Borrelia burgdorferi SEROPREVALENCE IN DEER ACROSS TRAVIS COUNTY, TEXAS: RELEVANCE FOR LYME DISEASE ECOLOGY

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ABSTRACT

Borrelia burgdorferi Seroprevalence in Deer across Travis County, Texas: Relevance for Lyme Disease Ecology (May 2014).

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The goal of this study is to evaluate the role of deer in the transmission of Lyme disease (LD) in Travis County, Texas. This disease is transmitted by the bite of an infected Ixodes tick. LD was first identified in Texas in 1984. Many diagnosed cases are not reflected in official statistics due to restrictive reporting criteria. In 2009 the case definition of LD was revised and currently the CDC differentiates in between probable and confirmed cases for this disease. In most recent years, Texas is the only state in the US with a recurrent ratio 2:1 of probable versus confirmed cases. LD is largely unrecognized in Texas so it is often misdiagnosed by physicians who are not familiar with its clinical presentation. This can be attributed to many different causes. In order to better understand the ecology of this disease in southern US, the main goal of this study is to evaluate the *Borrelia burgdorferi* (causative agent) seroprevalence in the deer population in Travis County, which is the area with the most reported cases of LD in Texas.

DEDICATION

This thesis is dedicated to my loving parents, Garry and Annette Castellanos, whose continued support and encouragement has paved the path for success. Without their guidance I would not be where I am today.

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I wish to thank Dr. Maria Esteve-Gassent for her countless hours of reflecting, reading, encouraging, and most of all patience throughout the entire process of my undergraduate research. I would also like to thank Dr. John Morrill DVM, PhD with Orion Research and Management Services and UT Medical Brach for providing the collection of sera utilized in this study.

NOMENCLATURE

LD	Lyme disease
CDC	Center for Disease Control and Prevention
ELISA	Enzyme-Linked Immuno-Sorbed Assay
WTD	White-tailed deer

CHAPTER I

INTRODUCTION

In 2012, the CDC reported 30,831 cases of Lyme Disease (LD). This is the most prevalent arthropod-borne disease in North America [1] and it is estimated that over 300,000 people become infected every year [2]. The increase in LD cases during the last decade has prompted its classification as an emerging infectious disease. Several hard tick species in the genus *Ixodes* are recognized as common vectors of the spirochete *Borrelia burgdorferi*, the causative agent of LD. *Ixodes scapularis* and *I. pacificus* are the known competent vectors in the US while *I. persulcatus*, *I. ricinus* and *I. ovatus* are the documented vectors in Eurasia [3-7].

The transmission of LD happens in a complex system subjected to shifts in ecological processes that influence vector biology and the epidemiology of *B. burgdorferi* infection in reservoir hosts and humans [8-12]. The spirochete is maintained in the environment by different vertebrate hosts with varying degrees of competence. In the forests of eastern North America the white-footed mouse, *Peromyscus leucopus*, is its main reservoir [13, 14]. On the other hand, white-tailed deer (*Odocoileus virginianus*, WTD) are the primary reproductive host for *I. scapularis* in the US, but they are reservoir-incompetent for *B. burgdorferi* [9, 15]. A number of studies have suggested that vertebrate biodiversity affect the risk of contracting human LD [16-20].

LD is a multisystemic disease, which can be characterized by three different stages. The first stage is the **localized infection** characterized by the typical rash known as *Erythema migrans* (EM). This is the most common symptom in LD patients and is identifiable by a target-shaped

rash and accompanied by flu-like symptoms. This first stage of the diseases happens after an incubation period of 3-32 days. The rash is the only way to detect LD without a diagnostic test and forms at the site of the tick bite in 70% of all reported cases [21-23]. The second stage of LD is known as the **disseminated LD**. In this stage, *Borrelia burgdorferi* disseminates to other parts of the body within days to weeks after the disease onset. The patients in this stage can show a wide variety of symptoms such as multiple secondary EM sites and complications with the involvement of the neurological and cardiac systems [21]. The third stage is known as the **persistent infection** (or chronic LD) and occurs after several weeks of disseminated infection and may prolong for several years. The pathogen continues to spread to the joints, nervous system, and cardiac system [4]. Depending on the species of borrelia, the frequency of the dissemination to the different sites varies. For example, *Borrelia burgdorferi* in North America is mainly arthritogenic, while European strains cause neuroborreliosis more frequently. Lyme arthritis is asymmetrical, occurs in large joints (i.e. elbows, knees, and ankle), and is recurrent for several years. In approximately 60% of the untreated patients, intermittent attacks of arthritis begin to occur months after the onset of illness, especially in the knees [21, 23].

