STANDARDIZATION OF A PAN-SPECIFIC TEST FOR THE DIAGNOSIS OF LYME DISEASE IN VETERINARY MEDICINE

A Senior Scholars Thesis

by

ERIN MCGREGOR

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Biomedical Sciences

STANDARDIZATION OF A PAN-SPECIFIC TEST FOR THE DIAGNOSIS OF LYME DISEASE IN VETERINARY MEDICINE

A Senior Scholars Thesis

by

ERIN MCGREGOR

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by:

Research Advisor:	Maria Esteve-Gassent
Associate Director for Honors and Undergraduate Research:	Duncan MacKenzie

May 2012

Major: Biomedical Sciences

ABSTRACT

Standardization of a Pan-Specific Test for the Diagnosis of Lyme Disease in Veterinary Medicine. (May 2012)

Erin Mcgregor
Department of Veterinary Medicine and Biomedical Sciences
Texas A&M University

Research Advisor: Dr. Maria Esteve-Gassent
Department of Veterinary Pathobiology

Lyme disease (LD) is the most prevalent tick borne disease in the US with a total of 22,572 confirmed human cases reported to CDC in 2010. LD is caused by the infection of a mammalian host with the bacterial pathogen *Borrelia burgdorferi,* through the bite of an infected tick. Currently, there is no pan-specific test available for the diagnosis of the disease. Our hypothesis is that a non-species specific competitive ELISA test, also known as pan- specific ELISA test, will help improve the diagnosis of LD not only in Veterinary Medicine but will also help evaluating the sero-prevalence of this disease in different species of animals. Our objectives for this project are to 1) evaluate the antibody level against *B. burgdorferi* whole cell lysates and the Borrelial recombinant proteins P66 and OspC of a collection of dog serum samples by traditional ELISA and 2) determine the immune-reactivity of Texan dogs serum samples to Borrelia by Immunoblot assay, so as to determine the cut off values for the ELISA assay, This evaluation will help establish the basic parameters for the final competitive ELISA and will determine the validity of P66 as potential antigen to be use in the final assay. We

will achieve our objectives by i) purification of OspC and P66 ii) evaluate the immune reactivity of the serum collection to P66 and OspC and Borrelia whole cell lysates by ELISA and by Immunoblot and iii) designate the starting conditions of the competitive ELISA for diagnostics of Lyme disease. It has been concluded that IFA is not an effective testing method because of the high volume of false negatives that are associated with it. By performing Western Blots and ELISAs as confirmatory tests, the number of cases being reported will increase because of the decrease in false negatives, thus allowing for surveillance of the disease to increase.

DEDICATION

This thesis is dedicated to my loving parents, who have been my rocks, and to my amazing animals that I have had the opportunity to love and be loved by; Chinook, Bear, Dusty, Lucky, Gizmo, Midnight, and Tink.

ACKNOWLEDGMENTS

I would like to take this opportunity to acknowledge the many people who made this project possible. Dr. Alfonso Clavijo and Sandy Rodgers from Texas Veterinary Medicine Diagnostic Laboratory (TVMDL) for their help collecting dog and horse samples from different counties in the state of Texas necessary to carry out this study, the AgriLife-TVMDL seed grant funding the project entitled "Improving diagnostic methods for Lyme disease, and epidemiology of human and animal infections in TX", that will provide the financial support to conduct the experiments outlined in this thesis. I would also like to thank The Texas A&M Honors Program for their support through the Undergraduate Fellowship program and giving me the opportunity to partake in such an excellent program. Special thanks to Dr. May Bogges for her valuable help and knowledge during the statistical analysis of the data generated in this thesis. I would also like to give a very special thanks to my amazing PI, Dr. Maria Esteve-Gassent "Loles".

NOMENCLATURE

LD	Lyme disease
EM	Eythema migrans
rOspC	Recombinant uter embrane protein C
Amp	Ampicillin
Kan	Kanamycin
PASN	Post absorption supernatants
ELISA	Enzyme linked immuno sorbed assay
BSA	Bovine serum albumin
PBS	Phosphate buffer saline
ROC	Receiving operating characteristic
WB	Western blot

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Lyme disease (LD) is a vector borne disease, meaning it is transmitted from an insect to a human or other animal. It is the most common vector borne disease in North America, with a total of 22,572 confirmed human cases reported to CDC in 2010. LD is caused by infection of mammalian host with the bacteria *Borrelia burgdorferi* acquired through the bite of an infected *Ixodes* tick (2, 11, 14, 22, 28). Currently there is very limited epidemiological data available to represent the number of confirmed animal cases nationwide (5) since Lyme disease is not an animal reportable disease. Therefore the epidemiological data available come from studies done nationwide in collaboration with pharmaceutical companies and using their commercially available kits (5). In 2010, out of 500 LD tests performed at TVMDL, 40% of the positive tests were from Texas while the other 60% were from out of state. There were 69 positive animal cases that were confirmed for Lyme, and occurred mostly in East Texas. When analyzing the confirmed number of human cases in Texas over the last 10 years (personal communication from Texas State Department of Health Services), most of them occurred in the metropolitan areas of East Texas. There have also been several human cases reported in Western Texas and near the panhandle, where few to no animal cases have been reported. The lack of information and awareness of LD in rural areas and a better understanding in the

---

This thesis follows the style of Infection and Immunity.

metropolitan areas could explain the reporting differences between the two areas. Consequently, by developing surveillance programs in the State of Texas, we could determine which are areas of high risk for this disease by determining the sero-prevalence in the wildlife together with the detection of *Borrelia burgdorferi* DNA in sampled ticks (4, 7, 13, 18, 19, 24).

