PREVALENCE OF *ANAPLASMA SPP.* INFECTION IN PUERTO RICAN CATTLE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Prevalence of *Anaplasma spp.* in Blood Samples of Puerto Rican Cattle.

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Anaplasma spp., the causative agent of Anaplasmosis in bovine species, is a rickettsial parasite that is transmitted through tick-vectors. The species of Anaplasma that are known to infect cattle are *Anaplasma centrale*, *Anaplasma marginale*, and *Anaplasma phagocytophilum*. Of the three, *A. marginale* and *A. phagocytophilum* infections are usually the most significant and acutely detrimental to the health and production of the cattle population. Once infected, individuals remain carriers for life. This study aims to evaluate the prevalence of the various strains of *Anaplasma spp.* in blood samples of Puerto Rican Cattle. Collaboration with the USDA Cattle Tick Fever Research Laboratory provided our team with extracted DNA from Puerto Rican Cattle blood samples. Using conventional polymerase chain reaction (PCR), the 16S rRNA gene region was amplified from 198 cattle-DNA samples. After the samples were screened for positive *Anaplasma spp*., the positive amplicons were then sequenced and analyzed using the "Basic Local Alignment Search Tool" (BLAST®) for the various species. The analysis of these samples will allow for better understanding of the diversity of the parasite in the tested Puerto Rican area and may serve as a paradigm for future research that may promote ecological control of Anaplasmosis and formation of effective preventive technique

DEDICATION

I dedicate this thesis to my research advisor, Dr. Maria Esteve-Gasent, who has gone above and beyond to provide me with a positive research experience. With her mentorship, I have been able to grow not only as a researcher but as a human. I cannot thank her enough for her guidance and patience even during the hardest times, and for her continued trust and encouragement. I would not have been able to succeed as much as I have this year at Texas A&M without her.

Dr. Esteve, I will forever be in your gratitude and I want you to know that you are so much more than a professor to me. I look forward to many years of updates and conversations no matter where the world takes us. From the bottom of my heart, thank you.

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Finally, thanks to my mother and father for their encouragement, interest, and support in my research. Dad, I cannot wait to tell you all about when you get better.

CHAPTER I

INTRODUCTION

Bovine anaplasmosis, also known as "yellow fever", is a non-contagious vector borne, infectious disease that commonly occurs in tropical and subtropical areas around the globe.[1,2,3,4] Despite its global occurrence, this disease is endemic in cattle only in Mexico, Central America, South America, and the Caribbean.[1,2] The disease is triggered by an obligate intraerythrocytic Proteobacteria in the genus *Anaplasma*.

The Pathogen: Family Anaplasmataceae

Anaplasma species belongs to the Class Alphaproteobacteria and falls under the Order Rickettsiales, Family Anaplasmataceae. There are currently six species of *Anaplasma* within the Family Anaplasmataceae: *Anaplasma marginale*, *Anaplasma centrale*, *Anaplasma ovis*, *Anaplasma phagocytophilum*, *Anaplasma bovis,* and *Anaplasma platys* (Table 1).[3] Of these species, *A. marginale* is most known to cause severe bovine anaplasmosis globally.[2]

Table 1. Characteristics of *Anaplasma* species

Species	Disease	Hosts	Vectors	Distribution	Infection Location
A. bovis	Bovine anaplasmosis	Cattle, buffaloes	*Amblyomma spp., Rhipicephalus spp. Hyalomma spp.*	Africa, Asia, Europe, South America, United States	Monocytes
A. centrale	Mild form of bovine anaplasmosis	Cattle	*Rhipicephalus spp.*	Tropical and subtropical areas around the globe.	Erythrocytes
A. marginale	Bovine anaplasmosis	Cattle	*Ixodes spp. Dermacentor spp. Rhipicephalus spp.*	Tropical and subtropical areas around the globe.	Erythrocytes
A. ovis	Ovine anaplasmosis	Sheep, goats, deer, and other wild ruminants	*Dermacentor spp. Rhipicephalus spp.*	Africa, Asia, Europe, United States	Erythrocytes
A. phagocytophilum	Human granulocytic anaplasmosis, equine anaplasmosis, anaplasmosis in dogs and cats, anaplasmosis in cattle and other ruminants.	Broad host range: Cattle, humans, horses, rodents, birds and other ruminants	*Ixodes spp.*	Global	Granulocytes
A. platys	Canine thrombocytopenia	Dogs	*Rhipicephalus spp.*	Global	Platelets