Ecology of Lyme disease: ticks and reservoir hosts

Ixodes spp. are ticks that have a three stage life cycle which include a larval, nymphal, and adult stage. The tick has one blood meal during each of these stages then drops off to molt to the next stage, which takes several months (Fig. 1).

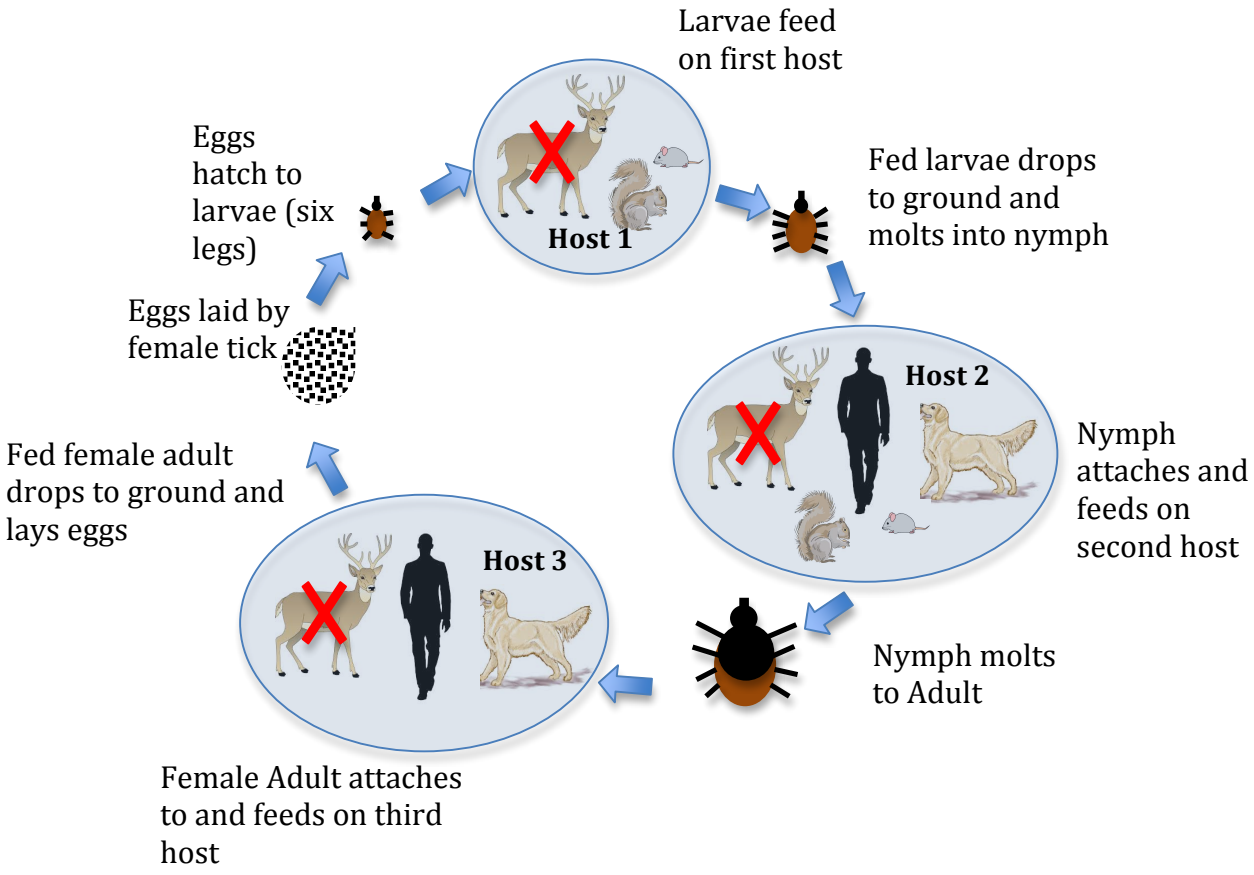

Figure 1. Tick Life Cycle. Infectious cycle of the European *Borrelia burgdorferi* sensu lato genospecies. This genospecies is the only pathogenic genospecies present in the US and Europe. Both rodents and birds are reservoirs. A red cross indicates a non-reservoir host. (Adapted from "Lyme borreliosis" Stanek, 2012)

