TICK-BORNE PATHOGENS IN PUERTO RICAN LIVESTOCK: A MOLECULAR APPROACH

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PAYTON BLACKBURN

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ABSTRACT

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Payton Blackburn
Department of Veterinary Medicine and Biomedical Sciences
Texas A&M University

Research Advisor: Dr. Maria Esteve-Gasent
Department of Veterinary Medicine and Biomedical Sciences
Texas A&M University

Tick-borne pathogens (TBPs) are a significant source of health and economic burden in the Puerto Rican livestock industry. Previous research suggests that upwards of U.S. $6.7 million is lost annually to TBP related infestations. Additionally, changes in climate have been known to disrupt tick distribution and the prevalence of TBPs. This project seeks to evaluate the presence and distribution of various TBPs in ticks sampled from Puerto Rican livestock, post-Hurricane Maria in 2017. To accomplish this goal, our team collaborated with USDA-APHIS officials in Puerto Rico. To this end, USDA-APHIS veterinarians collected tick samples from livestock (cattle and horses) from different premises across the island. Samples were submitted to our laboratory and were screened for pathogens using both conventional PCR and real-time quantitative PCR. The results of this study show 81.1% of the cattle premises to be positive for *Anaplasma/Ehrlichia* spp., and 24.2% of the cattle premises to be positive for *Babesia bovis* and *Babesia bigemina*. We also detected *Babesia caballi* in 9.8% of the horse premises. The results of this study will inform stakeholders of the TBP diversity affecting Puerto Rican livestock, as well as the distribution of those pathogens throughout the island. This information will be useful in future programs focused on effective eradication and management of TBP transmitting ticks.

DEDICATION

This thesis is dedicated to my family. To my mom and dad, who are my constant encouragement and the reason why I have been able to achieve all of my accomplishments. And to my brothers, Luke and William, who are my supporters, my co-conspirators, and my friends. I love you all so much!

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NOMENCLATURE

CFTRL	Cattle Fever Tick Research Laboratory
PCR	Polymerase Chain Reaction
TBD	Tick Borne Disease
TBP	Tick Borne Pathogens
USDA-APHIS	United States Department of Agriculture, Animal and Plant Heath Inspection Services

CHAPTER I

INTRODUCTION.

Tick-Borne Diseases: The Problem

Tick-borne diseases are a major health concern for both humans and animals. In addition to being capable of transmitting a great variety of pathogenic organisms, ticks cause irritation, allergy, and other important health concerns. Thus, they are one of the most important vectors of diseases affecting livestock (Jongejan et al., 2004). Since the late 1980's it has been accepted that ticks and tick-borne diseases cause significant economic impact on livestock production, with over 2 million cattle heads susceptible to infection by a number of tick-borne pathogens (Dallwitz et al., 1987). Because healthy animals lead to a productive and profitable livestock industry, ticks and tick-borne diseases are of the upmost importance economically. Small ranchers and farming communities in impoverished areas are particularly at risk for economic damages related to tick borne disease infestations (Jongejan et al., 2004). Animals who develop tick-borne diseases often display symptoms that lead to decreased market value, together with the negative impact they have in both milk and meat production (Pérez de León et al., 2010). To make matters worse, the treatments for these diseases can be costly and labor intensive. Because of this, significant time and effort have been devoted to eradicating tick vectors responsible for transmitting the diseases in the first place (Crom., 1992).

History of Tick Eradication Efforts in Puerto Rico

The first tick eradication efforts in Puerto Rico began in 1936, and were focused on clearing the island of the tick species *Rhipicephalus (Boophilus) microplus*. The program was modeled after mainland United States' successful *R. microplus* eradication efforts, which took

place between 1906 and 1943, and is described below in further detail. Puerto Rico's eradication efforts were centered on regular dipping of cattle into pools of acaricide every 14 days. By 1954, the island was officially declared free of *R. microplus*, and remained free of the vector for 24 years until it was reintroduced in 1978. At that point, a second eradication program was established, which has continued to the present day (Crom., 1992). The current program includes acaricide treatments and vaccination.

The Vectors

Rhipicephalus (Boophilus) microplus

Rhipicephalus microplus is a species of the hard-shelled family *Ixodidae*, that infects many different species of animals. It is colloquially known as the Cattle Fever Tick, and is the main vector responsible for transmission of the pathogen responsible for the disease known as Bovine Babesiosis, or "Cattle Fever." *R. microplus* is a one-host tick, meaning it remains on the same animal during the majority of its lifecycle, only leaving at the end stage of life to complete the egg laying process. Because of its wide range of hosts, and ability to transmit disease causing pathogens, it is a major burden on the world's livestock industry. Additionally, *R. microplus* is broadly distributed across the globe and has been found in both tropical and subtropical environments (Spickler., 2007).

Eradication from the United States

Between 1906 and 1943 the United States organized a nationwide effort to eradicate *R. microplus* and *R. (B.) annulatus* from the country with the objective to eliminate bovine babesiosis. This effort was accomplished primarily through extensive use of acaricides and pasture rotation. Texas A&M's College of Veterinary medicine, then School of Veterinary Medicine, played an essential part in the eradication of this disease. The work of Dr. Marc

Francis, the college's first dean, proved that the disease was transmitted by ticks and led to a subcutaneous method of immunizing the cattle against bovine babesiosis (McCrady., 2010**)**. These accomplishments have led to him being referred to as "The Father of the Texas Cattle Industry." In 1943 the USDA officially declared the United States free of both *R. microplus* and *R. annulatus,* thus eliminating the presence of bovine babesiosis. This absence is maintained through strict quarantine regions along the U.S./Mexico border as well as continuous surveillance of at-risk herds. These activities have been active and coordinated under the United States Department of Agriculture (USDA) until today (https://www.ars.usda.gov/plains-area/kerrville-tx/knipling-bushland-us-livestock-insects-research-laboratory/, March 28th, 2020). Because of the detrimental economic effects of reintroduction of *R. microplus* into the United States, importation of foreign cattle is tightly controlled, and requires dipping the livestock into pools of acaricide and passing a period of quarantine (Pérez de León et al., 2012) together with significant certification and compliance by the farmers so as to import cattle into the US.

Rhipicephalus sanguineus

Rhipicephalus sanguineus, also known as the brown dog tick, is a species of hard tick also in the family *Ixodidae*, that commonly parasitizes dogs. It is a one-host vector, and is the only known tick capable of completing its entire lifecycle indoors. It is primarily found in warmer climates, and is capable of transmitting many pathogens that are responsible for canine diseases. It has also been found to be capable of transmitting, *Rickettsia rickettsii*, the causative agent of Rocky Mountain Spotted Fever in humans (Dantas-Torres., 2010). Although, *R. sanguineus* is not necessarily a major concern in the livestock industry, it is still an important vector to consider when discussing the overall impact of TBDs, since it affects companion animals commonly found in livestock production areas.

Dermacentor nitens

Dermacentor nitens, also known as the tropical horse tick, is the most common tick species that parasitizes equines. It is also a natural vector for transmission of *Babesia caballi* and *Theileria equi*, the causative agents of Equine piroplasmosis. *Dermacentor nitens* is distributed across many tropical and subtropical regions and remains an important ectoparasite in equine health (Gondard et al., 2017).