Anaplasma marginale

Anaplasma marginale is the most prevalent pathogen of bovine anaplasmosis .[4] The first genome sequence that was completed for *A. marginale* was the St. Maries strain.[2] Since then

many other strains have also been sequenced and published based on genome organization of the major surface proteins, *msp1a* and *msp4*.[2,4,5,6]

Anaplasma species are intra-erythrocytic pathogens. To gain access to the hosts erythrocytes, this organism uses surface proteins, which are critical for the ability of *A. marginale* to cause bovine anaplasmosis. The major surface proteins are the main source of genetic variability between strains of *A. marginale*, this is because the proteins are subjective to selective pressures from interaction with both invertebrate and vertebrate hosts. Among such proteins, three are particularly relevant- MSP1a, MSP4, and MSP5.[4,5,6] These proteins are derived from duplication of a single gene common to all strains of *A. marginale.* The *msp1a* gene, that codes for the MSP1a protein, is highly variable between strains and is used as an indicative marker for the geographic location of these strains. Despite the variability observed in the *msp1a* gene sequence, the protein sequence is relatively conserved during the replication of *Anaplasma* in both, the cattle host and the tick vector. This is mostly due to the essential role of MSP1a to adhering to the hosts' erythrocytes and the gut cells of the vectors.[4,5,6] When looking at the topology of the MSP1a protein, we find a conserved C-terminal region and a variable N-terminal region.[4]

Anaplasma marginale can be transmitted through different routes of transmission such as tick bite (biological), injection through other non-vector insect bites or puncture with contaminated equipment (mechanical), and transplacentally.[4,7,8] Once a host is infected with this species of *Anaplasma* the pathogen invades the erythrocytes, and replicates within the cytoplasm of the cells. The infected erythrocytes are ultimately destroyed resulting in hemolytic anemia, one of the most common signs of infection.[4]

Anaplasma phagocytophilum

Anaplasma phagocytophilum was first identified in 1940 as a tick-borne pathogen associated with disease in Scotland sheep.[9,10,11] The pathogen is transmitted by tick species of the genus *Ixodes,* and is capable of infecting a diverse host range. Because of the preferred tick-vector, there has been no cases of trans-ovarial transmission of the pathogen and *A. phagocytophilum* has only been known to be transmitted trans-stadially.[13,14] Similar to *A. marginale*, this species of *Anaplasma* is also highly variable and consists of strains that differs across countries. The major surface proteins of this species are similar to those found in *A. marginale*.[12] On the other hand, unique to *A. phagocytophilum* is that this pathogen infects granulocytes rather than erythrocytes.[12,13,14,15] Once infected, the host can develop a reduction in neutrophil function as well as leukopenia and neutropenia, ultimately leading to an immunosuppressant host. Interestingly, *A. phagocytophilum* strains can coinfect cattle with *A. marginale*, due to the use of different cell types for replication. Studies have shown that, infected cattle with both pathogens showed no clinical signs and a reduced level of parasitemia with *A. marginale*. [4,16]

Anaplasma centrale

Anaplasma centrale is a less pathogenic subspecies of *A. marginale.*[3] Both species share $CD4^+$ T-cell epitopes and may be the contributing factor for *A. centrale* cross protection against virulent strains of *A. marginale*.[17] Consequently, *A. centrale* has been used as a live blood vaccine against anaplasmosis caused by *A. marginale*. However, due to the variability in strains of *A. marginale*, *A. centrale* is not effective against all geographically diverse and highly virulent strains of *A. marginale* especially in countries such as Zimbabwe, Paraguay, and Argentina.[18]

The Disease: Bovine Anaplasmosis

Economic importance

Anaplasmosis is responsible for over $300 million dollars of damages in the United States' cattle industry and over $875 million dollars in Latin America. [4,13] Infection can affect cattle along their life mostly when the disease is introduced in a heard. Nevertheless, the presence of disease and severity is age dependent. Thus, disease is extremely rare in calves under the age of 6 months, while calves between the age 6 months and 1 year are more likely to develop a mild form of anaplasmosis.[4] In contrast, cattle over 2 years of age develop an acute version of disease that is often fatal with mortality rates ranging between 29% and 49%.[1,4,19,20] Once cattle become infected with *Anaplasma spp.* they remain life-long carriers.