Therefore, these ticks are known as 3-host ticks due to the fact that they tend to feed on three different hosts at each developmental stage. The life cycle of a tick can vary between 2 to 6 years depending on different environmental factors such as climate, host availability, etc [24, 25]. Larva hatch from eggs laid by the female. They are not important vectors of LD because transmission of *B. burgdorferi* does not occur transovarially. Larvae need to feed on infected intermediate hosts (small rodents) in order to acquire the spirochetal pathogen. After the larva feeds and drops to the ground, it molts into an 8-legged nymph. Therefore, the transmission of *B.*

burgdorferi occurs trans-stadially, which means that the bacteria can survive the molting process from larvae to nymph to adult. Larvae feed on small to medium mammals and birds while the nymphal stage tends to feed on bigger animals such as medium size mammals, deer, dogs, and of course humans. Consequently, the nymphal stage is mostly associated with the transmission of LD for different reasons. One of them is the fact that they could have acquired the infection during their first blood mean, they are hard to see and there could be higher numbers of them [26] questing at the same time. Nymphs feed on small mammals as well as on some larger mammals such as deer. They are active from early summer to early autumn in northeastern models. In the adult stage, the ticks mainly feed on larger mammals including deer, horses, dogs and humans, and they are most active from autumn through winter, until early spring [25]. Humans are considered accidental hosts and are a dead end for the transmission of *B. burgdorferi* while the white footed mouse is the most important reservoir for *B. burgdorferi* [26].

Deer are important for maintaining tick populations because they provide the perfect environment to feed sufficient numbers of adult ticks, and will allow the mating of male and females, necessary to generate the next generation of ticks [8]. However, they are not competent reservoirs for the disease agent [15, 25], since spirochetemia has only been described in this mammalian host anecdotally [12, 27]. A typical habitat for the transmission of LD includes wooded areas with decaying vegetation on the ground in order to maintain humidity for the survival of ticks and have a sufficient amount of vertebrate hosts. Moreover, recent studies have shown that the level of biodiversity will also affect the maintenance of the enzootic cycle as well as the risk of disease transmission to humans [8, 14, 16, 28-31].

LD is caused by the bacterial spirochetes *Borrelia burgdorferi senso lato*, which comprises a total of 18 genospecies of which a limited number are responsible of disease in humans (*B. burgdorferi*, *B. garinii*, *B. afzilii*, *B. valsiana*, *B. bissett*i and *B. spielmani*) [32-34]. Only *B. burgdorferi sensu stricto* has been shown to cause disease in humans in the US, while *B. garinii* and *B. afzelii* have been proven to cause Lyme borreliosis in Europe. LD causing borrelials belongs to the eubacterial phylum and with planar wave morphology. It is classified as a gram negative bacteria due to the presence of inner and outer membranes, even though it does not present the typical outer membrane lipopolysaccharide (LPS) of gram negative bacteria species. In addition, these Borrelia species depend on the host for most of its nutrition requirements [21]. Borrelia is transmitted in the saliva and possibly through the regurgitation of gut material during the tick bite [35]. *B. burgdorferi* has a unique and very fragmented genome with one linear chromosome and up to 21 plasmids (12 linear plasmids, and 9 circular plasmids) [36]. These spirochetes have antigenic surface lipoproteins, of which there are three main outer surface proteins (Osp): OspA, OspB, and OspC [21, 37]. These proteins are good genetic markers when testing for the presence of the bacteria and can be used in diagnosis of LD. When transmission occurs there is a phenotypic switch in these proteins. OspA is down-regulated while OspC is up-regulated [37, 38].

Diagnostics of Lyme Borreliosis

A range of laboratory techniques have been established for direct detection of *B. burgdorferi* sensu lato These assays provide evidence for the incidence of intact spirochetes or spirochete components such as DNA or protein in tick vectors, reservoir hosts, or patients. Four different approaches have been used in the clinical laboratory: microscope-based assays, detection of *B.*

burgdorferi- specific proteins or nucleic acids, and culture. Of these, culture of *B. burgdorferi* sensu lato certainly offers the best confirmation of active infection and has been increasingly used as a diagnostic modality by many researchers. The convenience of cultured organisms has also allowed study of the structural, molecular, antigenic, and pathogenic properties of the different *B. burgdorferi* sensu lato species. Nevertheles this is not widely used in diagnostics due to the fact that it is timely consuming. Consequently, for the confirmation of LD in human and veterinary medicine, serological test such as ELISA and Immunoblot assay have extensively been used [32, 39-42].

Table 1: Probable and confirmed human Lyme disease cases in Texas 2006-2011.

Year	Total	Probable (%)	Confirmed (%)	Avg. precipitation (inches)*
2006	**29**	NA	NA	30.32
2007	**87**	NA	NA	40.82
2008	**153**	48 (31.70)	105 (68.30)	26.96
2009	**276**	188 (68.12)	88 (31.88)	30.25
2010	**142**	87 (61.26)	55 (38.74)	31.95
2011	**74**	46 (62.16)	28 (37.84)	14.15
2012	**75**	42 (56%)	33 (44%)	25.31

NA: Not available.
* Average has been calculated based on yearly average given by 10 stations hosting State Climatologists in Texas. 31.602 inch is considered normal average precipitation for Texas. http://climatexas.tamu.edu/index.php/data/full-network-estimated-precipitation/86-data/749-data-from-ncdc

