MOLECULAR DETECTION OF PATHOGENTIC *EHRLICHIA SPP.* IN TEXAS

An Undergraduate Research Scholars Thesis

by

BRANDEN R. NETTLES

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ABSTRACT

Molecular Detection of Pathogenic *Ehrlichia spp.* in Texas. (May 2015)

Branden R. Nettles
Department of Veterinary Pathobiology
Texas A&M University

Research Advisor: Dr. María D. Esteve Gasent
Department of Veterinary Pathobiology

Ehrlichiosis is becoming one of the increasingly common tick borne illnesses in the US with a steady increase in reported cases from 200 in the year 2000, to 1,549 in 2013 [1]. This study aims to analyze the prevalence of *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis and *Ehrlichia canis*, the causative agent of canine monocytic ehrlichiosis (and potential human pathogen) in ticks throughout the state of Texas. To determine the distribution of *E. chaffeensis* and *E. canis* in the area of study, we collected ticks from different wildlife management areas, deer hunting stations, veterinary clinics, and animal shelters in different counties across the state of Texas. The sampling was conducted during the time period extending from September 2011 to September 2014. All ticks were identified to species and their DNA was purified individually. The tick species mostly observed in this area are: *Rhipicephalus sanguineus*, *Dermacentor variabilis*, *Amblyomma americanum*, *A. cajennense*, *A. maculatum*, *A. inornatum*, *Ixodes scapularis*, and *I. affinis*. Each tick sample was tested by PCR, utilizing primers specific to *E. chaffeensis* and *E. canis*. Positive PCR results were confirmed by sequencing. We evaluated the percentage infection of each tick species for each *Ehrlichia* species. In addition, we correlated infection with geographic location to determine the distribution of these bacterial pathogens in the state of Texas.

DEDICATION

I want to dedicate this thesis to my research advisor Dr. María D. Esteve-Gassent. Even though she has had a lot on her plate over the past year, she still made time to work, not only with me, but also with everyone else in her lab, and even students from outside of her lab, her classes, and on her study abroad trips. She is one of the best professors I have had during my undergrad career at Texas A&M. She deserves every award and accolade she receives, and definitely much more.

Loles, moltes gràcies per haver estat en tot moment al meu costar fent tot el possible en ajudarme, te'l agradeix moltissim, gràcies de tot cor!

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CHAPTER I

INTRODUCTION

Causative agent

Ehrlichia spp. is a genus of the *Alphaproteobacteria* family *Rickettsiales*. These bacteria are gram-negative, obligate parasites of arthropods [2]. There are five species of *Ehrlichia* that cause disease in humans and/or animals and are generally distributed worldwide (Table 1) [3]. *E. ruminantium*, the agent of Heartwater disease in ruminants, has not yet been introduced to the United States; however, the risk is present as the tick *Amblyomma maculatum* has shown competency *in vitro* of maintaining the disease cycle [4].

Table 1. Common characteristics of pathogenic *Ehrlichia* species

Pathogen	Disease	Distribution	Vectors	Reservoir	Disease-presenting Host
E. chaffeensis	Human monocytic ehrlichiosis (HME), canine ehrlichiosis	USA, Africa, South America, Asia	*Amblyomma americanum*	White-tailed deer	Humans, dogs
E. canis	Canine monocytic ehrlichiosis (CME), ehrlichioses in humans	Worldwide	*Rhipicephalus. sanguineus*, *Dermacentor variabilis*	Dogs, wild canids	Dogs, humans
E. ewingii	Human ewingii ehrlichiosis (HEE), canine ehrlichiosis	USA, Africa, Asia	*A. americanum*	White-tailed deer, dogs	Humans, dogs
E. muris	Murine splenomegaly	Eurasia	*Haemaphysalis spp.*, *Ixodes spp.*	Small rodents	Humans*
E. ruminantium	Heartwater in ruminants,	Africa, Caribbean	*Amblyomma spp.*	Cattle, sheep, goats and some wild ruminants	Ruminants, dogs, humans

* as described by [3]

E. chaffeensis is the causative agent of human monotcytic ehrlichiosis (HME). The primary reservoir associated with this pathogen is the White-tailed deer, *Odocoileus virginianus*, and is vectored by the Lone-star tick, *Amblyomma americanum* [3, 5, 6]. *A. americanum* ticks have a wide distribution across the United States and Mexico, extending from the eastern regions of

Mexico to parts of the Midwest and Eastern Coast [7]. The Lone-star tick has also shown to be a competent vector of other disease causing bacteria, including: *E. ewingii*, the agent of Human ewingii ehrlichiosis (or human granulocytic ehrlichiosis) and canine ehrlichiosis; *Anaplasma phagocytophilum*, the agent of human granulocytic anaplasmosis; and *Borellia lonestari*, the causative agent of a Lyme-like disease that has been described in many of the southern states that has been named STARI (Southern Tick-Association Rash Illness) [3, 7].

E. canis is the causative agent of canine monocytic ehrlichiosis (CME). The primary reservoir, as well as dead-end host, for the pathogen is both domestic and wild canids (Figure 1). The Brown dog tick, *Rhipicephalus sanguineus*, and the American dog tick, *Dermacentor variabilis*, are the vectors of *E. canis* [3, 6]. Ehrlichiosis is a great example of a vector-borne disease with zoonotic potential. *E. chaffeensis* is most often considered to be a zoonotic agent, rather than *E. canis* [6], since it has been clearly observed and studied in humans. However, there is evidence coming from Latin America and other developing countries that support *E. canis* as being a competent zoonotic agent [8, 9]. There is increasing evidence showing the importance of dogs in the enzootic cycle of HME with a significant increase in the chance of infection for humans in close contact with infected dogs [10].

Although the impact on the human population of *E. canis* is still undetermined, studies suggest the possibility for it to resemble the other Rickettsiales pathogens (*Anaplasma phagocytophilum*, *E. ewingii,* and *E. chaffeensis*) in terms of zoonotic potential [11]. For instance, in developing countries or in low-income regions in developed countries, dogs that are not routinely treated for ectoparasites and that live in close proximities with their owners increase the probability of the

dog acquiring the agent of ehrlichiosis as well as facilitating its transmission to humans [9]. Various regions of the world face this scenario, in which the culture as well as the socio-economic status of given populations play an important role in disease transmission [10].