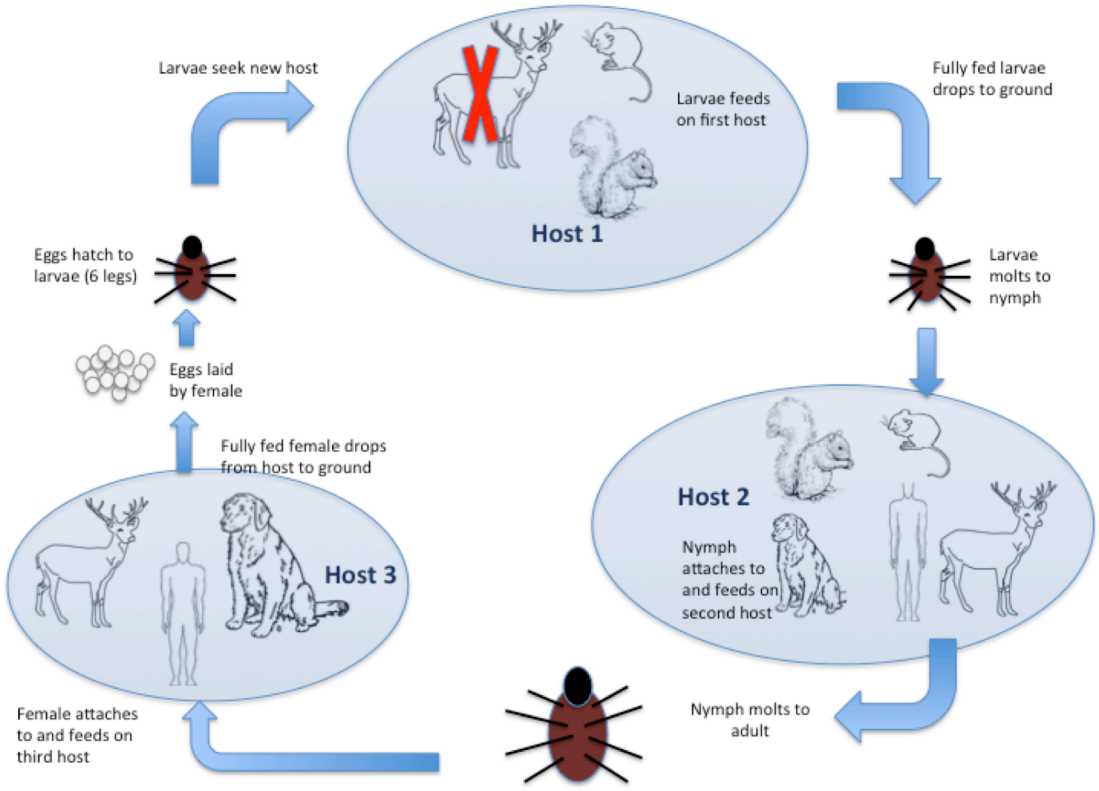


FIG 1. Tick Life Cycle. Infectious cycle of the European *Borrelia burgdorferi* sensu lato genospecies *B. burgdorferi* sensu lato is the only pathogenic genospecies present in the US and Europe, both rodents and birds are reservoirs. A red cross indicates a non-reservoir host. (Adapted from "Lyme borreliosis"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

Carriers, signs and symptoms

Lyme disease is a very common problem in several regions around the world (22). Lyme disease is transmitted by the bite of *Ixodes* ticks. *I. scapualaris* is the competent vector for the transmission of LD in East US while *I. pacificus* is mostly associated with the transmission of the disease in Western US (32). Furthermore, *I. ricinus* will be the competent vector transmitting the disease in Euroasia. The regions mostly affected by LD have similar environmental conditions that allow ticks to thrive (1).

The life and transmission cycle of a tick begins when a fully developed female tick drops off the host and lays eggs (FIG. 1). Once the eggs hatch the larval stage of the tick seeks a new host, these larvae are not infected with the infectious Borrelia responsible of LD, and they acquire it after feeding on an infected small mammal, usually a small rodent. After feeding on the first host, the larvae drop to ground and develop into the nymph stage. The nymph then attaches to the second host and feeds. At this point, nymphs can infect larger animals, including humans, dogs, horses, deer, etc. The nymph molts to an adult, attaches to the third and final host and feeds (FIG 1). LD can be transmitted to any of these hosts (23). In North America, the only species of Lyme borrelia known to cause human disease is *Borrelia burgdorferi* sensu stricto. In Europe, there are at least five species of Lyme borrelia (*B. afzelii, B. garinii, B. burgdorferi, B. spielmanii,* and *B.a bavariensis)* that can cause the disease, thus leading to a wider variety of possible clinical manifestations in Europe than in North America. *B afzelii* and *B garinii* infections account for most Lyme borreliosis cases in Europe, whereas *B garinii* is predominant in Asia. Moreover, *B afzelii* is mostly associated with skin manifestations,

B garinii seems to be the most neurotropic, and *B burgdorferi* seems to be the most arthritogenic.(1).

LD is characterized by various neurological, dermatological, cardiovascular and musculoskeletal problems. (30, 34) These symptoms can vary by region. For example, arthritis is more common in American patients while neuroborreliosis is more common in European patient. Erythema migrans (EM) is the only way to characterize Lyme without a diagnostic test (1). EM is a red, "bulls-eye" shaped rash that appears on some patients infected with Lyme. Only 68% of the US patients develop this symptom and therefore better understanding of the distribution of the pathogen in different areas of the country will eventually improve the awareness of the disease in the population and the medical community. The symptoms of the disease also differ based on the stage of the disease (2, 3, 10, 14). The first stage, **early-localized stage**, occurs between 1-4 weeks after infection. During this stage, patients may experience flu-like symptoms, in addition to the development of EM. The second stage, **early disseminated**, extends from 1 to 4 months after the infection has occurred. Additional rashes, partial paralysis, conjunctivitis, inability to concentrate and other flu-like symptoms characterize the stage. The final stage, **late disseminated** or **Chronic Lyme disease**, can be developed after a few months to a few of the establishment of the infection. During this stage, the disease can cause severe damage to the joints, nerves and brain. Inflammation, severe fatigue, partial nerve paralysis, neurological problems and chronic arthritis also accompany this stage of LD. Consequently, by establishing surveillance programs with a better diagnostic tool, the time at which the diagnosis of the disease is done will

significantly impact the success of the disease treatment the patients will receive (1). Moreover, surveillance programs will help introducing awareness campaigns in the local and medical population, increasing the early diagnostic of the disease and the prevention of tick bites by an informed society (4, 7).

Various testing methods

With EM being the only physical characteristic of Lyme disease, accurate testing methods are very important when it comes to diagnosing the disease. There are various laboratory-testing methods, which include: culturing of bacterial pathogen from biopsy samples, PCR from biopsy or from blood (serum and/or plasma), serolocial tests such as ELISA (enzyme immunoassay), IFA (immunofluorescent-antibody assay) and Western Blot (immunoblot, IB) (1). The commercially available serological diagnostic kits are very expensive and mostly detect chronic cases with clinical signs, but are not efficient in detecting acute and/or subclinical cases (15, 25, 27, 29, 33, 34). Currently, there are also no reliable tests to detect the causative agent of the disease. PCR has been proven to be the most effective of these methods (1, 26, 34); however, it isn't used as often as other methods due to the lengthy process and the fact that it is only giving valuable information when biopsy tissue is used. Culture methods from blood, serum and/or plasma are very rarely used because of limitations when it comes to isolate the bacterium from these samples. ELISA and IB have very close testing sensitivities, but IB is more specific. These two methods are used more often in the diagnostic laboratory than PCR. Variations among these tests have both advantages and disadvantages. Many companies today are trying to develop new and improved methods that are more specific and

sensitive than the methods mentioned above (5, 33). With Lyme disease being such a common disease it is imperative to find an effective and efficient method to diagnose those infected with the disease. The Lyme serological diagnostics techniques mostly used in veterinary medicine are the IFA and the ELISA test using the C6 epitope of the variable surface antigen VlsE. The IFA is known for its subjective interpretation and its low sensitivity, while the ELISA C6 test is known for it high sensitivity (9, 16, 21). Nevertheless, there are limitations to all this tests like the fact that each one uses species-specific antibodies and they can detect animals infected for at least 3 weeks to up to 6 months. Consequently we foresee the necessity for better diagnostic test that can be used in sero-surveillance programs to control and prevent Lyme disease.