Lyme disease in Texas

In recent years, our research team has observed that most of the positive canine LD cases were diagnosed during the cooler months as opposed to what is typical for this disease in Northeastern US (manuscript in preparation). On the other hand, human LD cases did not follow any temporal trend being diagnosed equally in summer as they were in winter months. In addition, most of the

confirmed humans cases of LD in Texas over the last 10 years (Texas Department of State Health Services, DSHS), occurred in major metropolitan areas and primarily in Eastern Texas as was observed with the animal cases (Figure 2). Oddly, where few to no animal cases have been reported in rural West Texas and the Panhandle, human cases have been reported. The differences in reporting between rural and metropolitan areas could be explained by a lack of information and awareness about LD in more rural areas and a better understanding of the disease in major metropolitan urban areas. In addition, in 2009 the case definition of LD was revised and in the current definition for epidemiologic surveillance, the CDC differentiates in

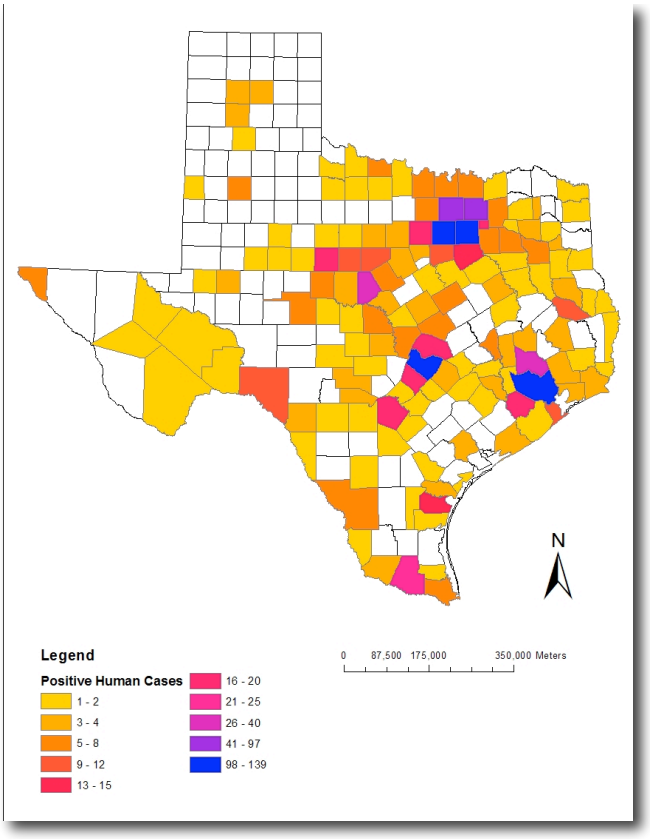

Figure 2: Distribution of human Lyme disease reported cases during 2001 to 2012. Data were acquired from the Texas State Department of Health Services (DSHS) and were mapped using GIS Arc 10 at the ZIP code level. Background colors represents the average precipitation for the state of Texas where the green represents wettest regions while red corresponds to the dryer regions of the state.

between confirmed and probable cases for this disease. Taking this into account, since 2009 Texas is the only state in the US in which the ratio of probable versus confirmed cases is repetitively 2:1 (Table 1). This scenario leads us to initiate a study of the ecological bases for the distribution of this disease in Southern US, by looking into the distribution of infected ticks, and the mammalian hosts involved in the maintenance of tick populations such as the white tail deer.

Hypothesis

While WTD is not a competent reservoir for *B. burgdorferi*, it does play a role in the maintenance of the *I. scapularis* tick population, as commented above. In addition, different studies have shown, that even though *B. burgdorferi* does not survive in WTD, this animal species does develop antibodies towards this bacterium that can be evaluated by means of an ELISA test [12, 27, 43-46]. In collaboration with Dr. John Morrill DVM, PhD with Orion Research and Management Services and UT Medical Brach, we had access to a WTD serum bank collected during population management hunts in Travis County since year 2001. Therefore we had access to 12 years worth of samples from the same region. Our hypothesis is that by evaluating the sero-prevalence to *B. burgdorferi* in a WTD population in a particular region of the State of Texas during a long period of time, will allow us to determine differences in the circulation of this pathogen in the region of study and correlate this with the fluctuations of human LD reported cases. In addition it will provide more information regarding the enzootic cycle of *B. burgdorferi* in Southern US, where it has not been studied.

CHAPTER II

METHODS

In this study, the *B. burgdorferi* seroprevalence in a serum bank of WTD samples collected from 2001-2012 in the Travis County, Texas were analyzed by means of ELISA and Immunoblot assay to determine whether there are temporal and spatial distribution patterns for this pathogen in one of the regions with higher density of human LD reported cases.