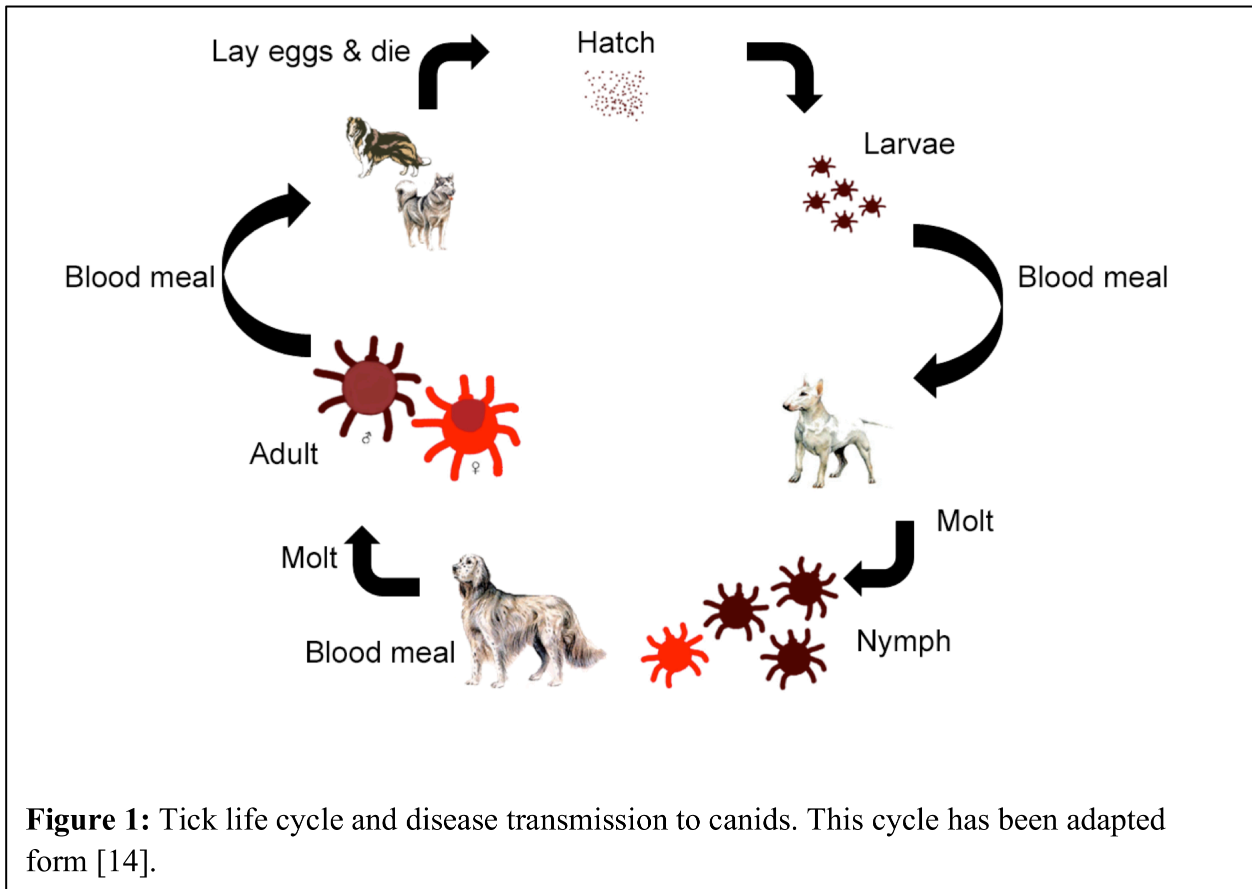

Figure 1: Tick life cycle and disease transmission to canids. This cycle has been adapted form [14].

Venezuela is widely considered to be a developing country in South America*. The socio-economic status of the population in this and other developing countries, allows for a close proximity of dogs to people. *Rhipicephalus sanguineus* has a wide distribution, as it is normally associated with domestic environments [6]. This makes for easy transmission of the vector from

* http://data.worldbank.org/country/venezuela-rb#cp_wdi

dogs to humans, as mentioned above [9]. If the pathogen, *E. canis*, were introduced, this cycle would facilitate the spread of HME and CME in these areas. The US boasts a wide biodiversity, including many different types of ticks, all having the potential to vector *Ehrlichia spp.* In addition, the US and Mexico share their natural history along the trans-boundary region, which allows for the maintenance of these diseases [10].

To have an understanding of the infection and disease cycle, the spatial distribution of the pathogen must be known; however, there is little data about the distribution of these pathogens in many parts of the world [6]. There have been no previous studies showing the spatial distribution of these agents in the state of Texas. A previous study, using results from the SNAP® 3Dx® or 4Dx® test by IDEXX Laboratories, Inc., Westbrook, ME, showed the national distribution of *E. canis* based on passive reporting from veterinarians that use that specific clinical test [12]. This study did not look at the vectors of these two pathogens nor did it account for cross reactivity between *E. canis*, *E. ewingii*, and *E. chaffensis*.

Disease pathology and diagnostics

Monocytic ehrlichiosis is a vector-borne zoonotic infectious disease of humans and canids [13]. The disease is similar in both species. 60% of human monocytic ehrlichiosis cases could be totally asymptomatic, or they could present with nonspecific symptoms, like a cold [14]. After a few days, up to a couple of weeks, the illness will classically be noted by fever, headache, myalgias, arthalgias, and chills [7]. Some cases of ehrlichiosis require admittance to the hospital, especially in populations who are older, have underlying diseases, and/or immunosuppressed [14]. These various nonspecific symptoms may last a few days before giving way to more severe

symptoms. These could involve various organ systems including the gastrointestinal system (nausea, vomiting, anorexia, or abdominal pain), respiratory system (cough or dysapnea), and the central nervous system (confusion or ataxia) [7]. The physical exam of a patient suffering from ehrlichiosis is often unremarkable. Fever, mild tachycardia, pallor, and petechiae could all be common findings. Laboratory results often show thrombocytopenia, leukopenia, elevated hepatic transaminase levels, and anemia [7]. The canine disease presents itself in a similar fashion to the human illness; there are few differences between the two forms, apart from the canine disease having specific stages: subclinical, acute and chronic; where as the human disease is typically characterized as mild or severe [3, 15].