The Diseases

Bovine Anaplasmosis

Bovine anaplasmosis is an infectious hemolytic disease caused primarily by the bacterium *Anaplasma marginale*. This intracellular pathogen of the Anaplasmaceae family, parasitizes red blood cells and leads to extravascular hemolytic anemia. It is most commonly found in areas with a tropical or subtropical climate. This includes regions of the United States, Central and South America, the Caribbean, Africa, Asia, and Australia (Suarez and Noh, 2011). Because of its widespread geographical distribution, it is a major burden on animal health as well as the world's livestock industry. While cattle are the main species susceptible to the disease, other notable species at risk include water buffalo, bison, and certain species of deer (Aubry et al., 2010). Cattle can be exposed to the disease through a number of ways, the most common of which being the mechanical transfer of blood via infected tick-vectors. Less often, other modes of blood exposure such as ear tagging, shared needle use, and transplacental infection can lead to transmission of the disease. Clinically, bovine anaplasmosis is characterized by symptoms such as fatigue, anemia, emaciation, jaundice, and, in severe cases, death. Cattle of all ages are susceptible to the disease; however, younger animals tend to display fewer clinical symptoms (Aubry et al., 2010). Once an animal has been infected with the pathogen, they remain carriers of

the disease for life and are capable of acting as reservoirs for the spread of the pathogen to healthy cattle. Diagnosing the disease is usually done through serological analysis, visualization of the organism in a blood smear, or PCR. In some cases, the disease can be treated with antibiotics such as tetracycline; nevertheless, this therapy is expensive and, in chronic infection it tends to be ineffective (Aubry et al., 2010).

Bovine Babesiosis

Bovine babesiosis, also known as Cattle Fever, is a tick-borne parasitic disease that is primarily caused by the protozoans *Babesia bovis* and *Babesia bigemina,* transmitted by *R. microplus* and *R.) annulatus,* and *Babesia divergens* transmitted by the three-host ticks *Ixodes ricinus.* Both *B. bovis* and *B. bigemina* species are common in tropical and subtropical environments, while *B. divergens* affects cattle in European and North African countries (Schnittger et al., 2012). As mentioned above, bovine babesiosis is primarily transmitted by the tick species *R. microplus* and *R. annulatus*, and has been a major economic concern in livestock all over the world. The United States' 40-year campaign to eradicate the tick-vectors responsible for the disease (*R. microplus* and *R. annulatus*) was successful at eliminating the disease from U.S. cattle herds. It is estimated that this freedom from babesiosis saves the livestock industry approximately three billion U.S. dollars annually (Pérez de León et al., 2012; Schnittger et al., 2012). Clinically, babesiosis is characterized by high fever, hemolysis, anemia, loss of appetite, lethargy, and decreases in milk production (Suarez and Noh, 2011). The severity of the disease is influenced by the age and immunological status of the host prior to infection. Young calves are generally more resistant to infection and are less likely to be symptomatic, while older cattle are more susceptible to the disease and tend to present with more severe symptoms. These severe cases may include neurological and respiratory symptoms and are usually caused by *B. bovis*,

which tends to be more virulent than *B. bigemina* (Schnittger et al., 2012). The disease is often diagnosed through visualization of the organisms in blood smears, serological analysis, or PCR (Urdaz-Rodríquez et al., 2009). In cattle whose infections are detected early, treatment includes anti-parasitic drugs and supportive treatment such as blood transfusions. However, severe cases of babesiosis are less likely to respond to treatment, as such the most effective and economic way to control the disease is through successful eradication of the tick-vectors responsible for its transmission (Pérez de León et al., 2012).

Equine Granulocytic Anaplasmosis

Equine granulocytic anaplasmosis is a tick-borne disease that causes thrombocytopenia in equines. Its causative agent is the bacterium *Anaplasma phagocytophilum* (formerly *Ehrlichia equi*). This disease is primarily transmitted by the flowing tick species: *Ixodes* spp., *Dermacentor* spp., *Rhipicephalus* spp., and *Hyalomma* spp. (Dziegiel et al., 2013). While the pathogenesis surrounding equine granulocytic anaplasmosis is unclear, it is suspected that decreased platelet numbers are a result of destruction by the host's uncontrolled immune system. Clinically, equine granulocytic anaplasmosis is characterized by initial weakening and increased temperature, followed by an aversion to movement, stiffness, gait changes, and sometimes lameness. Less frequently, symptoms such as high fever, bleeding from mucosa, weight loss and enlarged lymph nodes may be present. Equine granulocytic anaplasmosis is typically diagnosed via visualization of the organism in blood smears, as well as PCR analysis. Treatment of the disease primarily consists of antibiotics such as tetracycline as well as supportive therapies like blood transfusions. (Dziegiel et al., 2013)

Equine piroplasmosis

Equine piroplasmosis, also known as equine babesiosis or equine malaria, is a tick-borne disease that primarily affects horses, donkeys, zebras, and mules. Its causative agents are the protozoans *Babesia caballi* and *Theileria equi*. This disease is endemic to many tropical and temperate climates, and is transmitted by tick-vectors in the genera *Hyalomma*, *Rhipicephalus*, and *Dermacentor*. Equine piroplasmosis is a major burden on the world's equine industry, with infections resulting in losses from treatment cost, loss of activity, abortions, and death (Onyice et al., 2019). A major concern is the spread of this disease to non-endemic environments due to the widespread movement of horses between regions. Clinically, equine piroplasmosis is characterized by three manifestations: acute, subacute, and chronic. In the acute form, which is the most common presentation of the disease, common symptoms include fever, malaise, increased respiration, and peripheral edema. In the subacute form, clinical symptoms include discoloration of mucus membranes, weight loss, and gastrointestinal irritation. Lastly, the chronic form is characterized by nonspecific symptoms such as decreased performance, mild loss of appetite, and slight weight loss (Onyice et al., 2019). Options for diagnosing equine piroplasmosis include visualization of the organism in a blood smear, serological analysis, and also PCR. In the United States, current treatment options are limited to USDA approved equine piroplasmosis protocols, which include high doses of antiprotozoal drugs aimed at complete clearance of the infection. In positive cases from horses located in regions endemic to the disease, treatment is focused on limiting the manifestation of clinical symptoms rather than complete clearance of the infection (Onyice et al. 2019, https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/equine/ep/equine-piroplasmosis March 28th, 2020)

Lyme borreliosis

Lyme borreliosis is a bacterial tick-borne disease that is a concern for humans and animals in the cooler climates of the northern hemisphere. It is caused primarily by the bacterium *Borellia burgdorferi*. Borreliosis occurs in many vertebrate animals such as cats, dogs, and horses. While other vertebrate species, such as cattle, may become infected, they often lack clinical signs and symptoms of the disease. The primary tick vectors of borreliosis are the hard-shelled ticks in the genus *Ixodes*. This includes *I. scapularis*, which is common in the American northeast and Midwest, and *I. pacificus*, which is commonly found on the pacific coast. Clinically, Lyme borreliosis presents with symptoms of lameness, fever, anorexia, arthritis, and lethargy. In pregnant brood mares, abortion and increased foal mortality is also a concern (Butler et al., 2005). These symptoms are a result of the persistent auto immune responses against *B. burgdorferi* in the body's attempt to clear the animal of the infection. Because borreliosis is a multisystem disease and can present with a broad range of symptoms, diagnosis can be difficult. Visualization of the organism in blood smears is often inconclusive due to the pathogen's tendency to remain in the skin or synovial fluid of joints (Straubinger., 2020). However, a positive diagnosis can be made through serological analysis or PCR. Once the disease is confirmed, treatment options include a regimen of antibiotics such as tetracycline and doxycycline. Extended periods of antibiotic therapy are often required to fully clear the animal of the infection. A summary of the above tick-borne diseases can be found below in Table 1.