Borrelia burgdorferi strains and growing conditions

B. burgdorferi B31 A3 virulent isolate was used throughout this study. In order to obtain an antigenic profile similar to that observed in the natural infection, we grew this bacterium at room temperature (RT) and pH 7.6 to mimic the unfed tick conditions. Once the cultures reached a cell density of $1\text{-}2\times10^7$ spirochetes/ml a subculture was transferred to 37ºC, 1% CO_2, and pH 6.8 mimicking the conditions in the tick upon feeding. To run the ELISA tests using whole cell lysates, *B. burgdorferi* was grown in 500ml cultures shifted from RT/pH 7.6 to 37ºC/pH 6.8 and 1% CO_2. After cultures reached a cell density of $3\text{-}5\times10^7$ spirochetes/ml, cells were harvested, washed three times with HBSS buffer (HyClone, Thermo Scientific Inc.), quantified, and lysed using 0.1mm glass beads in 2ml screw cap tubes in a BeadRuptor 24 (Omni International, Inc). After the lysis cycle, the glass beads were sedimented by quick centrifugation and the supernatants were stored at -20ºC in 1ml aliquots until use in the ELISA assays

Evaluation of the immune-reactivity of the serum collection to *B. burgdorferi*

In order to evaluate the *B. burgdorferi* sero-prevalence in the serum bank, and Enzyme Link Immuno-Sorbed Assay (ELISA) was performed. Even though this assay is routinely used in our laboratory for the detection of serum antibodies to *B. burgdorferi* in dogs and mouse samples it was adapted to WTD serum samples in this study. To this end, 96- well MaxiSorbed ® plates (Nunc, ThermoScientidic, Ltd.) were coated with whole cell lysates of *B. burgdorferi* at a final density of 10^7 cells/well in carbonate buffer (pH9.4) the recombinant protein Bb lysate 500ng over night at 4ºC. Unbound proteins were washed with Phosphate Buffer Saline containing 0.1% Tween-20 (PBS-T) and plates were blocked with 3% Bovine Serum Aalbumin (BSA) in PBS-T over night at 4ºC. After blocking, plates were washed three times in PBS-T. Following washes, serial dilutions of the test serum ranging from 1:200 to 1:1600 were added to the plates in triplicates, and incubated for 1h at room temperature. After washing unbound antibodies, plates were incubated for 1hr with 1:3000 dilution of anti-deer-HRP labeled antibodies (Rockland Immunochemicals). Plates were washed three times followed by the addition of, *o*-phenylenediamine dihydrochloride substrate (OPD, Pierce, Thermo Scientific, Lt.). After a 20-minute incubation in the dark, plates were read at a wavelength of 450nm and analyzed using the BMG LABTECH OMEGA plate reader and software. A blank group was kept in each plate. Values were analyzed and sero-prevalence evaluated in the space and time.

Use of Immunoblot assay to confirm sero-positve samples

Each serum samples suspected of being positive by ELISA was confirmed by running the commercially available immunoblot assay *B. burgdorferi* Marblot^{TM} Strip Test System (Trinity Biotech) following the manufacturer's recommendations. Briefly, numbered strips (coated with

B. burgdorferi antigen) were removed with blunt forceps and avoiding touching their surface. For each sample or control strip (Positive, Negative and weakly reactive) a channel in a 12-strip plate was filled with 2 ml of 1X sample Diluent/wash Solution (tris buffered saline) provided in the kit. After strips were equilibrated for 5 minutes, 20 μL of each of the suspected positive samples were added to the appropriately marked channel and incubated at room temperature for 30 minutes. Strips were washed three times by adding 2 mL of sample Diluent/Wash Solution to each channel of the strip incubation tray and incubated for 5 minutes shaking. Two mL of 1:1,000 dilution of the anti-deer- AP conjugated IgG antibody (COMPANY) was added to each strip containing well and incubated for 30 minutes at room temperature. Strips were then washed three times and 2 mL of Color Developing Solution was added to each channel. All strips were incubated for 6 minutes to allow color development. Strips were then washed with 2 mL of deionized water, air-dried and evaluated. A group of negative deer samples were used in order to determine cross reactive bands.

Data Analysis

ELISA as well as immunoblot tests were analyzed with the help of a statistician (Dr. May Bogges, Arizona State University) in order to determine cut off values for the ELISA as well as number of bands visualized in the immunoblot assay so as to consider a test positive. With the help of GIS experts we will represent the positive cases in the space and time considering different climate variables such as average precipitation, relative humidity, ecological factors, etc. In addition we will plot the seroprevalence a long the years in order to evaluate whether there is a trend that can explain the humans incidence of LD in the past 10 years.