Diagnosis is difficult from the symptomology alone, risk factors such as patient history and the epidemiologic setting must be taken into account as well [14]. Although finding morulae in infected monocytes and macrophages is possible, it is a rare event and their absence should not count against a ruling of ehrlichiosis [7]. The isolation of the specific bacteria that cause these diseases is burdensome, detailed, and time consuming; thus it is not a resourceful diagnostic tool for determining ehrlichial infections [7, 15]. The most reliable methods for attaining a diagnosis for monocytic ehrlichiosis are immunoflorescent antibody testing (IFA) and polymerase chain reaction (PCR) [7, 15]. IFA requires an amount of time post infection for antibody levels to reach a detectable level, and even after an extended period of time, antibody levels might never reach the level required to diagnose an infection. PCR targets the 16S rRNA gene of the microbial genome. A nested PCR has been generated with greater sensitivity to the various *Ehrlichia spp.* [7]. There are enzyme-linked immunosorbent assay (ELISA) based diagnostic tool available to veterinary practitioners to aid in the determination of exposure to various pathogens, such as *E.*

canis and *E. ewingii* (e.g. Idexx SNAP® 4Dx® Plus test, Idexx Laboratories, Inc., Westbrook, ME). These tests however do not determine an active infection, nor do they give any quantitative information. These rapid tests are not available currently in human medicine.*

Hypothesis

The detection of these pathogens in their vector tick can help show in what geographical locations these pathogens are in circulation. Furthermore, the ticks that vector *E. chaffeensis* and *E. canis*, in particular *A. americanum*, are known to transmit other tick-borne pathogens, such as *A. phagocytophilum*, various *Ehrlichia spp.*, and *B. lonstari* as mentioned above [13]. However, there has been a lack of research done in regards to the presence of *Ehrlichia spp.* and other bacterial pathogens co-infecting ticks in wild populations in Texas. Nevertheless, previous studies have evaluated *A. phagocytophilum* and *Borrelia burgdorferi* co-infecting in ticks, as well as the presence of antibodies in dogs of a particular area in Minnesota [6]. To complement this previous study, our efforts will allow for an ecological study of collected vectors to show the spatial distribution of *E. canis* and *E. chaffeensis* in the state of Texas and the potential co-infection of ticks with the spirocheatal pathogen *B. burgdorferi*. Our hypothesis is that with the data from this, and future studies, disease ecology and risk maps can be generated allowing the implementation of preventive measures, improved diagnosis and quicker treatment in endemic and non-endemic regions.

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* https://www.idexx.com/small-animal-health/products-and-services/snap-4dx-plus-test.html

CHAPTER II

METHODS

Tick collection and identification

Table 2: Sampling effort

Year	Tick species	n			
		Nymphs	Adults		Total
			♂	♀	
2011	*Amblyomma americanum*	0	7	4	11
	A. cajennense	1	12	3	16
	A. maculatum	0	0	1	1
	Ixodes scapularis	0	6	17	23
	Dermacentor variablils	0	0	3	3
	Rhipicephalus sanguineus	0	1	2	3
2012	*A. americanum*	6	47	71	124
	A. cajennense	5	10	9	24
	A. maculatum	1	1	5	7
	I. scapularis	4	27	58	89
	D. variablils	0	6	18	24
	R. sanguineus	13	122	78	213
2013	*A. americanum*	0	1	2	3
	A. cajennense	0	12	11	23
	A. maculatum	0	9	1	10
	I. scapularis	1	2	8	11
	D. variablils	0	6	8	14
	R. sanguineus	12	38	28	78
2014	*A. americanum*	0	1	0	1
	A. cajennense	1	1	0	2
	A. maculatum	0	0	0	0
	I. scapularis	0	0	9	9
	D. variablils	2	9	1	12
	R. sanguineus	2	33	34	69
	R. pusillus	5	1	4	10
Total		53	352	375	780

*Nymphs are presented as pools with no more than 5 specimens in each pool
** *R. pusillus* are part of the *R. sanguineus* complex, the samples represented in this study were collected from Spain [18]

The ticks used for this study were part of a collection acquired by the Lyme Lab at Texas A&M University. There is an existing library of passively collected ticks since 2011. This collection consists of samples that were submitted by the general public to the laboratory with information about where and when the tick(s) was collected as well as if it was taken from a host. These ticks were identified by morphology using a dichotomous key [16, 17], modified for use for the

Veterinary curriculum at Texas A&M University. The identified ticks were placed in a 70% ethanol solution and submitted for DNA extraction. When tick morphology was not enough to determine the species, 12S rRNA PCR (Table 3) and sequencing of the PCR product was used to determine the species (See below). A small part of the collection came from Valencia, Spain. They were identified as *R. pusillus* via PCR. This is considered a subspecies of the *Rhipicephalus sanguineus sensu lato* tick species [18].

DNA extraction

DNA was then extracted from whole ticks using the High Pure PCR Template Preparation Kit (Roche, Indianapolis, IN) following manufacturers recommendations with modifications to adapt the protocol to ticks. Ticks were placed in screw cap microcentrifuge tubes containing 1.4mm ceramic beads or 2.8mm ceramic beads for engorged ticks. Two hundred microliters of Phosphate-Buffered Saline, 200µl of Tissue Lysis Buffer, and 40µl of Proteinase K were added to the tube; the tubes were then placed in a bead mill BeadRuptor 24 (Omni International, Inc., Kennesaw GA) and homogenized for 5 minutes at a 5.65 m/s intensity (equivalent to 210 x *g*). The supernatant was separated into a clean microcentrifuge tube, and 200µl of Binding Buffer was added to each sample. This mixture was incubated for 10 minutes at 70ºC. After incubation, 100µl of isopropanol was mixed into the solution. This solution was then transferred to a High Pure Filter Tube and Collection Tube assembly. This assembly was then centrifuged for 1 minute at 8,000 x *g*. After centrifugation the Collection Tube and flow-through were discarded, and a new Collection Tube was assembled with the same Pure Filter Tube. Five hundred microliters of Wash Buffer was added to the reservoir of the Pure Filter Tube, and the assembly was centrifuged for 1 minute at 8,000 x *g*. The washing procedure was then repeated. After the

second washing centrifugation, the assembly was centrifuged at full speed for 10 seconds to remove any residual ethanol, and the Collection Tube is then discarded. The High Pure Filter Tube is then assembled with a clean microcentrifuge tube, and 100µl of pre-warmed Elution Buffer (70ºC) was added to the High Pure Filter Tube. This assembly was centrifuged for 1 minute at 8,000 x *g*. Following elution; the extracted DNA was analyzed using a Nanodrop (Thermo Scientific, Inc. Willinton, DE) for quantity and quality. The extracted DNA was then stored at -20ºC until ready for further analysis.