Hypothesis

Our hypothesis is that a non-species specific competitive ELISA test, also known as pan-specific ELISA test, will help improve the diagnosis of Lyme disease in both human and veterinary medicine. More over, this type of test will help in the establishment of surveillance programs in which a number of different animal species can be tested without the development of a significant number of specific reagents. Our objectives for this project is to 1) evaluate the antibody level against *B. burgdorferi* whole cell lysates and the Borrelial recombinant proteins P66 and OspC of a collection of dog serum samples by traditional ELISA and 2) determine the immune-reactivity of Texan dogs serum samples to Borrelia by Immunoblot assay, so as to determine the cut off values for the ELISA assay. This evaluation will help establishing the basic parameters for the final competitive ELISA and will determine the validity of P66 as potential antigen to be use

in the final assay. The immune-reactivity to OspC will help us determine which animals in the population studied are in the early stages of Lyme and whether or not the sero-reactivity towards P66 can be used to detect animals with Lyme disease regardless of the stage of infection in which they are.

CHAPTER II

METHODS

The methods described below are used for the preparation and usage of both recombinant proteins P66 (rP66) and OspC (rOspC) together with the standardization of specific ELISA and Immunoblot tests utilized to evaluate dog and deer samples for LD.

Protein expression and purification

Protein induction

Both rP66 and rOspC were previously cloned (Dr. Esteve-Gassent, unpublished data) in the expression vector pET23a™ (Novagen) and in the expression *E. coli* Rosetta™ strain (Novagen). The first step to purify the rP66 and rOspC was to induce their expression in *E. coli*. To this end, a culture of the expression host *E. coli* strain Rosetta™ encoding rP66 or rOspC were started in LB broth media containing ampicillin 100µg/ml (Amp_{100}) and chloramphenicol 20µg/ml (Can_{20}) at 37ºC, shaking overnight. A 1:100 dilution of each of the cultures were used to start 1 liter of LB Broth containing Amp_{100} and Can_{20}. Large cultures were shaken for 4-5 hours at 37ºC or until OD_{600nm}=0.5 to 0.8. Then, the expression of the recombinant protein was induced by adding 1 ml of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the cultures, and continue shaking for 2 hours at 37°C. After the 2 h induction, cells were harvested by centrifugation for 20 minutes at 4000 rpm and 4ºC. Pellets were stored at -80ºC until use.

Protein purification

After thawing on ice the pellets of Rosetta™ cells expressing either rP66 or rOspC, 25 ml of Lysis Buffer (50mM sodium phosphate, 8 M urea, 300 mM NaCl, 20 mM imidazole pH 7.4) was added to resuspend the cells. Cells were disrupted by utilizing a French press (Thermo scientific). Each pellet was French pressed 3 times to ensure complete lysis of the cells. After the French Press, lysates were centrifuged for 20 minutes at 4000 rpm and 4ºC. Pellets were saved at -80°C until purification of the protein was confirmed. Supernatants were mixed with 5 ml of Nickle beads (His60 Superflow™ resin, Chlontech) previously equilibrated with Wash Buffer (50mM sodium phosphate, 8 M urea, 300 mM NaCl, 40 mM imidazole pH 7.4) and incubated overnight at 4°C with continuous gentle shaking. After the overnight binding, beads were clean by centrifugation for 5 minutes at 500 rpm and 4ºC to remove any unspecific binding. Post absorption supernatants (PASN) and washing fraction were saved at -80ºC until purification of each recombinant protein was confirmed. Beads with the recombinant His tag containing proteins were allowed to pack at room temperature in a chromatography column (BioRad). While the beads settle, two sets of 25 elution collection tubes were prepared. (Tubes should be labeled E1-E25). Each one of the proteins was eluted by adding 10 ml of the elution buffer (50mM sodium phosphate, 8 M urea, 300mM NaCl, 300mM imidazole pH 7.4) to the column. Approximately 20 ml of elution buffer was needed to ensure the adequate purification of the recombinant proteins. Fifty µl of each elution fraction was combined with 50 µl of 2X Final Sample Buffer (2x FSB) to check

for the fraction in which the recombinant protein eluted. Elution fractions were saved at -80°C until use in the ELISA assays.

SDS-PAGE gel

In order to determine in which elution fractions the recombinant proteins were mostly present, an aliquot of the induction pellet, PASN, wash fractions and elution fractions were separated in a 12% SDS-PAGE gel. Gels were stained in Coomassiee Blue (0.25% cooomassiee brilliant blue dye R250, 45% methanol, 10% acetic) for 1 hr at room temperature. After destaining (45% methanol, 10% acetic acid), the more concentrated elution fractions were selected and prepared for purification. The gels were stored (10% ethanol, 5% glycerol) and dried for further reference.

Protein clean up and concentration

The fractions containing the recombinant proteins were cleaned and concentrated by using the Amicon filtration system (Millipore) with cut off pores of 3kDa or 5kDa to ensure the retention of the proteins of interest. The concentrated protein was further cleaned by dialysis to ensure the elimination of the denaturing agent Urea and the Imidazol used to elute the protein during the purification steps. To this end, the fractions being cleaned are placed in a dialysis cassette (Slide-A-Lyser® Cassette, Thermos Scintific) and in 500ml of dialysis buffer (50mM sodium phosphate, 300mM NaCl) for 2 hours at room temperature and continuous stiring. After 2 hours, the concentrated fractions were recovered and stored at -80°C. Aliquots were used to determine protein concentration.

Protein quantification

Protein concentrations was determined by using the Pierce BSA Assay Kit (Thermo Scientific) so as to adjust the protein concentration to the one needed to run the ELISA test. Briefly, BSA (Bovine Serum Albumin) protein standards were made with concentrations ranging from 0 to 250µg/ml. The test proteins were diluted 1:5 and 1:10 and 25µL of each protein dilutions and standards were placed in triplicates in a 96 well plate (Corning). After adding 200 µl of the Working reagent plates were incubated for 30 minutes at 37ºC and in the dark. After incubation, protein concentration was measured in a plate reader (BMI LABTECH OMEGA) and protein aliquots adjusted to the right concentrations for the ELISA test.

Enzyme linked immuno sorbed assay

To determine the level of specific antibodies in the dog sera (samples were obtained from TVMDL years 2011-2012) to *B. burgdorferi* lysates, rP66 and rOspC Enzyme Linked Immunosorbed Assays (ELISA) were done. To this end, 96 well plates (Nunc, Thermo Scientific) were coated with the appropriate antigen at a concentration of 500ng/well (rP66 and rOspC) and 10^7 cells/well (*B. burgdorferi* lysate) in carbonate buffer pH 9.4 at 4ºC overnight. After coating, plates were washed three times with Phosphate Buffer Saline containing 0.5% Tween 20 (PBS-T). Plates were then blocked with 200µL of PBS containing 3% BSA at 4ºC overnight. After blocking, plates were washed three times in PBS-T and incubated for 1hr with serial two-fold dilutions ranging from 1:400 to 1:25,600 of each of the animal samples in PBS-T containing 1% BSA. No sample serum was used as the blank. After washing the plates 3 times with PBS-T,

1:2,000 dilution of the anti-dog HRP- conjugated IgG antibody was added to each well. The plates were shaken for 1 hour at room temperature. The plates were then washed 3 times in PBS-T and incubated with 100µl/well of the OPD buffer (Thermo Scientific), for 30 minutes in the dark (in a drawer wrapped in foil). Plates were read at a wavelength of 450nm and analyzed by using the BMI LABTECH OMEGA computer program and plate reader.