CHAPTER III

RESULTS

In this study, the *B. burgdorferi* seroprevalence in a serum bank of WTD samples collected from 2001-2010 in the Travis County, Texas were analyzed by means of ELISA. As shown in Table 2, 975 WTD serum samples were analyzed. The number of samples varied depending on the year, with a minimum of 46 samples in 2008 to a maximum of 199 in 2006. This gives us an average of 97.5 samples per year.

TABLE 2. Number of WTD serum samples tested in this study.

Year	Number of Samples
2001	88
2002	162
2003	72
2004	65
2005	68
2006	199
2007	79
2008	46
2009	82
2010	114
Total	**975**

The ELISA data will be analyzed with the help of a statistician (Dr. May Bogges, Arizona State University) in order to determine cut off values for the ELISA so as to consider a test positive. With the help of GIS experts we will represent the positive cases in the space and time considering different climate variables such as average precipitation, relative humidity, ecological factors, etc. In addition we will plot the seroprevalence a long the years in order to

evaluate whether there is a trend that can explain the humans incidence of LD in the past 10 years.

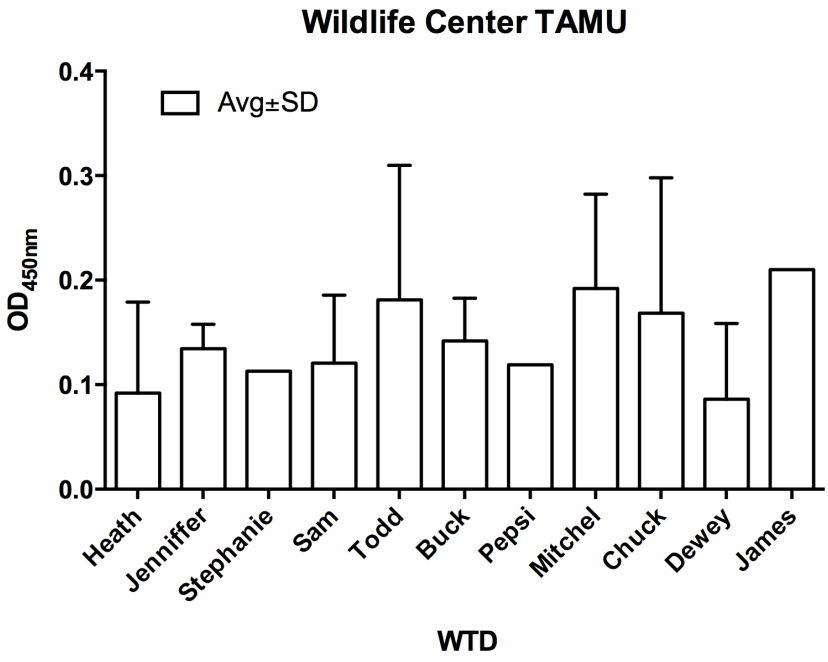

FIGURE 3. Immune reactivity of negative control WTD sera in the ELISA test utilized in our study. Each bar represents the average ± SD.

At the Texas A&M University Wildlife Center, 11 WTD have been held in a controlled environment. Serum samples from these animals collected in different years were used as negative controls, since there has been no report of tick infestation in the last 10 years. As shown in Figure 3, all animals except for three males (Todd, Mitchel, and Chuck) showed a very low reactivity with Bb whole cell lysates. The average of the OD_{450nm} readings of the negative control samples, plus three standard deviations (AVG + 3*SD = 0.370) was used as the baseline for the evaluation of WTD samples from Travis County. All OD_{450nm} values equal or lower than

0.370 are considered negative ($OD_{450nm} \leq 0.370$ = Negative) and all those higher are considered positive ($OD_{450nm} > 0.370$ = Positive).