Molecular detection of *Ehrlichia spp.*

In an effort to conserve time, as well as reducing the risk from working with biosafety level 2 agents; the positive controls for this study were synthetically generated. The specific genomes for *E. chaffeensis* and *E. canis* are available at GenBank through National Center for Biotechnology Information (NCBI). For this study regions specific to the 16S rRNA gene in both species were utilized. The region of this gene containing the fragments to be amplified was synthetized (Integrated DNA Technologies, Inc) and cloned in a vector that was later transformed into *Escherichia coli* culture. After transformation, plasmidic DNA was extracted from this culture, and used as the positive control for the PCR tests. Plasmidic DNA was extracted by using the PureYield™ Plasmid Midiprep System (Promega Corporation, Madison WI) following manufacturer's recommendations. Briefly, 250ml overnight cultures of *E. coli* TOP 10 cells containing the positive control vectors were harvested by centrifugation at 5,000 x *g* for 10 minutes. Cells were lysed and placed in a column assembly to extract and purify DNA. DNA was eluted in 600µl of Nuclease Free Water.

The PCR protocol for the detection of the 16S RNA gene of *Ehrlichia spp*. was previously determined by Wen *et al* [19]. This was standardized as a nested PCR reaction. It entails the use of a primary PCR common to both *E. canis* and *E. chaffeensis*, followed by a nested PCR to detect the specific species of *Ehrlichia*. The primary PCR primers are ECC and ECB, while the second, nested PCR for *E. canis* uses the primers HE-3 and ECA as shown in Table 3 [19]. On the other hand, the primer sequences for *E. chaffeensis* include the H

E-3 primer mentioned previously and the HE1 primer, which corresponds to a variable region of the 16S RNA gene specific to *E. chaffeensis* (Table 3) [20].

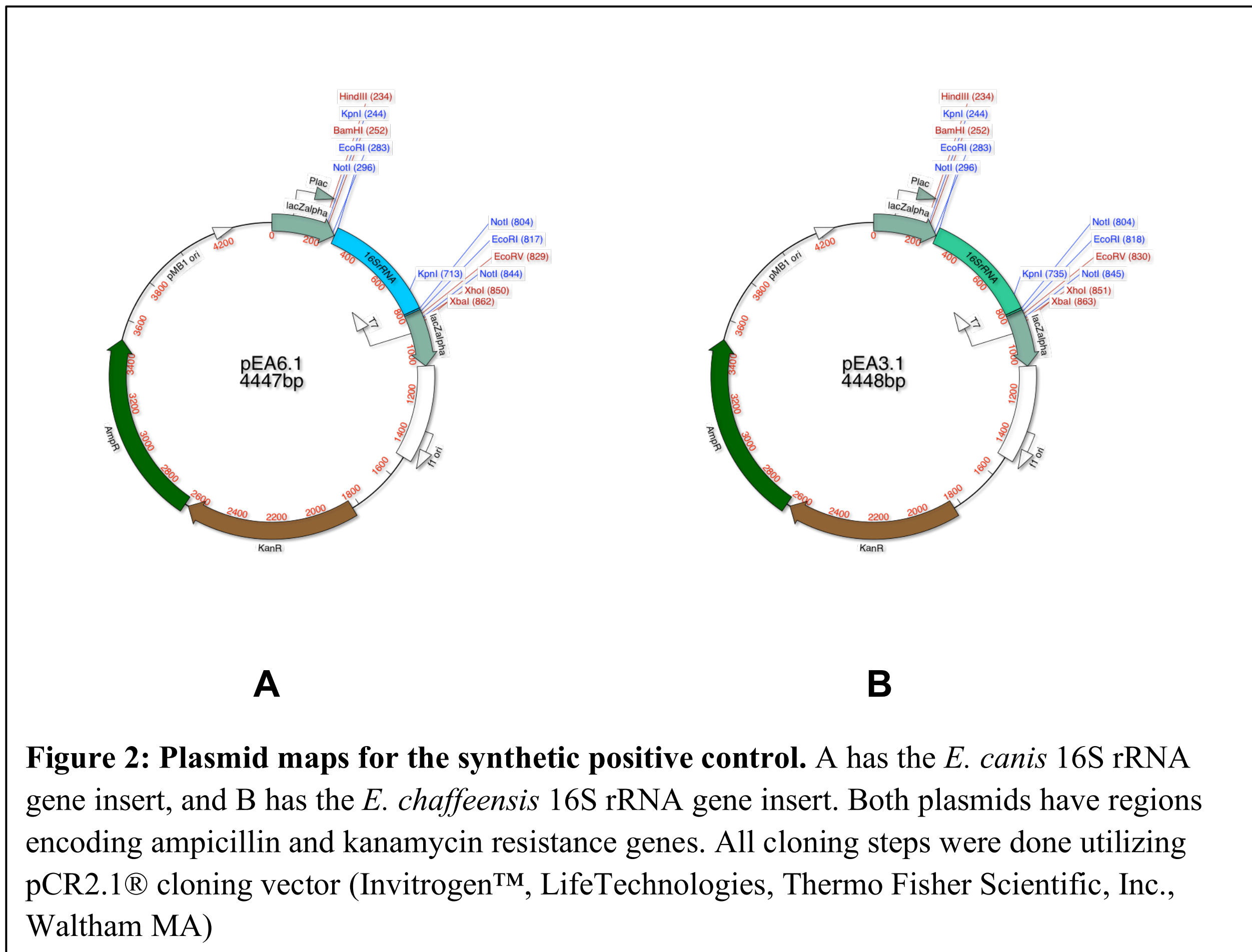

Figure 2: Plasmid maps for the synthetic positive control. A has the *E. canis* 16S rRNA gene insert, and B has the *E. chaffeensis* 16S rRNA gene insert. Both plasmids have regions encoding ampicillin and kanamycin resistance genes. All cloning steps were done utilizing pCR2.1® cloning vector (Invitrogen™, LifeTechnologies, Thermo Fisher Scientific, Inc., Waltham MA)

In the case that tick morphology analysis was not enough to determine species of a particular tick specimen, a tick PCR reaction was used. The primers Tick-F and Tick-R were used, corresponding with the 12S rRNA gene of the tick vector (Table 3) [21]. The PCR product was then subsequently send for sequencing (See below) to determine the tick species. Integrated DNA Technologies, California, produced all primers used in this study.