Table 1: Tick-borne disease summary

Disease	Pathogen	Known Tick-Vectors	Clinical Symptoms
Bovine Anaplasmosis	*Anaplasma marginale*	Many tick species, including *Rhipicephalus* spp.	Fatigue, emaciation, jaundice, anemia, fever, and lethargy
Bovine Babesiosis	*Babesia bovis* *Babesia bigemina*	*Rhipicephalus microplus* *Rhipicephalus annulatus*	High fever, hemolysis, anemia, and decreases in milk production.
Equine Granulocytic Anaplasmosis	*Anaplasma phagocytophilum*	*Ixodes* spp.	Stiffness, gait changes, high fever, bleeding from mucosa, weight loss and enlarged lymph nodes
Equine Babesiosis	*Theileria equi* *Babesia caballi*	*Dermacentor* spp. *Rhipicephalus* spp.	Fever, malaise, increased respiration, and peripheral edema
Lyme Borreliosis	*Borellia burgdorferi*	*Ixodes* spp.	Lameness, fever, anorexia, arthritis, and lethargy. Cardiac and neurological abnormalities are also common.

Hypothesis

Due to the impact of environmental perturbations on tick distribution and the documented presence of TBPs in the area, we hypothesize that a large number of the collected samples will screen positive for TBPs that are threatening to livestock production.

CHAPTER II

METHODS

Sample Collection

In collaboration with USDA-APHIS veterinarians in Puerto Rico, ticks were opportunistically collected from livestock in 60 of 78 total Puerto Rican municipalities. Ticks collected from each location were grouped by premise, and collectors recorded the number, location, and species of animals sampled as well as any notes regarding the animals' physical states or clinical symptoms. In total, 182 unique premises were sampled, generating a total of 2,184 tick specimens. Of these premises, 75 were dedicated to beef cattle, 20 were dairy cattle, and 82 were dedicated to horses; furthermore, 5 samples were obtained from dogs located in close proximity to the livestock. The collected ticks were stored in 50mL conical tubes containing 70% ethanol, and were shipped to the College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, for further processing and testing. All experiments were conducted under IBC permits 2016-051 and 2019-064.

Sample Preparation

Each of the 182 premises sampled provided tubes containing as little as 1 tick and as many as 96 ticks. Prior to maceration, all ticks from each premise were identified to the species level using a standard morphological key. In order to optimize the analysis efforts, premises containing more than 10 ticks were limited to a total of 10 randomly selected ticks for DNA extraction and subsequent analysis. In premises with less than 10 submitted tick specimens, all ticks were processed. In addition to the morphological key, the tick species of several premises were further confirmed through genetic analysis.

DNA Extraction

Following identification, DNA from ticks in each premise was extracted using conventional phenol-chloroform extraction protocol. Select ticks were removed from ethanol storage and fully dried, then placed in screw cap microcentrifuge tubes containing 1.4 mm ceramic beads (Omni International, Inc., Kennesaw, GA). Four hundred µL of Phosphate-Buffered Saline (PBS) was added to each tube; the tubes were then placed in a bead mill Bead Ruptor 24 (Omni International, Inc., Kennesaw, GA) and homogenized for 5 minutes at a 5.65 m/s intensity. Following homogenization, the contents of the tubes were transferred to 1.5 mL microcentrifuge tubes. Three µL of proteinase K (20 mg/mL) was added to each sample, and tubes were vortexed briefly and incubated at 55°C overnight. Following incubation, the subsequent procedures were performed under a fume hood. Six hundred µL of phenol (saturated buffer) was added to each tube. The samples were homogenized on a rocker for 5 minutes then centrifuged at 10,000 × *g* rcf and 4°C for 5 minutes. Next, 500µL of supernatant was collected from each tube and transferred to a fresh 1.5mL microcentrifuge tube. Five hundred µL of Phenol: Chloroform: Isoamyl alcohol (*v: v*, 25:24:1) was added to each tube. The samples were homogenized on a rocker for 5 minutes then centrifuged at 10,000 × *g* and 4°C for 5 minutes. Four hundred µL of the supernatant was collected from each tube and transferred to a fresh 1.5mL microcentrifuge tube. Four hundred µL of chloroform was added to each tube. The samples were homogenized on a rocker for 5 minutes then centrifuged at 10,000 × *g* and 4°C for 5 minutes. Three hundred µL of supernatant was collected and transferred to a final tube containing 30µL of Sodium Acetate 3M. Six hundred µL of ice-cold 100% ethanol was added to each tube. The tubes were inverted 10 times and incubated at -20°C overnight to allow DNA precipitation. The next day samples were centrifuged at 10,000 × *g* and 4°C for 10 minutes and

each DNA pellet was washed with 500µL of ice-cold 100% ethanol. Samples were then centrifuged at 10,000 × *g* and 4°C for 10 minutes. The supernatant was again discarded. The washing process was repeated twice with ice-cold 70% ethanol. Clean precipitated DNA samples were placed in an Eppendorf Vacufuge Plus for 45 minutes to allow evaporation of residual ethanol. The DNA pellets were resuspended with 50µL of distilled water and allowed to dissolve at 4ºC.

DNA sample storing

Once DNA was extracted and resuspended in distilled water, aliquots were prepared so as to keep one aliquot to perform pathogen detection and verification of tick species. The other aliquots were shipped to the United States Department of Agriculture, Agricultural Research Services (USDA-ARS) Cattle Fever Tick Research Laboratory (CFTRL) as vouchers for further studies. All DNA samples were stored at -20ºC until further used.

Confirmation of Tick Species

To confirm the results of morphologically identified tick species, primers specific to the *16S rRNA* gene were used as previously described (Black. W.C., Piesman J., 1994). Briefly, 3 µl of extracted DNA (>100 ng/µL) was used as template DNA in a 25µL PCR reaction containing 10pM forward primer 16S+1(5'CTGCTCAATGATTTTTTAAATTGCTGTGG3'), 10pM reverse primer 16S-1 (5'CCGGTCTGAACTCAGATCAAGT3'), 12.5µL of Supermix 2X (AccuStart II PCR Mix, Quanta Inc.) and 7.5µL of molecular grade distilled water. Molecular grade distilled water was also used as a negative control. The PCR reaction was performed in an Eppendorf MasterCycler Pro following the protocol: 95ºC for 5 minutes followed by 10 cycles of denaturalization at 92ºC for 1 minute, annealing at 48ºC for 1 minute and extension at 72ºC for 1.5 minutes. This was followed by 32 cycles of denaturalization at 92°C for 1 minute; annealing

at 54°C for 35 seconds; and extension at 72°C for 1.5 minutes. A final extension was carried out for 7 minutes at 72°C. Upon completion, PCR reactions were stored at 4ºC. All amplicons were separated in 2% agarose gel at 70 volts for 5 hours. Bands were visualized using a ChemiDoc™ Touch gel imager (BioRad Laboratories, Inc.) excised, cleaned using the Gel and PCR purification kit (Promega Inc.), and submitted for sequencing at Eurofins Genomics LLC as described below. Sequences were analyzed using MacVector© 17 and MacVector Assembler© (MacVector, Inc.) as described below.

Testing

Horse and dog sample testing

After identification and DNA extraction, the 82 horse premises and 4 dog premises were screened using the patent pending TickPath Layerplex qPCR technology (patent application No. 16/130,177) to amplify regions specific to the *Borrelia burgdorferi flaB, B. turicatae bipA, B. parkeri flaB, B. hermsii flaB* (genomic groups I and II), *Ehrlichia canis 16S rRNA, E. chaffeensis 16S rRNA, Anaplasma phagocytophilum msp2, Rickettsia rickettsii Rhhyp* genes, and pan-specific *Babesia* spp. *18SrRNA* gene (*B. canis, B. vogeli, B. gibsoni, B. bovis, B. microti, B. caballi*) as previously described (Modarelli et al., 2019a). The sensitivity and specificity values (and 95% confidence intervals) are 100% (86.8–100%) and 99.8% (99.4–99.9%) for the borrelial layer 100% (90.5–100%) and 99.1% (98.4–99.5%) for the rickettsial layer, and 100% (47.8–100%) and 100% (99.7–100%) for the *Babesial* layers. The qPCR was performed in a BioRad CFX 96 system (BioRad Laboratories, Inc.) following a thermal cycle of 95^{O}C for 3 min (1 cycle), and 40 cycles of amplification at 95^{O}C for 10 sec and 60^{O}C for 45 sec. Samples with a quantification cycle (Cq) ≤ 36.0 cycles were considered positive. The positive samples were then confirmed using conventional PCR.