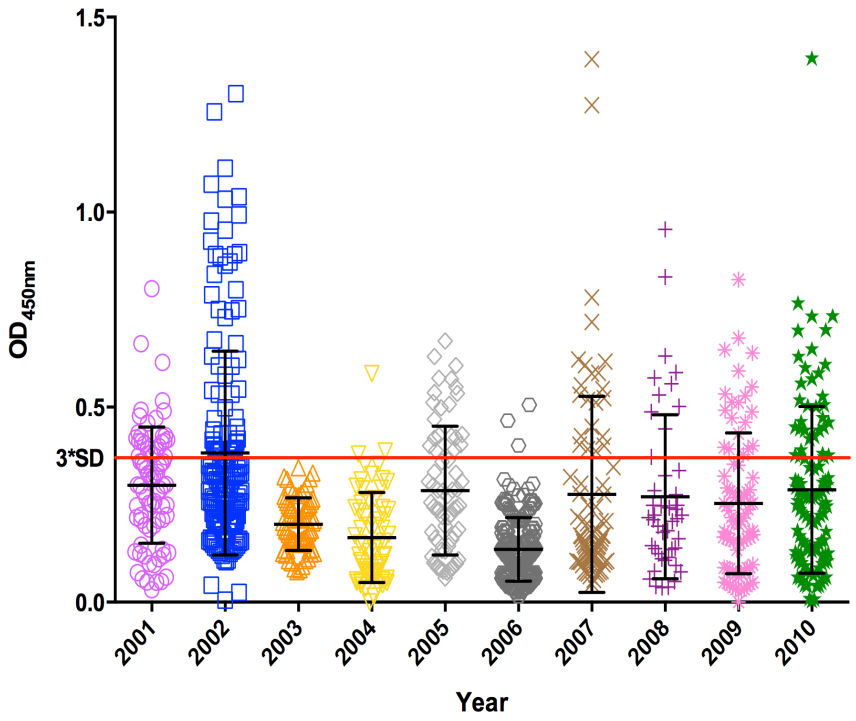

Figure 4. ELISA Analysis 2001-2010. Immune-reactivity of sera from sampled WTS per year from 2001 till 2010. All samples were analyzed utilizing the ELISA test described in material and methods. Avg ± SD are represented in black as the horizontal and vertical bars respectively.

The distributions of positive and negative serum samples are widely shown along the study period. In 2002 we detected the highest deviation of positive deer. On the other hand, from 2003 to 2005, the amount of positive deer samples lower. In 2007, we see another increase in positive samples and a gradual decrease through 2010.

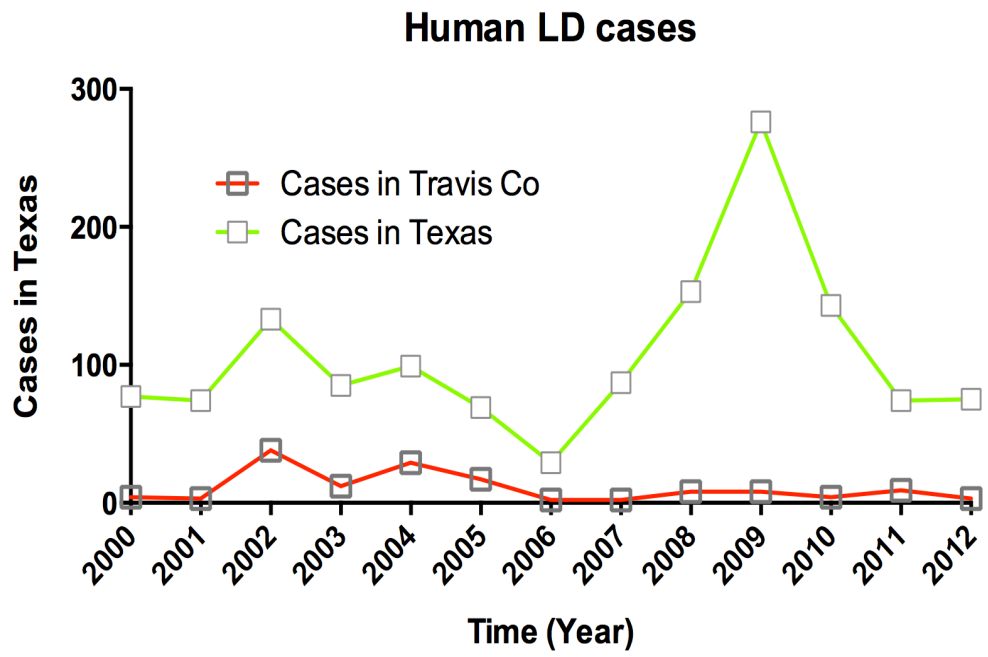

Figure 5. Human Lyme Disease Cases in Texas 2001-2012. Human LD cases reported to the CDC in the state of Texas are represented in green, those corresponding to Travis County are represented in red.

As shown in Figure 5, the lime line represents the total number of cases in the state of Texas each year from 2001-2012. The blue line represents those reported in Travis County during the same time period. The amount of reported cases from 2001 to 2002 has a slight increase with a gradual decrease through 2006. From 2006 to 2009 there is a stead and large increase in the number of reported cases. A rapid decrease happens from 2009 to 2010 and steadily continues to decrease until 2012. On the other hand other Dallas and Harris counties (Figure 6) do follow the trend observed in the state for the reporting of LD.