All PCR reactions were done in accordance with the laboratory's established PCR methods to avoid cross contamination of samples and reaction mixes. AccuStart™ PCR Supermix (Quanta BioSciences Inc., Gaithersburg, Maryland), containing: 3mM $MgCl_2$, 0.4mM each dNTP (dATP, dCTP, dGTP, dTTP), AccuStart Taq DNA Polymerase, and stabilizers, was used. The PCR mastermix was prepared in an Optimizer PCR Workstation. The primers were diluted using 10µL of the forward primer, 10 µL of the reverse primer, and 30 µL of water to a final concentration of 10pM. The reaction was then prepared with 12.5 µL of the PCR supermix, 2 µL of the diluted primer mix, and 8.5 µL of water. After the master mix was ready, the reaction tubes were taken out of the Optimizer PCR Workstation, and 2 µL of the sample DNA was added. The PCR was then ran with Eppendorf AG Master cycler® Pro machines following the specific protocols in Table 3. This process was then repeated for the nested PCR reaction to determine the individual *Ehrlichia* species.

Table 3: Primers

Organism and Gene	Forward Primer	Reverse Primer	PCR protocol
Ehrlichia spp. 16S rRNA	ECC: 5'-AGAACGAACGCTGGCGGCAAGCC-3'	ECB: 5'-CGTATTACCGCGGCTGCTGGC-3'	1-94ºC for 5 minutes 2-94ºC for 1 minute 3-60ºC for 1 minute 4-40 times to two 5-Final extension at 72ºC for 1 minute 6-4ºC o/n
Ehrlichia canis nested 16S rRNA	ECA: 5'-CAATTATTTATAGCCTCTGGCTATAGGAA-3'	HE-3: 5'-TATAGGTACCGTCATTATCTTCCCTAT-3'	1-94ºC for 5 minutes 2- 94ºC for one minute 3-60ºC for one minute 4-40 times to two 5-Final extension at 72ºC for 1 minute 6-4ºC o/n
Ehrlichia chaffeensis nested 16S rRNA	HE1: 5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3'	HE-3: 5'-TATAGGTACCGTCATTATCTTCCCTAT-3'	1-Three cycles of 94ºC for 1 minute 2-3 times one 3-55ºC for 2 minutes 4-70ºC for 1 minute 30 seconds 5-88ºC for 1 minute 6-55ºC for 2 minutes 7-37 times 5 8-Final extension 70º for 1 minute 30 seconds 9-4ºC o/n
Tick 12S rRNA	Tick-F: 5'-GAGGAATTTGCTCTGTAATGG-3'	Tick-R: 5'-AAGAGTGACGGGCGATATGT-3'	1- 95ºC for 5 min 2- 95ºC for 30 sec 3- 40ºC for 30 sec 4- 72ºC for 30 sec 5- 40 times to two 6- Final extension at 72ºC for 5 minutes 7- 4ºC o/n

*o/n corresponding to over night

After completion of PCR reactions, 5 µL of the product was mixed with OrangeG loading buffer and placed into wells of a 1% agarose gel containing 0.4µg/ml of ethidium bromide (Bio-Rad Laboratories Inc., Hercules, CA). The gel was placed in an electrophoresis cuvette filled with Tris-acetate buffer as the running buffer. The gel was run at 90 volts for 40-60 minutes. After electrophoresis the gels were imaged using ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA). Any positive bands were then cut directly from the gel and purified using the Wizard® SV Gel and PCR Clean-up System (Promega Corporation, Madison WI) per the manufacturers recommendations. The gel slice was placed in a 1.5ml microcentrifuge tube and dissolved. The DNA was then washed and eluted in Nuclease-Free Water. The purified product was then sent to Eton Biosciences Ltd. (San Diego, CA) for sequencing to confirm the PCR result.

Sequencing analysis

Sequences were cleaned individually before assembly using MacVector Version 13.0.7 (MacVector Inc., North Carolina) as follows. First, the 5'- and 3'- ends were removed from each sequence to avoid utilization of unclean and noisy sections obtained during sequencing. After cleaning the ends, each peak in the chromatograms was checked for accuracy of the corresponding nucleotide to make sure listed nucleotides were correct. Once all sequencing results were cleaned, the forward and reverse sequences were assembled using MacVector Assembler 13.0.7 (MacVector Inc., North Carolina). The consensus sequence produced was then used for further alignment analyses.

CHAPTER III

RESULTS

In this study only specimens from which we had information about the tick species, developmental stage, sex, date collected, location, host, and tests results were used. Therefore, all specimens from which we had partial information were not incorporated in the final analysis. Consequently 23 specimens were removed form the analysis. This group included 2 *I. scapularis*, 2 *A. americanum*, and 19 *R. sanguineus* (6 testing positive by PCR for *E. canis*). A total of 780 were studied in the following section (Table 4).

Molecular detection of *Ehrlichia canis*

Competent vectors for the causative agent of canine monocytic ehrlichiosis (*E. canis)* were tested. These included all 363 specimens of *R. sanguineus* ticks*,* 53 specimens of *D. variabilis* ticks, and 10 specimens of *R. pusillus* ticks (Table 4). There was a 5.87% positive rate in the library as a whole. The individual infection by species and by year is given in Table 5. Notice that *R. sanguineus* ticks were mostly infected (100%) with *E. canis* during year 2011, whereas *D. variabilis* and *R. pusillus* ticks were negative throughout the sampled years (Table 5).

Of the positive species, *R. sanguineus*, the nymphal stage showed a higher positive rate, 11.11%, than the adult stage, 7.44%.

Molecular detection of *Ehrlichia chaffeensis*

Competent vectors for the causative agent of human monocytic ehrlichiosis (*E. chaffeensis)* were tested. These included all 139 specimens of *A. americanum* ticks*,* 65 specimens of *A. cajennense* ticks, 18 specimens of *A maculatum* ticks, and 132 specimens of *I. scapularis* ticks (Table 4). There was an 18.93% positive rate in the library as a whole. The individual infection by species and by year is given in Table 5. Notice that *A. armericanum* ticks were mostly infected (24.19%) with *E. chaffeensis* during the year 2012, while *A. cajennense* and specimens *I. scapularis* (31.25% and 30.43%) were mostly positive during the year 2011. On the other hand, *A. maculatum* was mostly infected (30.0%) during the year 2013 (Table 5).