Western blot

The Western Blots were done following the Trinity Biotech *B. burgdorferi* Marblot™ Strip Test System. Briefly, remove the number of strips (coated with *B. burgdorferi* antigen) with blunt forceps. 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 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 1:2,000 dilution of the anti-dog AP conjugated IgG antibody 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. For the evaluation, we considered positive strips that show 3 or more bands in the region bellow the 40kDa band and 2 or more

bands in the region between 91kDa and 45kDa bands. A group of 13 negative dog samples (puppies from an in-house dog colony) were used to determine the evaluation criteria.

Statistical methods

Continuous variables will be described by median and interquartile range (IQR) or mean and standard deviation. The dichotomous variables were described by the number and percent positive (1) and negative (0). Logistic regression models were used to estimate the probability of a positive result as a function of antibody levels. These probabilities were used in a Receiver Operating Characteristic (ROC) analysis to determine the optimum antibody level cut-off for diagnosis that maximize the proportion correctly classified. Antibodies were compared for their ability to classify by performing a test of equality of ROC areas. All data manipulation and statistical analysis was performed in Stata (31).

CHAPTER III

RESULTS

Protein purification

SDS-PAGE gel

After growing the bacterial clone expressing each of the recombinant proteins we induced their expression for 2hrs at 37ºC. Once the induction was terminated, cells were disrupted by French Press and recombinant proteins were purified utilizing their binding to Ni chromatography through the 6*His tag engineered in their C terminus end. Each one of the eluted fractions together with the induction lysate and the post-absortion supernatant (resulting from the binding of the recombinant proteins to the Ni beads) were separated in a 12% separating SDS-PAGE gel. The SDS page gels helped us to determine which elution fractions were used in the downstream experiments, and therefore, they were cleaned and concentrated by dialysis and centrifugation. The gels provide a visual reference for which elution fractions contain the highest concentrations of protein. As shown in FIG. 2A, for rOspC, fractions 3 through 17 contain the highest concentrations of proteins whereas elution fractions 3 through 12 had the higher concentration of rP66 (FIG. 2B). These fractions were then pulled and dialyzed to remove the Urea and imidazole used in the purification process, followed by concentration via centrifugation (Amicon system, Millipore). The fractions 5-10 were pulled, and concentrated for rOspC and fractions 3-8 were used for rP66.

Protein quantification

After the protein was cleaned via dialysis and concentrated by filtration, the protein concentrations were determined by using the Pierce BCA Assay Kit. The average concentrations of rP66 and rOspC were approximately 600 µg/mL and 500 µg/mL respectively. FIG. 3 represents the standard curve obtained in the BCA assay for the quantification of both rP66 and rOspC. This particular curve had a fitness value (r^2) of 0.999641. The concentrations of each protein were required to dilute them accordingly to coat the plates for the subsequent ELISA test. A concentration of 5 µg /well was the standard concentration for this project.

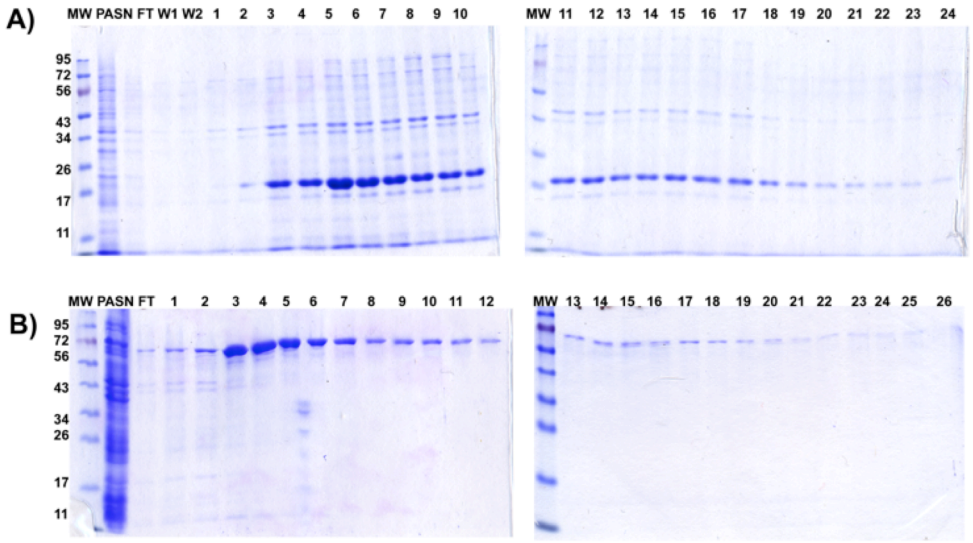


FIG 2. SDS-PAGE Gel. Recombinant OspC (**A**) and P66 (**B**) elution fractions were separated in a 12% SDS-Page Gel and stained with Coomassie brilliant blue. Molecular weigh (MW) marker in kDa is represented on the left side of each gel (EZ-marker, Thermo-Fisher). PASN: Post Absorption SuperNatant (containing unbound proteins to Ni columns); FT: Flow Though wash, W1 and W2: Wash fractions; 1-26 are the different elution fractions isolated where the recombinant protein got

Immunoreactivity of dog serum to *B. burgdorferi* antigens

After running ELISA tests using the different recombinant proteins (rP66 and rOspC) as well as the whole cell lysates, some discrepancies were found in between the IFA, the ELISA and Western blot (WB) tests. Out of the 30 IFA positive samples tested, 6 were diagnosed as negative using the ELISA and WB, which constitutes a 25% of false positive results when using the IFA diagnostic test. On the other hand, out of the 70 IFA negative samples studied, 42 were positive by ELISA and WB, which constitutes around 60% of false negatives in the pool of samples studied. FIG 4 shows a representation of the various results obtained from the ELISA test and FIG 5 represents various results obtained from the Western Blots.

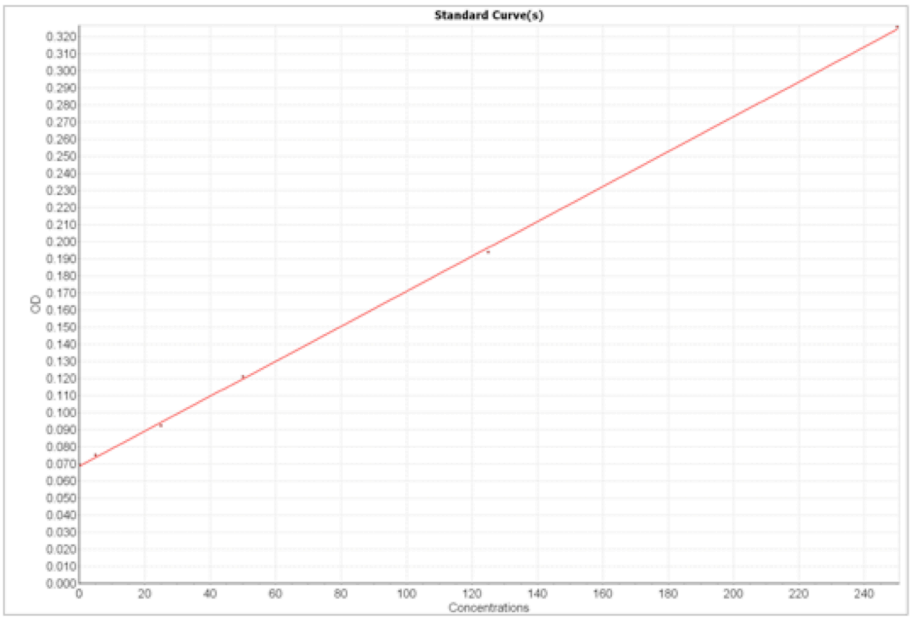


FIG 3.: BCA Assay Standard Curve.