Cycle of infection

Anaplasma species do not transmit trans-ovarially from the engorged female tick to the offspring (egg-larvae). Consequently, the infection cycle will start with the acquisition of the pathogen by an immature stage of the tick when it feeds on an infected host. Through the blood meal, the pathogen enters the gut of the tick where it begins to replicate. As mentioned above, *Anaplasma* species can survive molting of the ticks, showing trans-stadial transmission. Hence, the pathogen can travel to the salivary gland where it continues to replicate.[1,2,3,4] Once in the salivary gland, the tick can pass on the pathogen to the host. If this is an uninfected host, it will get infected during this second blood meal where 10^4-10^5 organism per salivary gland are released per subsequent transmission feeding.[19]

Upon transmission to the host, *A. marginale* has to infect the cytoplasm of erythrocytes to replicate and survive. Within the erythrocytes, membrane-bound inclusions form with around 4 to 8 rickettsiae.[2] After the erythrocytes of cattle are infected with this pathogen, there is an

incubation period in which the pathogen replicates in the infected blood cells, doubling every 24 hours.[21] The replication continues to the point where at least 1% of the host erythrocytes are infected. This process usually happens in a period of 3 to 8 weeks.[19] In order to determine whether an animal has an active *Anaplasma* infection, at least 15% of all erythrocytes must be parasitized. [23]Thus, for acute anaplasmosis to be characterized, rickettsemia level must exceed 10^9 infected erythrocytes per ml.[23] Hosts in the incubation stage do not normally display clinical signs, although a slight fever may occur in some instances. Once the infected erythrocytes are detectable, the host immune system begins sends out bovine reticuloendothelial cells to phagocytize the infected erythrocytes.[4,11] This stage can last anywhere from 4 to 9 days and the host will display signs of severe anemia. If the host survives the developmental stage of the disease, they then enter a period of recovery named the **convalescent stage**. This stage is characterized by a 2-3-month period where the host body tries to replenish erythrocytes levels back to normal. Once this stage is over the host enters the **carrier stage** where it becomes a carrier of the pathogen for life. [21]Despite not experiencing any clinical signs of disease in this stage, blood transferred from a carrier host to a susceptible one, will induce anaplasmosis in the susceptible host. [1,2,3,4,21]

Mode of Transmissions

Anaplasma spp. is commonly transmitted in three ways. The first method of transmission and the most efficient one is the biological transmission which includes the tick vectors. Infected erythrocytes are ingested by ticks where the bacteria then replicate in the tick's gut and salivary gland. The tick can then pass the bacteria to an uninfected host via saliva during feeding. The ability of a tick to obtain the bacterium from an infected host, depends on the onset of disease. In the acute phase of the disease, 95-100% of the ticks feeding in the infected animal will get

infected. On the other hand, the probability reduces to 27-84% when feeding from an animal in the chronic phase (Figure 1).[1] The second method of transmission is mechanical. The bacterium can spread through transfer of blood from infected hosts to non-infected hosts via contaminated needles, ear tagging, castration, and branding equipment.[10] The final method of transmission that has been identified is transplacental transmission which occurs when adult female cattle passes on the disease to its offspring.[11]