Confirmation of *Borellia* spp.

To confirm the presence of *Borrelia* spp. in the tested samples, we used primers specific to the *16S rRNA* gene as previously described (Bunikis et al., 2004). Briefly, 3μL of sample DNA (>100 ng/μL) was used as template DNA in a 25μL PCR reaction containing 10pM of forward primer rrs-rrlA-F (5' GGTATTTAAGGTATGTTTAGTGAG3') and 10pM of reverse primer rrs-rrlA-R (5'GGATCATAGCTCAGGTGGTTAG3'), 12.5μL of Supermix 2x (AccuStart II PCR Mix, Quanta Inc.), and 7.5μL of molecular grade distilled water. Molecular grade distilled water was also used as a negative control and *Borellia* spp. genomic DNA was used as a positive control. The PCR reaction was performed in an Eppendorf MasterCycler Pro using the following protocol: an initial denaturalization step for 3 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 1 minute at 74°C. Upon completion, PCR products were stored at 4°C.

Confirmation of *Rickettsia* spp.

To confirm the presence of *Rickettsia* spp. in the suspected positive samples, we used 4 oligonucleotide primers specific to the *16S rRNA* gene for the PCR and nested PCR as previously described (Dawson et al., 1994; Anderson et al., 1992). In the first PCR reaction, 3μL of sample DNA (>100ng/μL) was used as template DNA in a 25μL PCR reaction containing 10pM of forward primer ECC (5'AGAACGAACGCTGGCGGCAAGCC3') and 10pM of reverse primer ECB (5'CGTATTACCGCGGCTGCTGGC3'), 12.5μL Supermix 2x (AccuStart II PCR Mix, Quanta Inc.), and 7.5μL molecular grade distilled water. Molecular grade distilled water was also used as a negative control and *Ehrlichia chaffeensis* genomic DNA was used as a positive control. The PCR reaction was performed in an Eppendorf MasterCycler Pro following the protocol: 5 minutes at 94°C, followed by 40 cycles of 1 minute at 94°C, 1

minute at 60°C, 1 minute at 72°C. Upon completion, PCR products were stored at 4°C. In the second nested PCR, 1μL of the primary PCR product was used as template DNA for a 25μL PCR reaction containing 10pM of forward nested primer HE-1 (5'CCATTGCTTAT-AACCTTTTGGTTATAAAT3') and 10pM of reverse nested primer HE-3 (5'ACGCGCGGCC-GCTATAGGTACCGTCAT3'), 12.5μL of Supermix 2x (AccuStart II PCR Mix, Quanta Inc.), and 9.5μL molecular grade distilled water. PCR reaction was performed in an Eppendorf MasterCycler Pro following the protocol: 5 minutes at 94°C, followed by 40 cycles of 1 minute at 94°C, 1 minute at 60°C, 1 minute at 72°C. Upon completion, PCR products were stored at 4°C.

Confirmation of *Babesia/Theileria* spp. in horse premises

Finally, the presence of *Babesia caballi* and/or *Theileria equi* was confirmed using primers specific to the *BC48* gene and the *merozoite antigen 1 (EMA-1)* gene respectively, as previously described (Battsetseg et al., 2001). For the detection of *Babesia caballi,* 4 oligonucleotide primers were used for the PCR and nested PCR. In the first reaction, 3μL of sample DNA (>100ng/μL) was used as template DNA for a 25μL reaction containing 10pM of forward primer BC48F1 (5'-ACGAATCCCACAACAGCCGTGTT-3') and 10pM of reverse primer BC48R3 (5'-ACGAATTCGTAAAGCGTGGCCATG-3'), 12.5μL Supermix 2x (AccuStart II PCR Mix, Quanta Inc.), and 7.5μL molecular grade distilled water. Molecular grade distilled water was used as a negative control and *B. caballi* genomic DNA was used as a positive control. The PCR reaction was performed in an Eppendorf MasterCycler Pro following the protocol: 4 minutes at 96°C, followed by 40 cycles of 1 minute at 94°C, 2 minutes at 56°C, 2 minutes at 72°C, followed by a final extension of 5 minutes at 72°C. In the second PCR, the reaction mixture and amplification conditions were the same as in the primary PCR reaction,

except for the primers and template DNA. For the second reaction, 1µL of primary PCR product was used as template DNA, along with 10pM of forward nested primer BC48F11 (5'-GGGCGA-CGTGACTAAGACCTTATT-3') and 10pM of reverse nested primer BC48R31 (5'-GTTCTCA-ATGTCAGTAGCATCCGC-3'). For the detection of *Theileria equi*, 3µL of sample DNA (>100ng/µL) was used as a template in a 25µL PCR reaction containing 10pM of forward primer EMA5 (5'-TCGACTTCCAGTTGGAGTCC-3') and 10pM of reverse primer EMA6 (5'AGCTCGACCCACTTATCAC-3'), 12.5µL Supermix 2x (AccuStart II PCR Mix, Quanta Inc.). Molecular grade distilled water was used as a negative control and *T. equi* genomic DNA was used as a positive control. The PCR reaction was performed in an Eppendorf MasterCycler Pro following the protocol: 4 minutes at 96°C, followed by 40 cycles of 1 minute at 94°C, 2 minutes at 56°C, 2 minutes at 72°C, followed by a final extension of 5 minutes at 72°C. Upon completion, PCR products were stored at 4°C.

All amplicons were separated in 2% agarose at 70Volts for 5 hours. Bands were visualized using a ChemiDoc™ Touch gel imager (BioRad Laboratories, Inc.), excised, cleaned and submitted for Sanger sequence as described below.

Cattle sample testing

A total of 95 premises originating from cattle farms were screened for both *Anaplasma/Ehrlichia* spp. and *Babesia* spp. using different molecular techniques.

Detection of *Babesia* spp.

The 95 Cattle premises were screened for the presence of *Babesia* spp. using a Piroplex Real-Time PCR protocol targeting gene regions specific to *Babesia bovis* and *Babesia bigemina* developed by our team. Briefly, a duplex quantitative real time PCR was performed using Bbov.18S.472-F and Bbov.18S.546-R primers, and the probe Bbov.18S.503(FAM)-P to detect *B.*

bovis, together with Bbig.18S.1462-F and Bbig.18S.1509-R primers, and the probe Bbig.18S.1485(HEX)-P to detect *B. bigemina* in a single reaction (Table 2), using 2x iQ Multi Powermix (BioRad Laboratories, Inc.). The qPCR was performed in a BioRad CFX 96 system (BioRad Laboratories, Inc.) following a thermal cycle of 95^{O}C for 3 min (1 cycle), and 40 cycles of amplification at 95^{O}C for 10 sec and 60^{O}C for 45 sec. Samples with a quantification cycle (Cq) ≤ 36.0 cycles were considered positive. The positive samples were then confirmed using conventional PCR.

Confirmation of Babesia spp*. in cattle premises*

Samples positive and suspected positive for *Babesia* spp. were confirmed using conventional PCR. Primers targeting the *18SrRNA* gene were used in an initial PCR to replicate genus wide *Babesia* spp. as previously described (Sogin., 1990). Briefly, 3µL of sample DNA (>100ng/µL) was used as a template in a 25µL reaction containing 10pM of the forward primer A (5'-ACCTGGTTGATCCTGCCAG-3'), 10pM of the reverse primer B (5′-GATCCTTCTGCAGGTTCACCTAC-3′), 12.5µL of Supermix 2x (AccuStart II PCR Mix, Quanta Inc.) and 7.5µL of molecular grade distilled water. Molecular grade distilled water was used as a negative control and *B. bovis* and *B. bigemina* genomic DNA served as the positive controls. The PCR reaction was performed in an Eppendorf MasterCycler Pro following the protocol: initial denaturation at 94°C for 3 minutes, followed by 45 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 2 minutes, followed by a final extension at 72°C for 2 minutes.

