designated for markers. You may need more than 6µl of ladder, depending on well size and level of intensity needed to visualize the bands.

APPENDIX E

FAST DNA spin kit for soil

1. Introduction to the FastDNA® SPIN Kit for Soil and the FastPrep® Instruments

The FastDNA® SPIN Kit for Soil quickly and efficiently isolates PCR-ready genomic DNA directly from soil samples in less than 30 minutes. Designed for use with the FastPrep® Instruments from MP Biomedicals, plant and animal tissues, bacteria, algae, fungi spores and other members of a soil population are easily lysed within 40 seconds. These benchtop devices use a unique, optimized motion to homogenize samples by multidirectional, simultaneous impaction with lysing matrix particles. FastPrep® Instruments provide an extremely quick, efficient and highly reproducible homogenization that surpasses traditional extraction methods using enzymatic digestion, sonication, blending, douncing and vortexing. Samples are placed into 2.0 ml tubes containing Lysing Matrix E, a mixture of ceramic and silica particles designed to efficiently lyse all soil organisms including historically difficult sources such as eubacterial spores and endospores, gram positive bacteria, yeast, algae, nematodes and fungi. Homogenization in the FastPrep® Instrument with Lysing Matrix E takes place in the presence of MT Buffer and Sodium Phosphate Buffer, reagents carefully developed to protect and solubilize nucleic acids and proteins upon cell lysis. These reagents work together to allow extraction of genomic DNA with minimal RNA contamination.

Following lysis, samples are centrifuged to pellet soil, cell debris and lysing matrix. DNA is purified from the supernatant with a silica-based GENECLEAN® procedure using SPIN filters. Eluted DNA is ready for PCR, restriction digest, electrophoresis and any other desired application.

2. Kit Components and User Supplied Materials

2.1 FastDNA® SPIN Kit for Soil Components

Lysing Matrix E	50x 2.0 ml tubes
Sodium Phosphate Buffer	60 ml
MT Buffer	8 ml
PPS Solution	25 ml
Binding Matrix	66 ml
SPIN Modules	50 each
Catch Tubes	50 each
Concentrated SEWS-M	12 ml
DES	20ml
BBS Gel Loading Dye	200 µl
User manual	1 each
MSDS	1 each
Certificate of Analysis	1 each

Protocol for FAST DNA spin kit for soil

1. Add up to 500 mg of soil sample to a Lysing Matrix E tube.
   NOTE: See section 3.2 for important guidelines.

2. Add 978 µl Sodium Phosphate Buffer to sample in Lysing Matrix E tube.

3. Add 122 µl MT Buffer.

4. Homogenize in the FastPrep® Instrument for 40 seconds at a speed setting of 6.0.

5. Centrifuge at 14,000 x g for 5-10 minutes to pellet debris.

   NOTE: Extending centrifugation to 15 minutes can enhance elimination of excessive debris from large samples, or from cells with complex cell walls.

6. Transfer supernatant to a clean 2.0 ml microcentrifuge tube. Add 250 µl PPS (Protein Precipitation Solution) and mix by shaking the tube by hand 10 times.

7. Centrifuge at 14,000 x g for 5 minutes to pellet precipitate. Transfer supernatant to a clean 15 ml tube. NOTE: While a 2.0 ml microcentrifuge tube may be used at this step, better mixing and DNA binding will occur in a larger tube.

8. Resuspend Binding Matrix suspension and add 1.0 ml to supernatant in 15 ml tube.

9. Place on rotator or invert by hand for 2 minutes to allow binding of DNA. Place tube in a rack for 3 minutes to allow settling of silica matrix.

10. Remove and discard 500 µl of supernatant being careful to avoid settled Binding Matrix.

11. Resuspend Binding Matrix in the remaining amount of supernatant. Transfer approximately 600 µl of the mixture to a SPIN™ Filter and centrifuge at 14,000 x g for 1 minute. Empty the catch tube and add the remaining mixture to the SPIN™ Filter and centrifuge as before. Empty the catch tube again.

12. Add 500 µl prepared SEWS-M and gently resuspend the pellet using the force of the liquid from the pipet tip.
    NOTE: Ensure that ethanol has been added to the Concentrated SEWS-M.
    See section 3.1.

13. Centrifuge at 14,000 x g for 1 minute. Empty the catch tube and replace.

14. Without any addition of liquid, centrifuge a second time at 14,000 x g for 2 minutes to "dry" the matrix of residual wash solution. Discard the catch tube and replace with a new, clean catch tube.

15. Air dry the SPIN™ Filter for 5 minutes at room temperature.

16. Gently resuspend Binding Matrix (above the SPIN filter) in 50-100 µl of DES (DNase/ Pyrogen-Free Water).
    NOTE: To avoid over-dilution of the purified DNA, use the smallest amount of DES required to resuspend Binding Matrix pellet.
    NOTE: Yields may be increased by incubation for 5 minutes at 55°C in a heat block or water bath.

17. Centrifuge at 14,000 x g for 1 minute to bring eluted DNA into the clean catch tube. Discard the SPIN filter. DNA is now ready for PCR and other downstream applications. Store at -20°C for extended periods or 4°C until use.