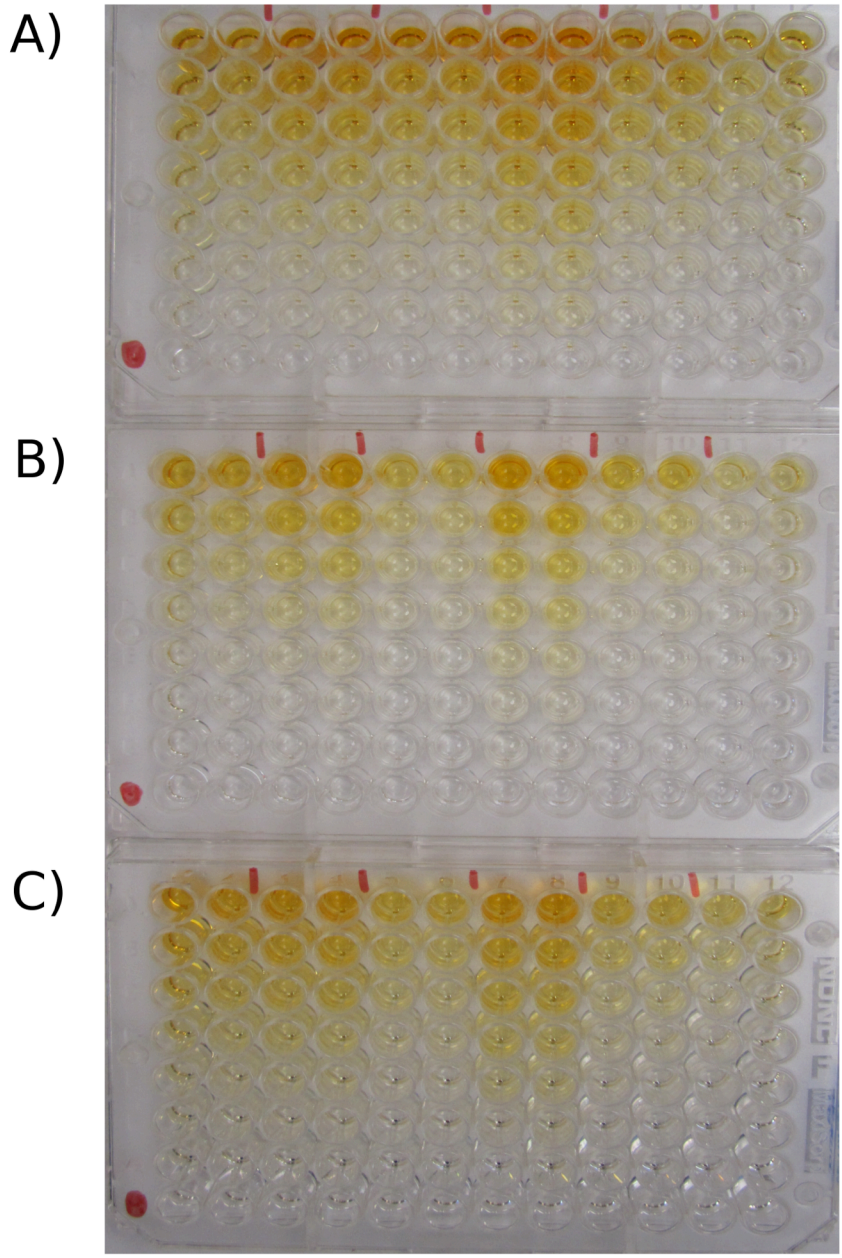


FIG. 4 ELISA Plates. Plates after incubating with different dog sera and were coated with rP66 (A), rOspC (B) and BB whole cell lysates (C).

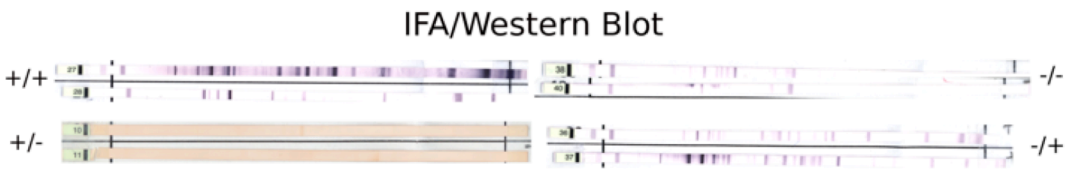


FIG 5 Western Blot Results. Representative immunoblot strips from animals that were positive for both IFA and Western blot (+/+), negative for both (-/-), and positive for one or the other (+/-; -/+). The commercially available human LD IgG kit was purchased and adapted for dog serum samples.

The ELISA and Western Blots had very similar results and are much more accurate than the IFA. The results of all three tests were analyzed using STATA 11 to determine which combination of test will provide better diagnostic of Lyme disease in dogs. FIG 6A represents the correlations of the positive and negative samples when tested by IFA and ELISA. The ROC model was used to determine an accurate cut off value for the ELISA test. Figure 6B,C and D display the ROC curves for the three borrelial antigens. The statistical test of ROC areas found no significant evidence of a difference between the three antigens (p value = .16). Nevertheless, the percent of correct diagnosis is below 70% in all cases, maintaining a 30% of misdiagnosis when using the 101 samples tested by ELISA thus far.

The same type of statistical analysis was done using the 80 samples tested by WB. Figure 7A represents the antibody levels of the positive and negative samples when tested by WB. The ROC model was used to determine an optimum cut off value for the WB test. Figure 7B,C and D display the ROC curves for the three borrelial antigens.

The statistical test of ROC areas found no significant evidence of a difference between the three antigens (p value = .86). Moreover, when determining cut off values for the ELISA test, all three antigens provided values that will correctly diagnose 70 to 78% of the cases and will improve with the testing of more samples. Consequently, P66 gave similar results to those observed when using either whole cell lysates or rOspC and therefore we suggest that P66 can be used as a potential new antigen for the development of a competitive ELISA for the diagnosis of LD in veterinary medicine regardless of the stage of infections in the tested animals.