For the samples positive for *B. bovis,* primers specific for this species were used in a nested PCR reaction as previously described (Pollard., 2017). Briefly, 1µL of the primary PCR product using A and B primers was used as a template in a 25µL reaction containing 10pM of

the forward primer Bbov660F (5'-GCCTGTATAATTGAGCATGG-3'), 10pM of the reverse primer Bbov1330R (5'-CAAGCATCAGTGTAGCG-3'), 12.5µL Supermix 2x (AccuStart II PCR Mix, Quanta Inc.), and 9.5µL molecular grade distilled water. Molecular grade water also served as the negative control for this reaction. The PCR reaction was performed in an Eppendorf MasterCycler Pro following the protocol: initial denaturation at 94°C for 2 minutes, followed by 45 cycles of 94°C for 15 seconds, 50°C for 30 seconds, 72°C for 1.5 minutes, followed by a final extension at 72°C for 7 minutes.

For the samples positive for *B. bigemina*, primers specific for this species were used in a nested PCR reaction as previously described (Holman et al., 2011). The reaction materials and amplification conditions were the same as the *B. bovis* specific reaction described above except for the primers used. This reaction contained 10pM of the forward primer Bbig200F (5'-GCGTTTATTAGTTCGTTAACC-3') and 10pM of the reverse primer Bbig1400R (5'ACAGGACAAACTCGATGGATGC-3'). Positive amplicons were separated by gel electrophoresis using a 2% agarose gel at 70V for 5 hours. Bands were visualized using a ChemiDoc™ Touch gel imager (BioRad Laboratories, Inc.), excised, cleaned and submitted for Sanger sequence as described below.

Detection of *Anaplasma* spp.

Conventional PCR was used to screen all 95 cattle samples for *Anaplasma* spp. Primers targeting the *16S rRNA* gene as previously described (Silaggi et al., 2017; Yu et al., 2020) were used in a 25µL PCR reaction containing 3µL of sample DNA (>100ng/µL), 5pM of the forward primer 16S-F (5'-CAGAGTTTGATCCTGGCTCAGAACG-3'), 5pM of the reverse primer 16S-R (5'-GAGTTTGCCGGGACTTCTTCTGTA-3'), 12.5µL Supermix 2x (AccuStart II PCR Mix, Quanta Inc.) and 7.5µL of molecular grade distilled water. Molecular grade distilled water was

also used as the negative control and *Anaplasma marginale* genomic DNA was used as a positive control. The PCR reaction was performed in an Eppendorf MasterCycler Pro following the protocol: initial denaturation at 95°C for 2 minutes, followed by 45 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes. Following completion of the reaction, PCR products were stored at 4°C. Positive amplicons were separated by gel electrophoresis using a 2% agarose gel at 80V for 5 hours. Bands were visualized using a ChemiDoc™ Touch gel imager (BioRad Laboratories, Inc.), excised, cleaned and submitted for Sanger sequence.

All primers and probes utilized in this study are summarized in Table 2 (gel-based PCR) and Table 3 (quantitative real time PCR), except those used in the TickPath Layerplex qPCR, which are provided in the manuscript (Modarelli et al., 2019a) and the patent application (#16/130,177)).

Table 2. Primers utilized for confirmatory PCR testing

Pathogen	**Gene Target**	**Primers**	**Primer Sequence**	**Reference**
Anaplasma spp.	rrs	16SANAF 16SANAR	5'-CAGAGTTTGATCCTGGCTCAGAACG-3' 5'-GAGTTTGCCGGGACTTCTTCTGTA-3'	Silaggi et al. (2017)
Borellia spp.	rrs-rrlA (23S/5S rRNA genes)	rrs-rrlA-F rrs-rrlA-R	5'-GGTATTTAAGGTATGTTTAGTGAG-3' 5'-GGATCATAGCTCAGGTGGTTAG-3'	Bunikis et al. (2004)
Ehrlichia/ Anaplasma spp.	16S rRNA	ECC ECB	5'-AGAACGAACGCTGGCGGCAAGCC-3' 5'-CGTATTACCGCGGCTGCTGGC-3'	Dawson et al. (1994)
Ehrlichia chaffeensis	16S rRNA	HE1 HE3	5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3' 5'-TATAGGTACCGTCATTATCTTCCCTAT-3'	Anderson et al. (1992)
Babesia caballi	BC48 (RAP-1)	BC48F1 BC48F3 BC48F11 BC48R31	5'-ACGAATTCCCACAACAGCCGTGTT-3' 5'-ACGAATTCGTAAAGCGTGGCCATG-3' 5'-GGGCGACGTGACTAAGACCTTATT-3' 5'-GTTCTCAATGTCAGTAGCATCCGC-3'	Battsatseg et al. (2001)
Theileria equi	Merozoite antigen 1 (EMA-1)	EMA-5 EMA-6	5'-TCGACTTCCAGTTGGAGTCC-3' 5'-AGCTCGACCCACTTATCAC-3'	Battsatseg et al. (2001)
Babesia spp.	18SrRNA	A B	5'-ACCTGGTTGATCCTGCCAG-3' 5′-GATCCTTCTGCAGGTTCACCTAC-3′	Sogin (1990)
Babesia bovis	18SrRNA	Bbov660F Bbov1330R	5'-GCCTGTATAATTGAGCATGG-3' 5'-CAAGCATCAGTGTAGCG-3'	Pollard (2017)
Babesia bigemina	18SrRNA	Bbig200F Bbig1400R	5'-GCGTTTATTAGTTCGTTAACC-3' 5'ACAGGACAAACTCGATGGATGC-3'	Holman et al. (2011)

Table 3. Primers utilized for Real-Time qPCR testing

Pathogen	**Gene Target**	**Primers**	**Primer Sequence**
Babesia bovis/bigemina	18SrRNA	Bbov.18S.472F / Bbov.18S.546R Bbig.18S.1462F/Bbig .18S.1509R	5'- ACCAATACGGGGCTACTGCTC- 3' 5′- GCCCTCCAATGGGTACTCG -3′ 5'-GCGCGCTACACTGATGCATC-3' 5'-CGCTGCACACTAAAGATTACCCAAC - 3'

Purification of Positive Amplicons

Positive amplicons were excised using sterile x-tracta tools (Sigma-Aldrich Inc.). The extracted gel slices were then cleaned using the Wizard® SV Gel and PCR Clean-Up Kit (Promega, Inc.) following manufacturer's recommendations. Briefly, excised gel slices were deposited into clean 1.5mL microcentrifuge tubes. Ten μL of Membrane Binding Solution was added to each tube per 10mg of gel slice. Tubes were then incubated at 60°C until the gel slices were completely dissolved. SV Minicolumns were inserted into collection tubes and the dissolved contents of each sample tube were transferred into a Minicolumn. The columns were then incubated for 1 minute at room temperature and centrifuged at 16,000 × *g* for 1 minute. The flow through was discarded and the Minicolumn was reinserted into the collection tube followed by the addition of 700μL of Membrane Wash Solution to each tube. The samples were recentrifuged at 16,000 × *g* for 1 minute and the flow through was again discarded. Five hundred μL of Membrane Wash Solution was added to each tube and the samples were centrifuged at 16,000 × *g* for 5 minutes. The flow through was discarded and the samples were centrifuged for an additional 1 minute with the microcentrifuge lid off to allow evaporation of residual ethanol. Each Minicolumn was transferred to a clean 1.5 mL microcentrifuge tube and 30μL of Nuclease-Free Water was added. The samples were incubated at room temperature for 1 minute then centrifuged at 16,000 × *g* for 1 minute. The Minicolumn was discarded and the collected DNA was stored at 4°C then submitted for confirmation using Sanger sequencing (Eurofin Genomics, LLC.).