Both the *A. maculatum* and *I. scapularis* specimens were only positive in the adult stage. *A. cajennense* specimens had a high positive rate as adults, 20.69% rather than as nymphs, 14.29%. However, *A. americanum* specimens had a higher positive rate as nymphs, 50.0%, to a 9.02% rate as adults.

Table 4. Total specimens tested

Species	Nymph	Adult ♂	Adult ♀	Total	Competency
Amblyomma americanum	6	56	77	139	*E. chaffeensis*
A. cajennense	7	35	23	65	*E. chaffeensis*
A. maculatum	1	10	7	18	*E. chaffeensis*
Ixodes scapularis	5	35	92	132	*E. chaffeensis*
Dermacentor variablils	2	21	30	53	*E. canis*
Rhipicephalus sanguineus	27	194	142	363	*E. canis*
R. pusillus	5	1	4	10	*E. canis*
Total	53	352	375	780	

Table 5. Positive specimens by year and species.

Year	Tick species	*E. canis*			Total	*E. chaffeensis*			Total
		Nymphs	Adults			Nymphs	Adults		
			♂	♀			♂	♀	
2011	*Amblyomma americanum*	NT	NT	NT	N/A	0	1	0	1
	A. cajennense	NT	NT	NT	N/A	1	4	0	5
	A. maculatum	NT	NT	NT	N/A	0	0	0	0
	Ixodes scapularis	NT	NT	NT	N/A	0	2	6	8
	Dermacentor variablils	0	0	0	0	NT	NT	NT	N/A
	Rhipicephalus sanguineus	3	1	2	6	NT	NT	NT	N/A
2012	*A. americanum*	NT	NT	NT	N/A	3	11	16	30
	A. cajennense	NT	NT	NT	N/A	0	3	2	5
	A. maculatum	NT	NT	NT	N/A	0	0	1	1
	I. scapularis	NT	NT	NT	N/A	0	4	5	9
	D. variablils	0	0	0	0	NT	NT	NT	N/A
	R. sanguineus	0	17	5	22	NT	NT	NT	N/A
2013	*A. americanum*	NT	NT	NT	N/A	0	0	0	0
	A. cajennense	NT	NT	NT	N/A	0	0	3	3
	A. maculatum	NT	NT	NT	N/A	0	3	0	3
	I. scapularis	NT	NT	NT	N/A	0	0	2	2
	D. variablils	0	0	0	0	NT	NT	NT	N/A
	R. sanguineus	0	0	0	0	NT	NT	NT	N/A
2014	*A. americanum*	NT	NT	NT	N/A	0	0	0	0
	A. cajennense	NT	NT	NT	N/A	0	0	0	0
	A. maculatum	NT	NT	NT	N/A	0	0	0	0
	I. scapularis	NT	NT	NT	N/A	0	0	1	1
	D. variablils	0	0	0	0	NT	NT	NT	N/A
	R. sanguineus	0	0	0	0	NT	NT	NT	N/A
	R. pusillus	0	0	0	0	NT	NT	NT	N/A

* Vectors not considered competent for a particular pathogen were not tested for it (NT)

** N/A corresponds to "Not Applicable"

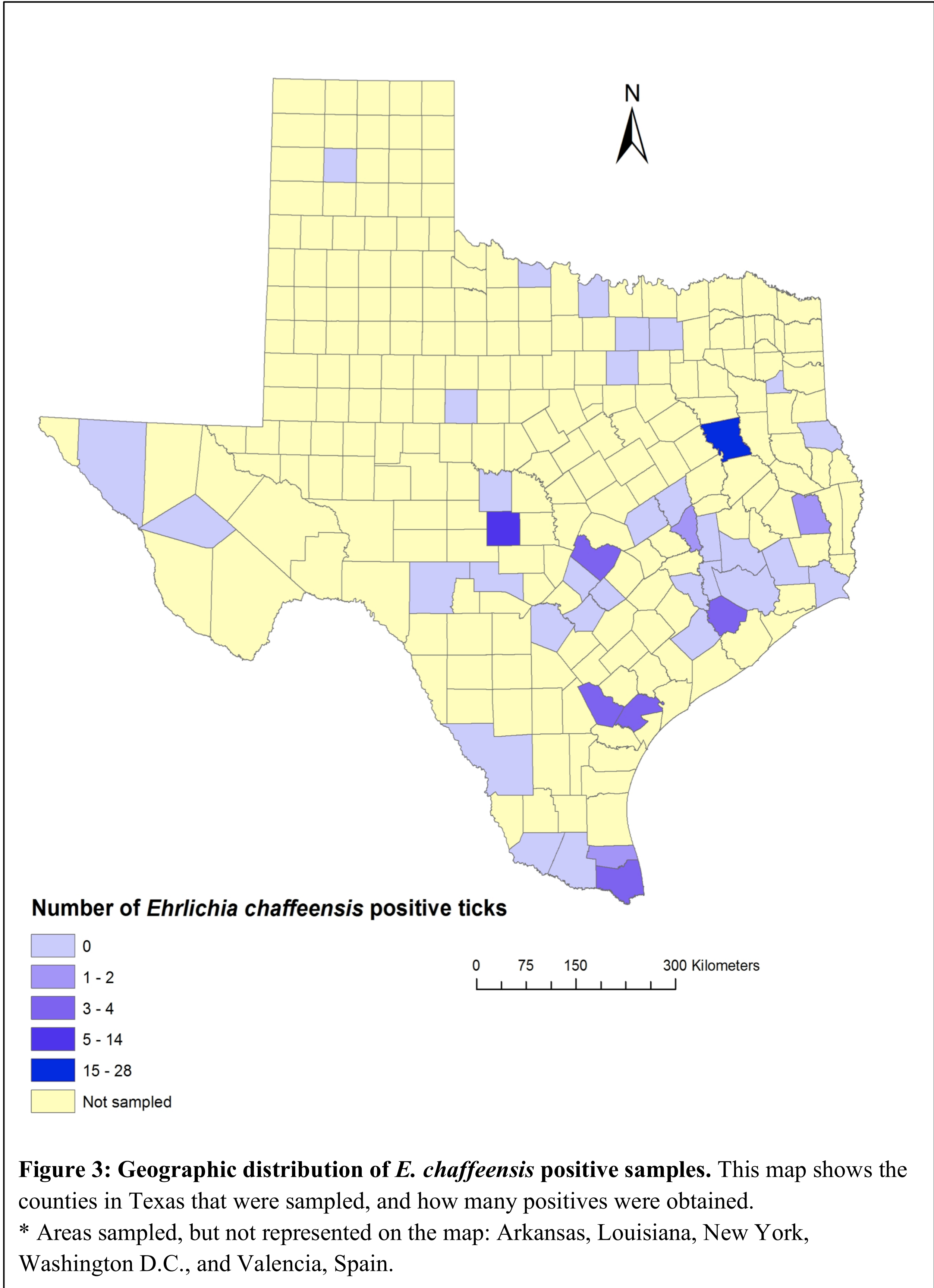

Figure 3: Geographic distribution of *E. chaffeensis* positive samples. This map shows the counties in Texas that were sampled, and how many positives were obtained.
* Areas sampled, but not represented on the map: Arkansas, Louisiana, New York, Washington D.C., and Valencia, Spain.