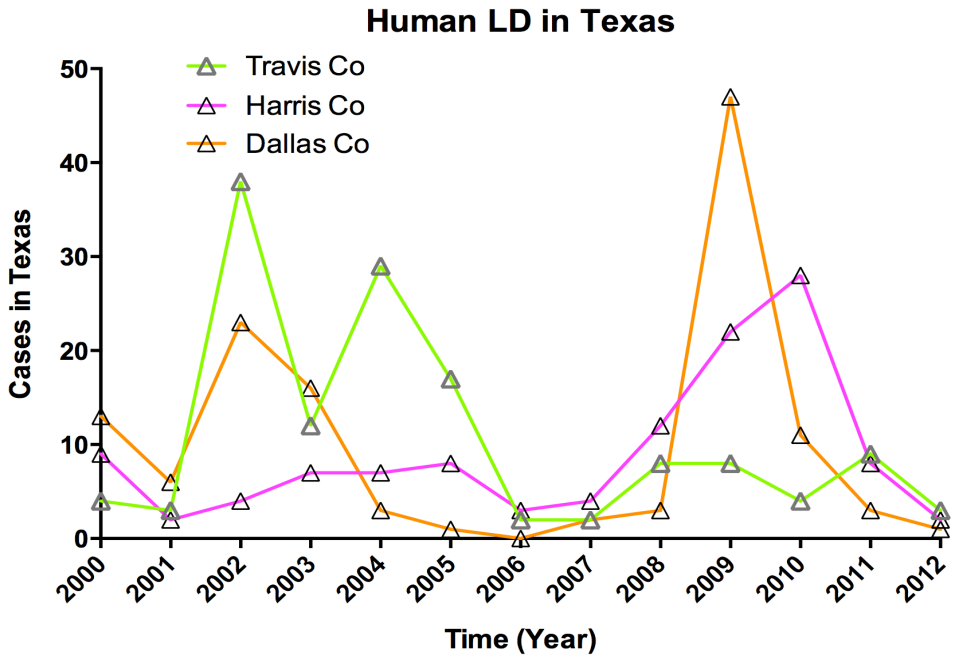

Figure 6. CDC confirmed Human Lyme Disease Cases in some counties in the state of Texas 2001-2012. Human LD cases reported to Travis County are represented in green, those reported in Dallas county in orange and those from Harris county are shown in pink.

CHAPTER IV

CONCLUSION

In this study we evaluated the presence of seropositive WTD to *B. burgdorferi* in a collection of 975 deer serum samples collected in Travis County since 2001. After determining the cut off value as the average OD_{450nm} of the control animals plus 3*SD we observed that, in the years 2002, 2005, 2007, 2008, 2009 and 2010 we have a significant number of deer seropositive samples for this spirochetal pathogen. The peak in 2002 and 2007-2009 coincide with a peak in the reported numbers of human LD cases. In addition, since we have an extended collecting of samples comprising more than 10 year of WTD samples we could observe that during the Texas drought through 2009-2012, the seropositive deer did not decrease in at least 2009 and 2010. This suggests that infected ticks might still be circulating in higher densities in this population of WTD. Our laboratory has acquired samples from the same location for years 2011 through 2013, that will be evaluated to determine whether or not the levels of seropositive deer decreases once the area of study recuperated from the drought (rainy seasons observed during spring and summer of 2013) [8, 13, 14, 29, 30, 47]. These results suggest that WTD could be sentinels for LD in the state of Texas as it has been observed in other stares in the country [8].

Interestingly there are very few studies on deer seroprevalence for *B. burgdorferi.* In addition, most of the studies have been performed in northeastern and midwestern states in the US as well as in Mexico [8, 12, 27, 43, 44, 46, 48-53]. To date, there are no studies done in Texas in regards to the role of WTD in the maintanance of the arthropod vector *I. scapularis*. Consequently, it is very difficult to compare results, or use any other study as reference, since the samples were

collected in states with a complete different ecology and during different times of the year [8, 12, 27, 43, 44, 46, 48-53]. In other parts of the country, studies similar to this one have been done, specifically New York and Pennsylvania. In this specific study a fluorescent bead-based multiplex assay was used to test sera from New York and Pennsylvania white-tailed deer for the presence of antibodies to OspA, OspC, and OspF. The significantly greater percentage of OspF seropositive deer suggests that the majority of sampled deer were chronically infected with *B. burgdorferi* [53].

Taken together, this study is the first in its nature that has been performed in Southern US. Results suggest that, as observed in other states, WTD are seropositive for *B. burgdorferi*, and that the numbers of seropositive individuals depend on climatic conditions (drought *versus* wet years). Unfortunately no correlation was observed with human cases. This could be due to the difficulty in the diagnostics of Lyme disease in non-endemic areas, where physicians and patients are less aware of this condition. Nevertheless, during the years when the samples were taken some localities where animals were captured underwent deforestation and destruction of natural habitats. Currently, other students in our laboratory are evaluating the impact of human disturbances on natural habitats on the prevalence o seropositive WTD in the collection analyzed. Therefore, with this study we have provided initial information regarding the role of WTD in the complex enzootic cycle of *B. burgdorferi*, the causative agent of Lyme disease in Southern US.

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