Sequence analysis

Once the positive samples were excised and purified, they were sent to Eurofin Genomics, LLC for Sanger sequencing. Along with the samples, aliquots of forward and reverse primers were also submitted. Once the results were received, MacVector© and MacVector Assembler© version 17.0.5 (MacVector Inc.) was used to clean and analyze the sequences. Forward and reverse sequences were aligned, and the consensus sequence obtained in Assembler© was compared to other sequences found in the online tool Basic Local Alignment Search Tool (BLAST®). All new sequences have been submitted to GenBank® and will be available upon publication of this thesis.

CHAPTER III

RESULTS

Tick Species Identification

The results of the morphological identification of ticks in each premise are as follows: From the 82 premises originating from horses, 42 were identified as morphologically similar to the tick species *Dermacentor albipictus* and 40 as *Dermacentor nitens*. From the 95 combined cattle premises, 94 were morphologically identified as the tick species *Rhipicephalus microplus* and 1 premise as the species *Rhipicephalus annulatus*. All ticks collected from dogs were each morphologically identified as *Rhipicephalus sanguineous*, the brown dog tick. Genetic confirmation of tick species using PCR reactions targeting the *16S rRN*A gene revealed that, despite morphological resemblances to *Dermacentor albipictus*, the ticks in horse premises randomly selected for PCR analysis were genetically similar to *Dermacentor nitens*. Due to the unforeseen events surrounding the COVID-19 virus in spring 2020, species level genetic confirmation of the single cattle premise containing ticks with morphological similarities to *Rhipicephalus annulatus* was unavailable at the time of publication for this thesis. Thus, all 82 horse premises were determined to be infested with ticks of the species *Dermacentor nitens*, and the 95 cattle samples were determined to contain 94 premises infested with *Rhipicephalus microplus* and one premise infested with *Rhipicephalus* spp. A summary of these results can be found in Table 4.

Table 4. Tick species detected in this study

Tick species	**Premises (n)**
Rhipicephalus (Boophilus) microplus	94
Rhipicephalus spp.	1
Dermacentor nitens	82
Rhipicephalus sanguineus	5

Pathogen Screening Results

TickPath Layerplex qPCR Results

Preliminary screening of the 82 horse premises using TickPath Layerplex qPCR technology showed the following: 8/82 (9.8%) positive for *Babesia* spp., 2/82 (2.4%) positive for *Rickettsia* spp., and 1/82 (1.2%) positive for *Borellia* spp. Confirmation via conventional PCR using the procedures described above yielded species level confirmation of the 8 horse premises positive for *Babesia* spp. Sequencing results concluded the presence of *Babesia caballi* in all 8 suspected positive premises (GenBank® accession numbers MT277577-MT277584). For the suspected rickettsial and borrelial positives, conventional PCR using the methods described above revealed the presence of commensal organism DNA and thus, the premises were recorded as negative. A summation of the pathogen presence in the 82 sampled horse premises is displayed in Table 5. TickPath Layerplex qPCR was also used to screen the premises collected from dogs located in close proximity to the livestock. Of the 5 premises collected and identified, one premise contained only male, non-engorged ticks, and thus was not included in the premises screened for pathogens. The remaining 4 dog premises were found negative for *Borellia spp*., *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp. and *Babesia spp*.

Table 5. Summation of the results of pathogens detected using the TickPath LayerPlex Real-Time quantitative PCR in horse premises.

Pathogen	**Premises (n) found positive by qPCR***	**Premises (n) confirmed via sequencing PCR***
Babesia spp.	8/82 (9.8%)	8/82 (9.8%)
Rickettsia spp.	2/82 (2.4%)	N/D
Borellia spp.	1/82 (1.2%)	N/D

* Positive samples/total samples tested. In parenthesis is represented the percent samples positive in this study. ND: Not Detected, samples were not confirmed to carry a pathogen.

Cattle pathogen detection

Preliminary screening of the 95 cattle premises using the methods described above yielded the following results. Overall, 23/95 (24.2%) were positive for *Babesia* spp. Of these 23 positive premises, 11 were detected as *Babesia bovis*, 5 as *Babesia bigemina*, and 7 as co-infections with both, *B. bovis* and *B. bigemina*. Confirmation of these suspected positives using conventional PCR procedures as described above revealed the following: of the 11 premises preliminarily found positive for *B. bovis*, 5 were confirmed (GenBank® accession numbers MT253097-MT253100, MT253102), of the 5 premises preliminarily found positive for *B. bigemina*, one was confirmed (GenBank® accession number MT253104), lastly, of the 7 premises preliminarily found to be co-infected with both *B. bovis* and *B. bigemina*, one premise was confirmed to be infected with both pathogens (GenBank® accession numbers MT253101 and MT253105), while two others were only able to be confirmed to be infected with *B. bigemina* at the time of this publication (GenBank® accession numbers MT253106 and MT253107). As stated above, the unforeseen events surrounding the COVID-19 virus in spring 2020 led confirmation of the remaining premises found preliminarily positive for *Babesia* spp. to be unavailable at the time of the publication of this thesis. These results are summarized below

in Figure 1 and Table 6, and a list of all GenBank® accession numbers can be found in Appendix A.

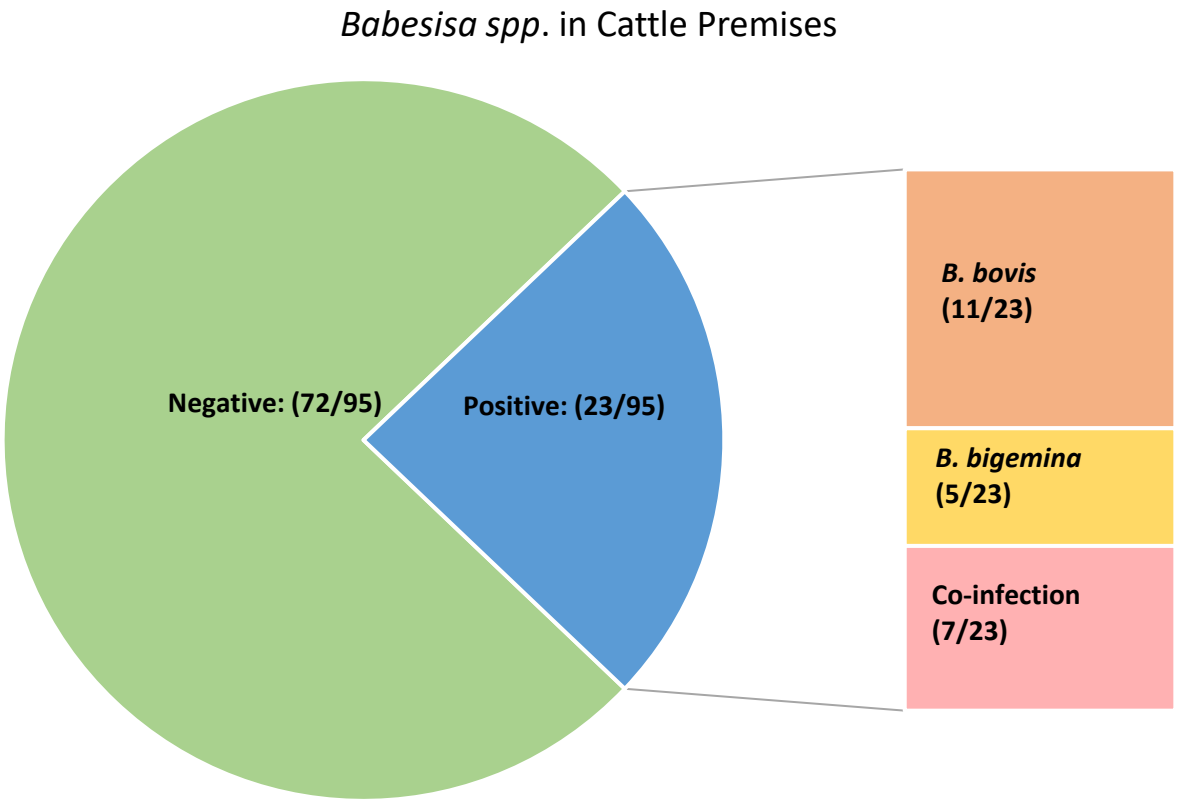

Figure 1. Summary of preliminary qPCR detection of *Babesia* spp. in 95 total cattle premises.