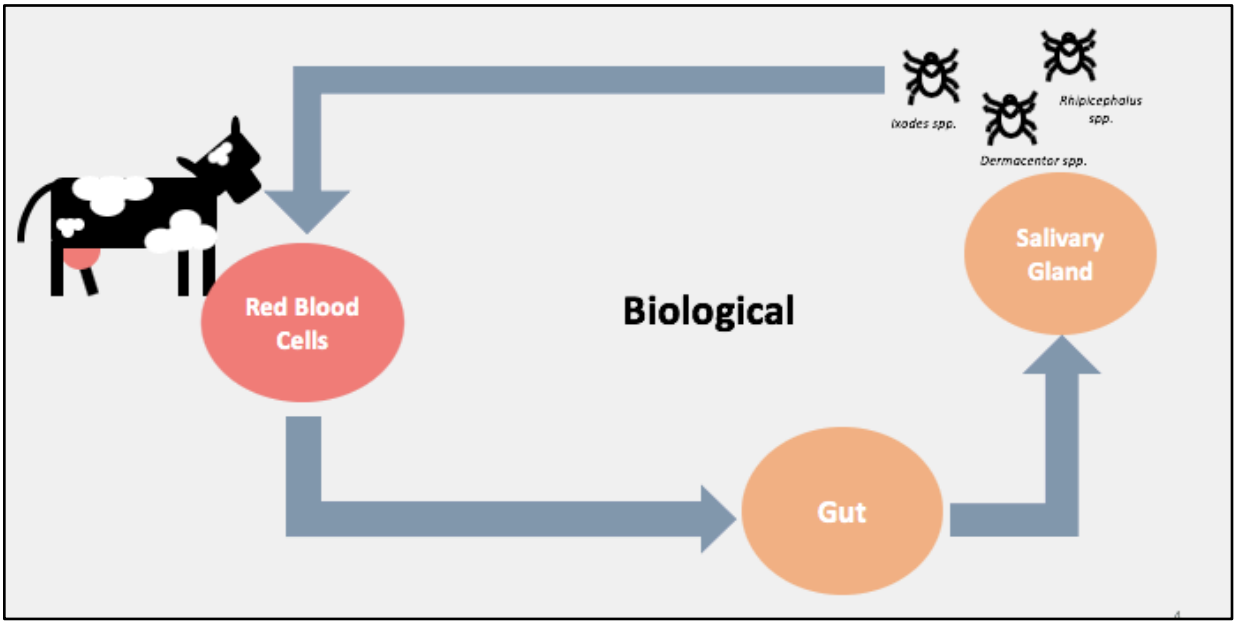

Figure 1. Biological transmission of *Anaplasma spp.*

Diagnosis

Clinical signs of anaplasmosis start to appear during the developmental stage of the disease. The primary clinical sign of anaplasmosis is anemia caused by loss of erythrocytes due to immune response activation. This sign can be identified by weakness and lethargy in the cattle. Other signs of disease that may occur are a jaundice appearance, reduce milk production,

colossal loss in body weight, fever, spontaneous abortions, and ultimately death due to hypoxia.[1,2,3,4,21] In order to diagnose bovine anaplasmosis laboratory tests must be used.

Blood Smears

Giemsa, Wright-Giemsa or Diff-Quick stained blood smears are used in detecting *A. marginale* and diagnosing bovine anaplasmosis during the acute phases of the disease. In this test thin blood smears are prepared for microscopic examination using standard staining protocols. The smears are examined for intra-erythrocytic inclusions. Though this test is routinely used in diagnosis of anaplasmosis due to simplicity, it lacks sensitivity.[1] This test is not useful for detecting the disease in sub-clinical cattle or carriers because of low number of infected cells in circulation, and can only detect $>10^4$ parasites/ml of blood.[1]

Serological Tests

Competitive ELISA is another diagnosis method for *A. marginale* infection and bovine anaplasmosis and is currently the most accurate. The test uses monoclonal antibody ANAF16C1 to recognize MSP5 in *A. marginale* and *A. centrale*. Serum antibodies that target MSP5 are detected and can determine infection by *Anaplasma spp.*[1,10,25] Currently cELISA is the most practical method of testing for bovine anaplasmosis in large number of cattle, it has a 95% sensitivity and a 98% specificity when used at a 30% inhibition cut off point.[26] Though this test is more sensitive than blood smears, it is limited in its ability to differentiate between *Anaplasma* species when coinfection occurs between *A. centrale*, *A. marginale*, and *A. phagocytophilum*. This is because these three species all contain the MSP5 antigen and produce the same antibodies. Another limitation is low sensitivity detection in early stages of infection.[1,11]

Polymerase Chain Reaction

PCR is another test that used to detect anaplasmosis in cattle. The detection limit of PCR is 24 infected erythrocyte per microliter of blood, which can lead to the misdiagnosis of carrier cattle as negative. The limitations of PCR are as followed: lower sensitivity in early infection, in addition to being costly by requiring trained personnel and specific laboratory equipment. [1]

Treatment and Prevention

Antimocrobial therapy

Antimicrobial therapy is one treatment method for bovine anaplasmosis. This treatment method incorporates tetracycline drugs as well as a variety of chemotherapeutic agent. The therapy does not eliminate persistent infection, and can sometimes have little to no effect on acute anaplasmosis.[1] Tetracyclines such as Oxytetracycline (OTC) and Chlortetracycline (CTC), are the most used and are effective at decreasing parasitemia levels and reducing clinical effects. Both OTC and CTC are FDA approved. The recommended use of OTC is parenteral use no more than four consecutive day in beef and non-lactating cattle, and the recommended use of CTC is continuous use in feed for control active anaplasmosis infection. This method of treatment can be expensive as it requires constant feeding, and it risks the development of drug resistant *Anaplasma*. [1,10]