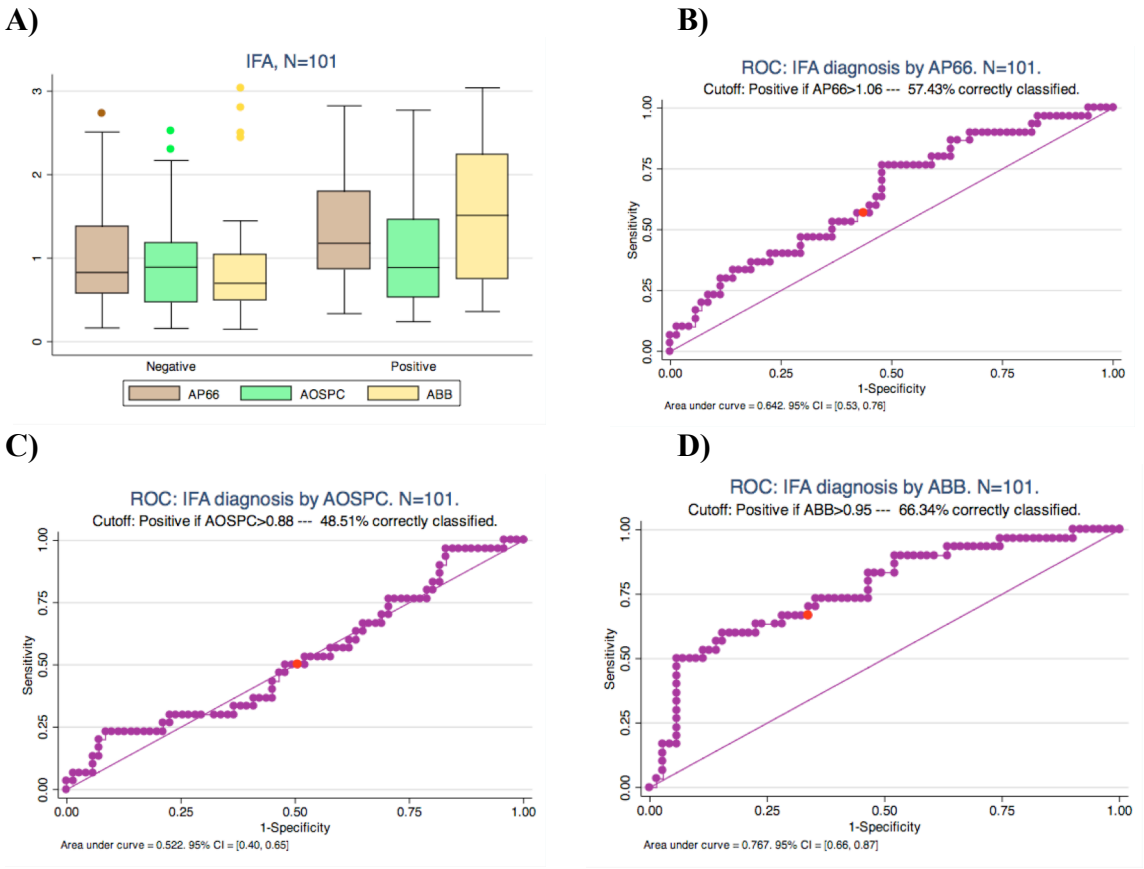


FIG 6. OD cut off determination based off of ELISA and IFA. OD cut offs based on both tests (A). Plot of ODs Cut offs based on P66 (B) Cut offs based on OspC (C) Cut offs based on BB (D).

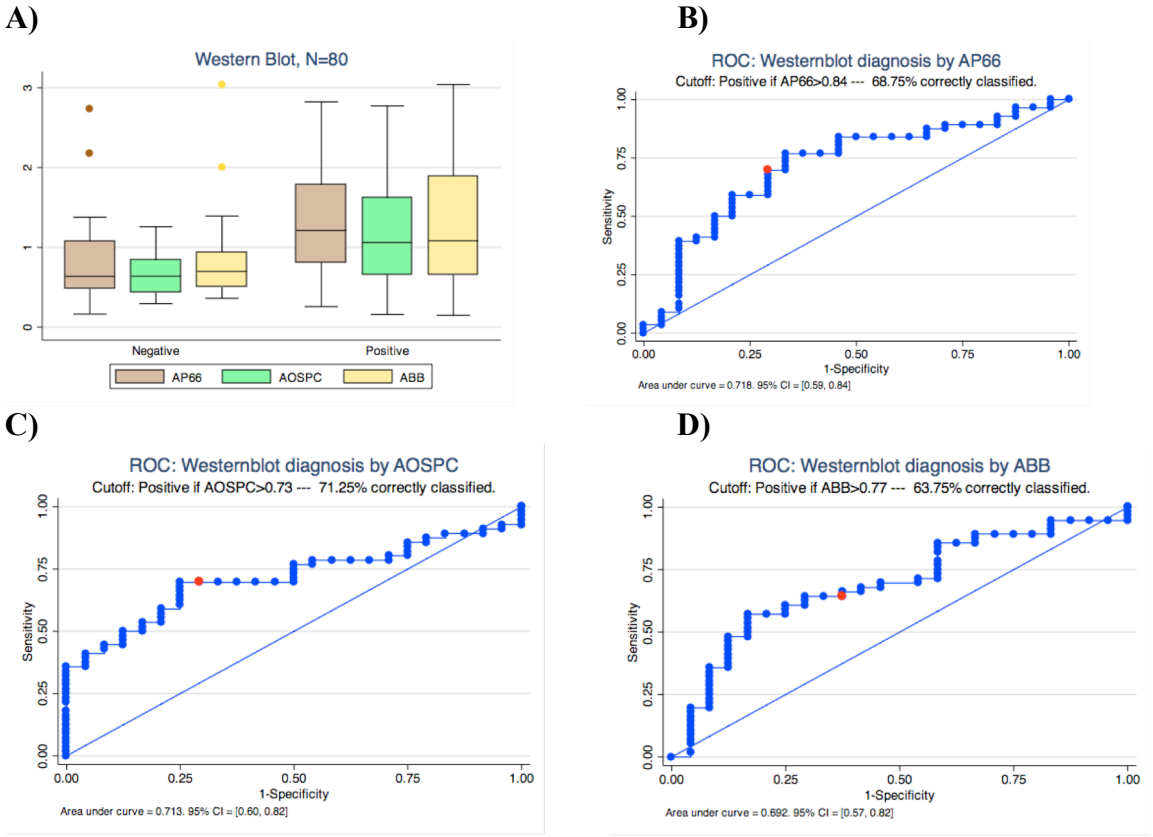
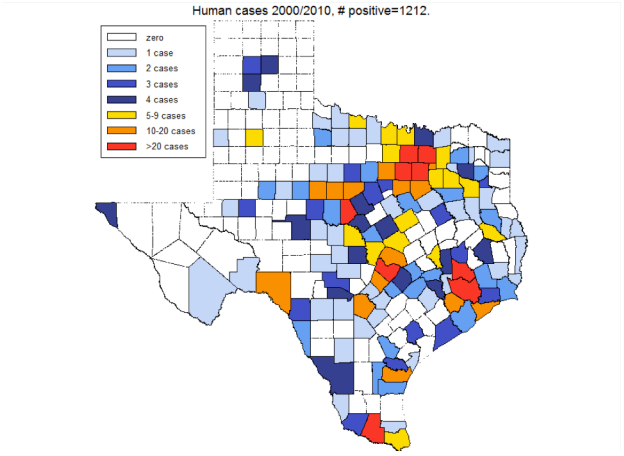


FIG 7. OD cut off determination based off of ELISA and IFA. OD cut offs based on both tests (A) Plot of ODs Cut offs based on P66 (B) Cut offs based on OspC (C) Cut offs based on BB (D)

Furthermore, we mapped the canine LD cases studied as well as the cumulative human LD cases from 2000-2010. In FIG.8A, we observed that the canine LD distribution in Texas is similar to that observed in humans (FIG. 8B) when using the same methodology for diagnostics (ELISA followed by Immunoblot assay). Moreover, the utilization of ELISA and Immunoblot allows a more sensitive diagnostics that results in a better mapping of the current canine LD cases in TX during the last trimester in the year 2011 and the beginning of year 2012.