Table 6. Summary of confirmed *Babesia* spp. in the 23 positive cattle premises

Pathogen	**Premises (n) found positive by qPCR**	**Premises (n) confirmed via sequencing PCR**
B. bovis	11	5
B. bigemina	5	1
Co-infection	7	1: Confirmed to be infected with both *B. bovis* and *B. bigemina* 2: Only confirmed to be infected with *B. bigemina*.

Anaplasma spp. *conventional PCR results*

Conventional PCR was used in the detection of *Anaplasma* spp. in all 95 cattle premises. The results are as follows: 77/95 (81.1%) were positive for *Anaplasma* spp. Of these 77 positive premises, 38 were sequenced. Within these 38 sequenced samples, 30 were confirmed as *Anaplasma marginale* (78.9%, GenBank® accession numbers MT253597-MT253626), 3 as *Anaplasma platys*-like (7.9%, GenBank® accession numbers MT253584-MT253586), and 5 as unspecified *Ehrlichia* spp. (13.2%, GenBank® accession numbers MT252985-MT252989). Through further PCR analysis as described above, we were not able to determine the exact *Ehrlichia* species present in these engorged ticks. These results are summarized in Figure 2.

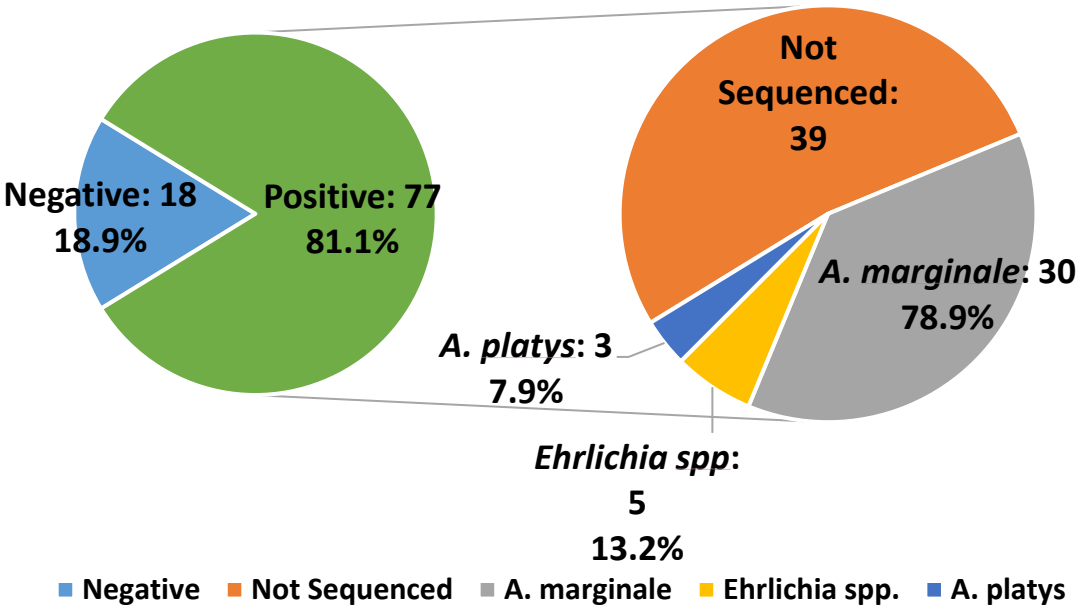

Figure 2. Summary of *Anaplasma/Ehrlichia* spp. detected in the 95 total cattle premises

CHAPTER IV

CONCLUSION

Overall Findings in Cattle

The findings of this study suggest a large presence of *Babesia* spp. and *Anaplasma* spp. in Puerto Rican Cattle herds, 24.2% and 81.1% respectively. Previous studies conducted in the Caribbean have reported similar findings in regards to TBP presence (Gondard et al. 2017., Li et al. 2015). Additionally, a study conducted in 2007 analyzed the seroprevalence of antibodies against *B. bovis* in Puerto Rican dairy cattle and found the overall seroprevalence to be 24.5% (Urdaz-Rodriquez. 2007). The same study also analyzed the seroprevalence of antibodies against *A. marginale* in Puerto Rican dairy cattle and found the overall seroprevalence to be 30.8% (Urdaz-Rodriquez. 2007). While this estimate of *A. marginale* presence is significantly lower than the findings of this study, there are several differences that must be taken into consideration. First, this study was aimed at evaluating genus wide presence of *Anaplasma* spp., rather than focusing on *A. marginale*. Second, our study included samples from both dairy and beef cattle, rather than focusing specifically on the animals within the dairy industry. Third, many of the premises of this study are pools of ticks from several animals, which increases the likelihood of sampling an animal infected with the pathogen. Additionally, other studies conducted on 8 neighboring islands in the Caribbean have found seroprevalences of antibodies against *A. marginale* and *Babesia* spp. ranging from 17 to 71% (Camus et al. 1994). Furthermore, our study is detecting the presence of the pathogen in engorged ticks feeding on animals. This is significantly different from serological studies, in which exposure to the pathogen is evaluated based on the animals immune response to the target pathogen, thus, animals that were exposed at

early age might not react to the pathogen using serological tests. Overall, our study's findings are in accordance with what has been previously observed in Puerto Rico, and therefore, we can confirm that both Anaplasma and Babesial pathogens are of significance in the health management of livestock in the island.

The detection of *Anaplasma platys*-like organisms in cattle is a finding of particular interest. *Anaplasma platys* is generally thought to be limited to canine hosts, although there have been a few reports of infestations in cattle (Chien et al., 2019, Dahmani et al., 2015). A previous study conducted in our own lab has shown similar findings of *A. platys*-like organisms infecting Puerto Rican cattle. In this previous study, the *A. platys*-like samples showed a greater genetic similarity to other *A. platys*-like samples found in cattle rather than the *A. platys* typically found in canine hosts (Mabizari., 2019). In both studies, only a segment of the *16S rRNA* gene was used to identify the pathogen, and the organism's entire genome was not sequenced. Thus, it is unclear how similar the *A. platys*-like samples are to the well-known *A. platys* that infects dogs. Because of this, the term "*A. platys*-like" is a more accurate description of the pathogen detected in cattle. These studies are the first reported cases of *A. platys*-like organisms found in Puerto Rican cattle. The implications of these results are not known, and more research is needed to understand if the presence of this pathogen has any impact on animal health, and therefore Puerto Rican livestock production as a whole.