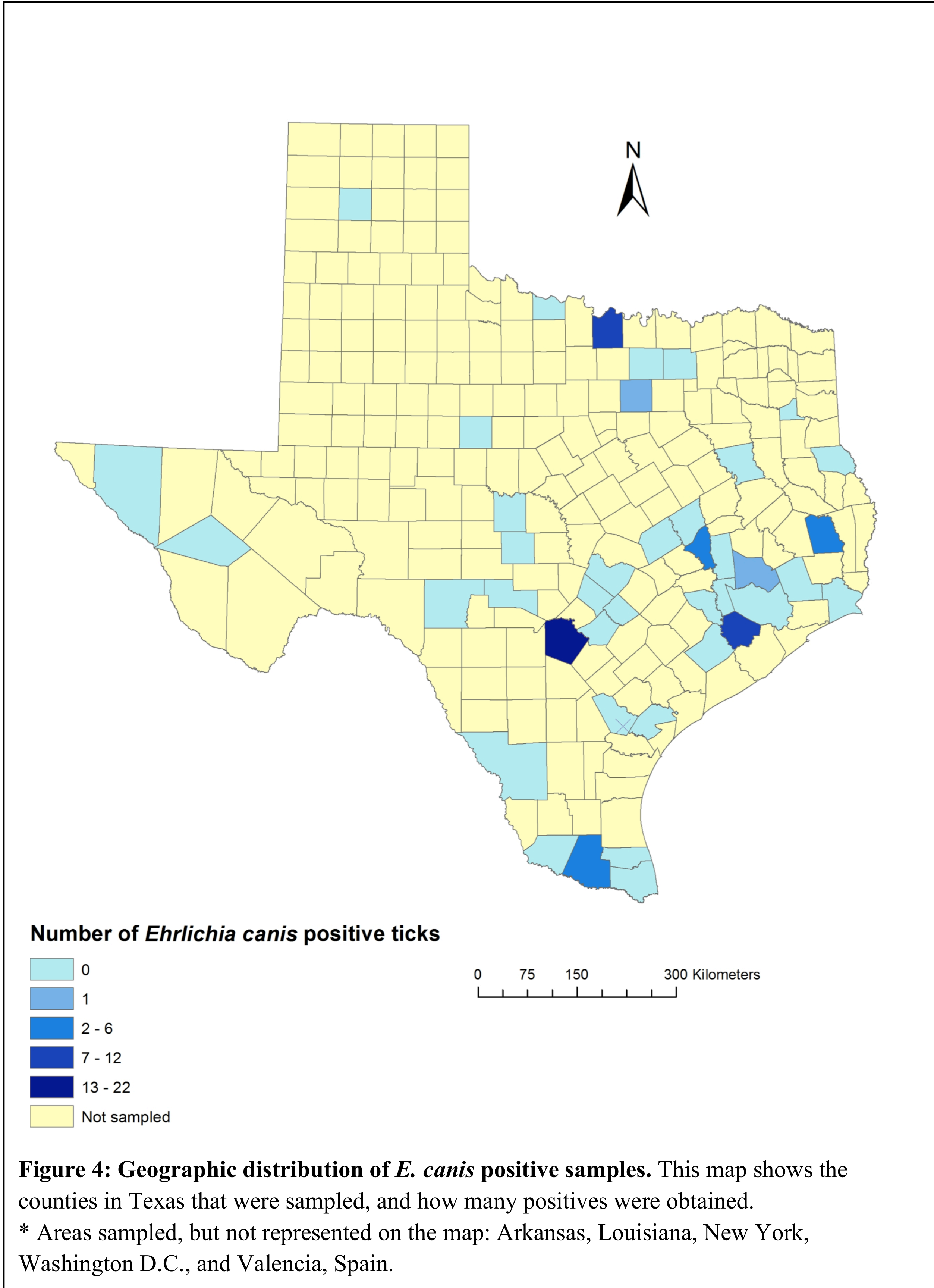

Figure 4: Geographic distribution of *E. canis* positive samples. This map shows the counties in Texas that were sampled, and how many positives were obtained.
* Areas sampled, but not represented on the map: Arkansas, Louisiana, New York, Washington D.C., and Valencia, Spain.

Co-infection of ticks with *Ehrlichia spp.* and *Borrelia burgdorferi*

This library was previously tested for the causative agent of Lyme disease, *Borrelia burgdorferi*. As both *B. burgdorferi* and *E. chaffeensis* are known to be zoonotic agents, the vectors tested for *E. chaffeensis* were analyzed for co-infection with *B. burgdorferi* as well (Table 6). After 2012, no ticks showed evidence of co-infection. During 2011, *A. cajennense* had the highest rate of co-infection (31.25%). Where as in 2012, *A. maculatum* had the highest rate of co-infection (85.71%).

Table 6. Co-infection of tested specimens with *B. burgdorferi*

Year	Tick species	*E. chaffeensis/B. burgdorferi*			Total
		Nymphs	Adults		
			♂	♀	
2011	*Amblyomma americanum*	0	0	0	0
	A. cajennense	1	4	0	5
	A. maculatum	0	0	0	0
	Ixodes scapularis	0	1	1	2
2012	*A. americanum*	0	0	1	1
	A. cajennense	0	3	2	5
	A. maculatum	0	0	1	1
	I. scapularis	0	1	5	6
2013	*A. americanum*	0	0	0	0
	A. cajennense	0	0	0	0
	A. maculatum	0	0	0	0
	I. scapularis	0	0	0	0
2014	*A. americanum*	0	0	0	0
	A. cajennense	0	0	0	0
	A. maculatum	0	0	0	0
	I. scapularis	0	0	0	0