A)



B)

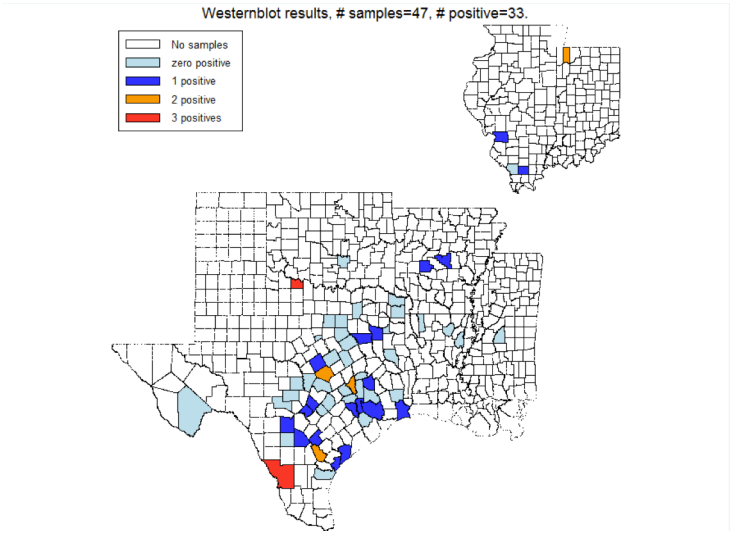


FIG.8. Distribution of human and canine LD in Texas. Cumulative human LD cases reported to CDC are represented in figure A (Data obtained through the Texas Department of State Health Servises). The counties with highest incidences of human LD were Travis with 127 cases reported followed by Dallas (125 cases) and Tarrant (118 cases). Canine LD cases analyzed by TVMDL by IFA and reanalyzed by our laboratory by means of ELISA and IB are represented in figure B. These results suggest that sero-surveillance of dogs and other species together with the analysis of sampled ticks will help determining the localization of areas of high risk of LD.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Many researchers have concluded that companion animals (dogs in particular) are very useful sentinels for the assessment of LD in a given area (9, 13, 17, 18, 20, 24). The maps generated in this study are a supportive evidence of this idea by showing the similar patterns of the disease in both humans and dogs in the state of Texas where the distribution of LD has not been studied until now. On the other hand, our study has been very helpful in order to determine which of the testing methods is most accurate for the diagnostics of LD in Texas. This study allows us to conclude that IFA is not an appropriate diagnostic tool for Lyme disease in veterinary medicine due to the high ratio of miss diagnostic observed in the population studied (false positive and false negative). This result was observed by others in different regions of Europe and the US where LD is endemic (12, 18, 24). Therefore our result support the fact that LD in Texas might be under reported due to lack of good diagnostic tools and awareness in the human and veterinary medicine community.

The CDC currently recommends using a two-tier approach when testing for LD serologically in humans; usually an initial ELISA or IFA followed by WB. Seeing this as a successful testing method used in human diagnostics, it should be as effective in veterinary diagnostics (6). After comparing the IFA tests with the confirmatory Western Blots and ELISAs, we have observed that IFA gives many false negatives (66%) as well as false positives (25%). The statistical analysis of all three testing methods (IFA,

Western Blot, and ELISA) led us to the conclusion that P66 is a good candidate for the development of a Competitive ELISA due to its sensitivity when measuring the antibody levels in dog serum sample. OspC and BB lysate are both good indicators; however, OspC antibodies are mostly representative of an early infection and therefore aren't as accurate (8).

The development of this test will result in better diagnostics to be used in veterinary medicine to detect vector borne zoonotic diseases, having a high impact not only on animal but also on human health. Specifically, with better diagnostic tests, the efficacy of available treatments in both animal and human medicine will increase considerably. As observed in the preliminary maps generated with only 80 dog serum samples we can start seeing a distribution of veterinary LD very similar to that observed in the human population, with the added value that the animal LD is closer to the natural areas where the infection happens. Additionally, by using the same diagnostic tools in both human and veterinary medicine comparative studies, more accurate predictions of distribution and dissemination of the disease in areas where this disease is not well understood can be made. Consequently, when using the same techniques, we observed that most of the human and animal cases are reported around the big metropolitan areas of Austin, Dallas Fort Worth and Houston and along the border of Mexico. This observation might be due to the fact that both MDs and DVMs in those urban metropolitan areas are more aware of the disease than those practicing in rural areas. Consequently, by developing a method that can be used in surveillance programs we can monitor the levels of LD in the environment, develop mathematical models and predict when the disease can emerge in

different areas. By understanding the distribution of the disease in companion animals as well as in wild life we can better understand the maintenance of the enzootic cycle of LD (7).

This thesis encompasses the first steps towards the development of a pan-specific test to be used in such surveillance programs. Together with the immune-reactivity of Texan dog serum, we are also studying the sero-reactivity of white tail deer are horses throughout the state. These preliminary studies will help us to understand the immune-reactivity of these animals in Texas and what antigens are best to use in this state. Taken together all the approaches and the results of the different direct ELISAS we will be able to outline the basic conditions to start the standardization of the pan-specific test proposed in this thesis.

In addition to this study, our laboratory is collecting ticks from different areas in Texas in order to correlate the presence of LD in human and animals to the areas in the state that might be maintaining the pathogen in its enzootic cycle, and can be consider as the areas with high risk for infection with the disease. A diagnostic test that is not host species specific will be of great value when establishing surveillance programs, since it avoids the generation of reagents for each specific animal species to be monitored, thus making the diagnosis of LD an easier process. The methodology we are trying to develop will decrease the amount of errors that occur from the current testing method. This project is still a work in progress. Due to time constraints we have only been able to test 100 samples of dog serum thus far, making it our animal model. However, other

animal (cattle, horses and WTD) sera will be analyzed following the same methodology. Nevertheless, this project has helped prove that Western Blot and ELISA are the better diagnostic tools, not only for human, but also for veterinary medicine. By testing a larger number of samples and a variation of species, this testing method will improve diagnostic tests and improve the surveillance of Lyme disease in states where the distribution of the disease and the maintenance of its enzootic cycles is not very well understood.

REFERENCES

1. **Aguero-Rosenfeld, M. E., G. Wang, I. Schwartz, and G. P. Wormser.** 2005. Diagnosis of lyme borreliosis. Clin Microbiol Rev **18:**484-509.

2. **Auwaerter, P. G., J. Aucott, and J. S. Dumler.** 2004. Lyme borreliosis (Lyme disease): molecular and cellular pathobiology and prospects for prevention, diagnosis and treatment. Expert Rev Mol Med **6:**1-22.

3. **Barthold, S. W., M. S. de Souza, J. L. Janotka, A. L. Smith, and D. H. Persing.** 1993. Chronic Lyme borreliosis in the laboratory mouse. Am J Pathol **143:**959-971.

4. **Blancou, J., B. B. Chomel, A. Belotto, and F. X. Meslin.** 2005. Emerging or re-emerging bacterial zoonoses: factors of emergence, surveillance and control. Veterinary research **36:**507-522.