The detection of *Ehrlichia* spp. is an interesting, but inconclusive finding. The molecular markers used in this study were unable to identify the pathogen beyond the genus level. It is possible that the organisms detected are a commensal part of the tick's microbiome, and thus the findings in this study cannot conclusively report more information than the fact that we have detected *Ehrlichia* spp. in engorged ticks collected from Puerto Rican cattle. However, it is

worth noting that similar studies using FRET-PCR on blood samples from cattle on four Caribbean islands have reported findings of an unspecified *Ehrlichia* spp. that shows similarities to *E. canis* (Zhang et al. 2015). Additionally, other studies conducted on *R. microplus* ticks collected from cattle in Brazil and French Polynesia have detected a novel cattle pathogen *Ehrlichia minasensis,* which has been shown to be closely related to *E. canis* (Cabezas-Cruz et al., 2012; Aguiar et al. 2014; Laroche et al. 2016; Cabezas-Cruz et al., 2016). Additional research is needed to further evaluate the presence of this unidentified *Ehrlichia* spp. in Puerto Rican cattle before conclusions can be drawn about its identity and impact on animal wellbeing.

Overall Findings in Horses and Dogs

The findings of this study suggest a notable presence of *B. caballi* (9.8%) in Puerto Rican horses. These results are not surprising since this pathogen is considered to be endemic in most countries with tropical and subtropical environments (Gondard et al., 2017). In fact, the detection level found in our study is on the lower side when compared with what has been reported in horses from neighboring Caribbean islands. A previous study conducted on thoroughbreds in Trinidad detected seroprevalences of 33.3% for *T. equi* and 68.8% for *B. caballi* (Asgarali et al., 2007). Accurate data regarding the prevalence of *B. caballi* and *T. equi* is important to preventing the spread of the pathogen via asymptomatic carriers to disease-free areas (Gondard et al., 2017; Wise et al., 2013). Thus, studies focused specifically on the presence of *B. caballi* and *T. equi* in Puerto Rican horses are needed to confirm the lower prevalence reported in this study, and better inform efforts to reduce the transmission of the disease to non-endemic areas.

This study also included five premises opportunistically collected from dogs located in close proximity to livestock. Of these five premises, four were screened for pathogens in the

genera *Babesia* spp., *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp., and *Borellia* spp. All four of the premises were found negative for pathogens within these genera. However, due to the small sample size and casual sampling, these results are not indicative of the actual prevalence of TBPs in Puerto Rican dogs. A larger study, with a focus on evaluating the presence of canine TBPs is needed to draw conclusions and inform stakeholders of the impact of these pathogens on canine and human health.

A "One Health" Approach to TBDs

As repeatedly noted, TBPs and the diseases they cause are incredibly important to both the world's livestock industry and human and animal health in general. Accurate data regarding their presence is essential to alleviating economic burdens faced by the agricultural industry within Puerto Rico and, in a broader scope, the global livestock industry as a whole. The concept of a "One Health" global health strategy has been presented as a tool for management of these diseases (Gondard et al., 2017; Dantas-Torres et al., 2012). This concept stresses the importance of integrated human, animal, and environmental health and encourages communication between veterinarians and physicians. The hope is that this unified approach will accelerate advancements in the diagnosis, treatment, and prevention of TBDs (Dantas-Torres et al. 2012). Because of the known impact of TBPs on the Puerto Rican livestock industry, the results of this study are relevant to understanding the extent to which these diseases are affecting livestock. This knowledge will aid in informing strategies to control tick infestation, and therefore the diseases themselves, leading to increased quality of both beef and milk production in the island.

In conclusion, this study has provided molecular identification of *Anaplasma* spp. and *Babesia* spp. species in Puerto Rican livestock. It has additionally confirmed the presence of a novel "*A. platys*-like" species in Puerto Rican Cattle. The presence of this species is cause for

additional studies on the epidemiological status of *Anaplasma* spp. in Puerto Rico. This study has also highlighted both the need for additional surveys of *B. caballi* and *T. equi* presence in Puerto Rican horses, and surveys focused on the presence of TBPs in dogs located in close proximity to livestock and humans.

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APPENDIX A

Table 5: Samples and GenBank® Accession Numbers

Premise:	**Host**	**Species:**	**GenBank® Accession Number:**
PRTS002	Beef Cattle	*Anaplasma marginale*	MT253597
PRTS003	Beef Cattle	*Anaplasma marginale*	MT253598
PRTS004	Beef Cattle	*Ehrlichia spp.*	MT252985
PRTS006	Beef Cattle	*Anaplasma marginale*	MT253599
PRTS007	Beef Cattle	*Anaplasma marginale*	MT253600
PRTS009	Beef Cattle	*Anaplasma marginale*	MT253601
PRTS010*	Beef Cattle	*Anaplasma platys* *Babesia bigemina*	MT253584 MT253104
PRTS015	Beef Cattle	*Anaplasma marginale*	MT253602
PRTS016	Beef Cattle	*Anaplasma marginale*	MT253603
PRTS017	Beef Cattle	*Anaplasma marginale*	MT253604
PRTS018	Beef Cattle	*Anaplasma platys*	MT253585
PRTS020	Dairy Cattle	*Anaplasma marginale*	MT253605
PRTS021	Beef Cattle	*Ehrlichia spp.*	MT252986
PRTS027	Beef Cattle	*Ehrlichia spp.*	MT252987
PRTS031	Beef Cattle	*Anaplasma marginale*	MT253606
PRTS032	Beef Cattle	*Anaplasma marginale*	MT253607
PRTS033	Beef Cattle	*Anaplasma marginale*	MT253608
PRTS035	Beef Cattle	*Anaplasma marginale*	MT253609
PRTS036	Beef Cattle	*Anaplasma marginale*	MT253610
PRTS039	Beef Cattle	*Anaplasma marginale*	MT253611
PRTS040	Beef Cattle	*Anaplasma marginale*	MT253612
PRTS047	Beef Cattle	*Anaplasma marginale*	MT253613
PRTS049	Beef Cattle	*Anaplasma marginale*	MT253614
PRTS052	Beef Cattle	*Anaplasma marginale*	MT253615
PRTS061	Beef Cattle	*Ehrlichia spp.*	MT252988
PRTS062	Beef Cattle	*Babesia bovis*	MT253097
PRTS079	Beef Cattle	*Anaplasma marginale*	MT253616
PRTS103	Dairy Cattle	*Anaplasma marginale*	MT253617
PRTS104*	Dairy Cattle	*Anaplasma marginale* *Babesia bovis*	MT253618 MT253098
PRTS110	Beef Cattle	*Anaplasma marginale*	MT253619
PRTS111	Beef Cattle	*Babesia bovis*	MT253099
PRTS129*	Beef Cattle	*Ehrlichia spp.* *Babesia bovis*	MT252989 MT253100
PRTS145	Beef Cattle	*Anaplasma marginale*	MT253620
PRTS146	Beef Cattle	*Anaplasma marginale*	MT253621
PRTS150*	Beef Cattle	*Babesia bovis* *Babesia bigemina*	MT253101 MT253105

PRTS158	Beef Cattle	*Anaplasma marginale* *Babesia bigemina*	MT253622 MT253106
PRTS175	Beef Cattle	*Anaplasma marginale*	MT253623
PRTS179	Beef Cattle	*Anaplasma marginale*	MT253624
PRTS180*	Beef Cattle	*Anaplasma marginale* *Babesia bigemina*	MT253625 MT253107
PRTS181*	Beef Cattle	*Anaplasma platys* *Babesia bovis*	MT253586 MT253102
PRTS183	Beef Cattle	*Anaplasma marginale*	MT253626
PRTS072	Horse	*Babesia caballi*	MT277577
PRTS099	Horse	*Babesia caballi*	MT277578
PRTS100	Horse	*Babesia caballi*	MT277583
PRTS116	Horse	*Babesia caballi*	MT277579
PRTS117	Horse	*Babesia caballi*	MT277580
PRTS123	Horse	*Babesia caballi*	MT277581
PRTS133	Horse	*Babesia caballi*	MT277582
PRTS148	Horse	*Babesia caballi*	MT277584

* Premises infected with more than one pathogen.