CHAPTER IV

CONCLUSIONS

There is limited information regarding the geographic distribution of pathogenic *Ehrlichia* species in the State of Texas and other southern regions in the United States. As such there is much work to be done to understand the ehrlichiosis cycle in Texas. This study was completed using passively collected ticks to obtain a general idea about the pathogenic landscape in Texas as it pertains to *E. chaffeensis* and *E. canis*. To this end the collection of ticks provided by citizens since the year 2011 was evaluated by molecular techniques. A total of 780 specimens were tested in this study showing a 5.87% infection with *E. canis* and 18.93% infection with *E. chaffeensis*. *Rhipicephalus sanguineus* was the tick species mostly infected with *E. canis*; this is in agreement with the fact that this tick species is the competent vector for *E. canis* transmission. On the other hand, *A. americanum* and *I. scapularis* were the two tick species with the highest infection rate with *E. chaffeensis*. This observation was also in accordance with the fact that both species have been described as a competent vector for this pathogen. Moreover the distribution of infected ticks is mostly in Central, East, and South Texas (Figure 3 and 4). The counties with most of the *E. chaffeensis* infected ticks were Anderson, Mason, Travis, Bee, Cameron, Refugio and Ford Bend. On the other hand *E. canis* infected ticks were found mostly in Bexar, Fort Bend, Montague and Brazos.

Currently, all samples that were positive by PCR are undergoing sequencing analysis to confirm their positive result for either *E. chaffeensis* or *E. canis*. Although not yet complete, some of the samples positive by PCR for *E. chaffeensis* have been confirmed by sequencing. In addition, a

subset of samples showed after sequencing analysis, equal percent identity to *E. chaffeensis* and *E. ewingii*. Further molecular analysis will need to be done to confirm this result. *E. ewingii* is also a zoonotic agent, and often can be indistinguishable from other *Ehrlichia spp.* both serologically and in disease manifestation [22]. These results suggest two possibilities. One being that the amplified region of the 16S rRNA gene is not specific enough to distinguish between the various species of *Ehrlichia*. The second possibility is that the host from which these tick specimens were feeding was infected with both species of *Ehrlichia*. Therefore, a more specific test will need to be considered to confirm the species infecting the tick specimens. For instance, other groups have used the outer membrane protein A (ompA) gene to differentiate between Rickettsiales [22-24]. Even though the separation of these species is important for molecular epidemiology purposes; it is beyond the scope of this project.

The results show a majority of tick specimens, including positive samples, coming from the years 2011 and 2012. This time corresponds with a significant drought in the state of Texas, as well as other regions across the United States[*]. This leads to speculate that the drought had an impact on tick populations as well as the subset of the population that was infected with *Ehrlichia spp.* The low rainfall would have had a negative impact on the over all tick populations; however, the results show that the drought also had a concentration effect on those ticks that were questing and feeding on or near humans and domestic animals. The data also shows a concentration effect on the ticks infected by *Ehrlichia spp.* and co-infected with *E. chaffeensis* and *B. burgdorferi*. The advantageous effect of some microorganisms on the physiology of their host has been well established (e.g. *Wolbachia spp.*). Herrmann *et al.* has

[*] http://droughtmonitor.unl.edu/MapsAndData/DataTables.aspx

shown that *Ixodes ricinus* nymphs that are infected with the agent of Lyme disease, *B. burgdorferi sensu lato* have a higher fat content than those without the pathogen [25]. Therefore, it is reasonable to speculate that *Ehrlichia spp.* could also have an advantageous effect on their tick hosts.

Texas has been shown to support a vast diversity of tick species [26]; each of these ticks has the capability of transmitting multiple tick-borne pathogens. As has been shown through this study, the possibility exists for a tick to be infected with more than one agent of disease (Table 5), in particular *E. chaffeensis* and *B. burgdorferi*. In previously studies, Breitschwerdt showed in dogs that multiple species of *Ehrlichia* could infect one individual [22]. Therefore, in the case of *R. sanguineus*, a domestic tick species that feeds on dogs, these types of situations suggest that dogs could be reservoirs for multiple *Ehrlichia* species that could potentially affect humans. This is of particular relevance in areas economically depressed in the state of Texas, such as the southern most counties near the border with Mexico. In these areas, 17.6% of the population is in poverty, and in some instances families live in closer proximity to domestic animals, especially dogs. Under these conditions, the spreading of these zoonotic ticks borne diseases exacerbates. For instance, although previously not considered a threat to humans, *R. sanguineus* has been shown to transmit the agent of Rocky Mountain spotted fever, *Rickettsia rickettsii*, in Mexicali, Mexico [23], in addition to the transmission of *E. canis* [8, 15]. Many urban and metropolitan areas of Texas have poor areas within them. Without access to proper tick preventive methods for the household, the introduction of a pathogen that could be vectored to humans by *R. sanguineus* could severely impact these populations.

Control and prevention of vector-borne infectious diseases can be jeopardized in transboundary regions of the world, due to the constant movement of people and livestock [27-30], Texas is the state that harbors the longest U.S. border line with northern states of Mexico. Not only are the various regulatory laws different in the United States and Mexico, but also there is free ranging wildlife that transports vectors across boundary lines without any form of regulation. These regions not only have an importance in the maintenance of the various disease cycles, but they also represent a region historically poorer compared to other areas of both countries. The socio-economic status can greatly affect the zoonotic potential in the area [10]. In our study, this is particularly relevant since both *E. chaffeensis* and *E. canis* infected ticks were found in counties along the border with Mexico.

Passive surveillance efforts such as the one utilized in this study could be of great value in vector borne disease research. For instance, they allow researchers to initiate studies in areas where there is a lack of information regarding the presence of a particular pathogen and frequency of infected vectors, as well as reservoir host densities. Moreover, the ticks collected by "citizen scientists" are of epidemiological importance since those are the ticks that are actively questing and feeding on humans and their pets in a particular geographic region. Thus, these efforts provide with valuable information regarding both tick and human activity, such as geographic locations and times of the year where ticks are found. All in all this information can be used latter on in the design of more comprehensive research studies, and subsequent surveillance programs.

In summary, this study has provided basic information in regards to tick species infected with the zoonotic pathogens *E. canis* and *E. chaffeensis* and their geographic distribution in the state of Texas. None of this information was available in the literature. Therefore, with such data we are equipped to design a more a structure ecological study in Texas to provide a more comprehensive look at *Ehrlichia spp.,* their vectors and reservoir host so as to determine their interaction and impact on disease transmission, and disease risk for humans and companion animals.

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