5. **Bowman, D., S. E. Little, L. Lorentzen, J. Shields, M. P. Sullivan, and E. P. Carlin.** 2009. Prevalence and geographic distribution of *Dirofilaria immitis*, *Borrelia burgdorferi, Ehrlichia canis*, and *Anaplasma phagocytophilum* in dogs in the United States: results of a national clinic-based serologic survey. Veterinary Parasitology **160:**138-148.

6. **Burbelo, P. D., A. T. Issa, K. H. Ching, J. I. Cohen, M. J. Iadarola, and A. Marques.** 2010. Rapid, simple, quantitative, and highly sensitive antibody detection for lyme disease. Clinical and Vaccine Immunology : CVI **17:**904-909.

7. **Carver, S., A. M. Kilpatrick, A. Kuenzi, R. Douglass, R. S. Ostfeld, and P. Weinstein.** 2010. Environmental monitoring to enhance comprehension and control of infectious diseases. Journal of Environmental Monitoring : JEM **12:**2048-2055.

8. **Day, M. J.** 2011. The immunopathology of canine vector-borne diseases. Parasites & Vectors **4:**48.

9. **Duncan, A. W., M. T. Correa, J. F. Levine, and E. B. Breitschwerdt.** 2005. The dog as a sentinel for human infection: prevalence of *Borrelia burgdorferi* C6 antibodies in dogs from southeastern and mid-Atlantic States. Vector Borne Zoonotic Dis **5:**101-109.

10. **Franz, J. K., and A. Krause.** 2003. Lyme disease (Lyme borreliosis). Best Practice & Research Clinical Rheumatology **17:**241-264.

11. **Goldings, E. A., and J. Jericho.** 1986. Lyme disease. Clin Rheum Dis **12:**343-367.

12. **Hamer, S. A., J. I. Tsao, E. D. Walker, and G. J. Hickling.** 2010. Invasion of the lyme disease vector *Ixodes scapularis*: implications for *Borrelia burgdorferi* endemicity. EcoHealth **7:**47-63.

13. **Hamer, S. A., J. I. Tsao, E. D. Walker, L. S. Mansfield, E. S. Foster, and G. J. Hickling.** 2009. Use of tick surveys and serosurveys to evaluate pet dogs as a sentinel species for emerging Lyme disease. American Journal of Veterinary Research **70:**49-56.

14. **Hengge, U. R., A. Tannapfel, S. K. Tyring, R. Erbel, G. Arendt, and T. Ruzicka.** 2003. Lyme borreliosis. Lancet Infect Dis **3:**489-500.

15. **Johnson, A. L., T. J. Divers, and Y. F. Chang.** 2008. Validation of an in-clinic enzyme-linked immunosorbent assay kit for diagnosis of *Borrelia burgdorferi* infection in horses. J Vet Diagn Invest **20:**321-324.

16. **Liang, F. T., A. L. Alvarez, Y. Gu, J. M. Nowling, R. Ramamoorthy, and M. T. Philipp.** 1999. An immunodominant conserved region within the variable domain of VlsE, the variable surface antigen of *Borrelia burgdorferi*. Journal of Immunology (Baltimore, Md. : 1950) **163:**5566-5573.

17. **Little, S. E., S. R. Heise, B. L. Blagburn, S. M. Callister, and P. S. Mead.** 2010. Lyme borreliosis in dogs and humans in the USA. Trends in Parasitology **26:**213-218.

18. **Mead, P., R. Goel, and K. Kugeler.** 2011. Canine serology as adjunct to human lyme disease surveillance. Emerg Infect Dis **17:**1710-1712.

19. **Ogden, N. H., and J. I. Tsao.** 2009. Biodiversity and Lyme disease: dilution or amplification? Epidemics **1:**196-206.

20. **Olson, P. E., A. J. Kallen, J. M. Bjorneby, and J. G. Creek.** 2000. Canines as sentinels for Lyme disease in San Diego County, California. J Vet Diagn Invest **12:**126-129.

21. **Philipp, M. T., L. C. Bowers, P. T. Fawcett, M. B. Jacobs, F. T. Liang, A. R. Marques, P. D. Mitchell, J. E. Purcell, M. S. Ratterree, and R. K. Straubinger.** 2001. Antibody response to IR6, a conserved immunodominant region of the VlsE lipoprotein, wanes rapidly after antibiotic treatment of *Borrelia burgdorferi* infection in experimental animals and in humans. J Infect Dis **184:**870-878.

22. **Piesman, J., and L. Gern.** 2004. Lyme borreliosis in Europe and North America. Parasitology **129 Suppl:**S191-220.

23. **Radolf, J. D., M. J. Caimano, B. Stevenson, and L. T. Hu.** 2012. Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. Nature reviews. Microbiology **10:**87-99.

24. **Rand, P. W., E. H. Lacombe, S. P. Elias, B. K. Cahill, C. B. Lubelczyk, and R. P. Smith, Jr.** 2011. Multitarget test for emerging Lyme disease and anaplasmosis in a serosurvey of dogs, Maine, USA. Emerg Infect Dis **17:**899-902.

25. **Rudenko, N., M. Golovchenko, L. Grubhoffer, and J. H. Oliver, Jr.** 2011. Updates on *Borrelia burgdorferi* sensu lato complex with respect to public health. Ticks Tick Borne Dis **2:**123-128.

26. **Schmidt, B. L.** 1997. PCR in laboratory diagnosis of human *Borrelia burgdorferi* infections. Clinical Microbiology Reviews **10:**185-201.

27. **Schwaiger, M., O. Peter, and P. Cassinotti.** 2001. Routine diagnosis of *Borrelia burgdorferi* (sensu lato) infections using a real-time PCR assay. Clin Microbiol Infect **7:**461-469.

28. **Seshu, J., and J. T. Skare.** 2000. The many faces of *Borrelia burgdorferi*. J Mol Microbiol Biotechnol **2:**463-472.

29. **Stanek, G., V. Fingerle, K. P. Hunfeld, B. Jaulhac, R. Kaiser, A. Krause, W. Kristoferitsch, S. O'Connell, K. Ornstein, F. Strle, and J. Gray.** 2010. Lyme borreliosis: Clinical case definitions for diagnosis and management in Europe. Clin Microbiol Infect.

30. **Stanek, G., and F. Strle.** 2003. Lyme borreliosis. Lancet **362:**1639-1647.

31. **Stata LP Corp.** 2011. 4905 Lakeway Dr College Station, TX 77845.

32. **Tsao, J. I.** 2009. Reviewing molecular adaptations of Lyme borreliosis spirochetes in the context of reproductive fitness in natural transmission cycles. Veterinary Research **40:**36.

33. **Wagner, B., H. Freer, A. Rollins, and H. N. Erb.** 2010. A fluorescent bead-based multiplex assay for the simultaneous detection of antibodies to *B. burgdorferi* outer surface proteins in canine serum. Vet Immunol Immunopathol.

34. **Wilske, B.** 2005. Epidemiology and diagnosis of Lyme borreliosis. Ann Med **37:**568-579.

CONTACT INFORMATION

Name:	Erin McGregor
Professional Address:	Dr. Maria Esteve-Gassent Department of Veterinary Pathobiology MS 4227 Texas A&M University College Station, TX 77843
Email Address:	ekm09@tamu.edu
Education:	B.S., Biomedical Science, Texas A&M University December 2012 Undergraduate Research Scholar