Killed vaccines

Inactivated vaccines were developed in the USA in the 1960s, and were effective in preventing clinical anaplasmosis in southern United States. The vaccines were recalled in 1999 due to company restructuring.[1,2,4] There is currently one vaccine that is distributed throughout 14 states however it is not USDA approved, and the USDA will not allow further distribution of the

vaccine elsewhere. The vaccine is highly expensive because it requires booster immunization, extensive purification, and live animals as an antigen source.[1,2,4]

Live vaccines

In live vaccines cattle erythrocytes are infected with a less pathogenic strain of A. *marginale* or *A. centrale*. [1,2,4] *A. centrale* is used as a vaccine in Africa, Australia, Israel and Latin America, but it does not provide effective protection in other geographical areas. The current problem with vaccines is the diversity of *A. marginale*. Because *A. marginale* species have diverse strains that are not cross protective, it is hard to develop a vaccine that will be effective to all geographical regions. However, there is continuous research on developing a vaccine that will be able to be effective against various genotypes of *A. marginale*. [1,2,4]

Anaplasma-free herds

In order to maintain an *Anaplasma* free heard, all cattle must be tested consistently for *Anaplasma* in addition to any newly added cattle. It is recommended that new cattle are tested twice in 3-weeks interval before they are added to the heard. Positive cattle must be eliminated when detected because they are capable of transferring the pathogen. [1]

Vector control

Vector control can be obtained with the use of acaricides, however is not preferred because it can contribute to environmental pollution as well as create a resistant tick population. An alternative method of control is keeping animals in environment that do not allow for survivals of ticks. [1]

Hypothesis

Based on the information presented above, and due to the abundance of Bovine Anaplasmosis in Tropical and Subtropical regions of the world, we hypothesized that Bovine production in Puerto Rico will have an average prevalence of Anaplasmosis caused mainly by *Anaplasma marginale*.

CHAPTER II

METHODS

Sampling

In partnership with the USDA-ARS Cattle Fever Tick Research Lab (CFTRL) in Mission, Texas we were able to locate a collection of cattle DNA samples from Puerto Rico, where concerns on tick-borne diseases affecting livestock production were investigated. The USDA laboratory collected 198 cattle blood samples from different regions in Puerto Rico. The CFTRL then performed phenol-chloroform extraction of the blood samples and sent the extracted DNA samples to Dr. Esteve-Gasent's laboratory in the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University in College Station, Texas.

Testing

Upon arrival, samples were processed to detect *Anaplasma* spp. by means of polymerase chain reaction (PCR) and confirmation of the results were done using Sanger sequencing. The steps developed to test all samples are described below. We followed standardized protocols implemented by Dr. Esteve-Gasent's laboratory personnel.

Step 1: Polymerase Chain Reaction and Gel Electrophoresis

Once the samples were received in College Station, Texas, conventional polymerase chain reaction (PCR) was performed on the samples. The oligonucleotide primers used during the PCR process for all samples were 16SANA-F and 16SANA-R (Table 2). The primers used targeted the *16s rRNA* gene region which is highly variable, and will allow the detection of all potential *Anaplasma* species expected to be present in cattle herds. All PCR reactions were done using established laboratory PCR procedures to prevent cross-contamination of samples. The

primers were diluted using 10µL of primer and 90µL of water for an end result of 1:9 ratio of primer to water and a final concentration of 10pM. The PCR reaction was prepared using 12.5µL of GoTaq® Green Master Mix, 1µL of both diluted forward and reverse primers (10pM final concentration), and 8.5µL of water. All master mix preparation steps were done in a PCR workstation to avoid cross-contamination. After master mix was distributed in individual PCR tubes, 1µL of sample DNA was added to each tube. This step was prepared outside of the PCR workstation and in a different room underneath a vented hood. Positive and negative (water) controls were used in all amplifications. PCR reactions were done in an Eppendorf Master Cycler Pro following the program outlined in (Table 2) and previously described by others.[34] After PCR amplification was completed, amplicons were separated using a 1% agarose gel at 80 volts for 45 minutes. Positive amplification will be determined based on the size of the positive control (468bp fragment)

Table 2. Primers

Primers	**Protocol**
Forward: 5'-CAG AGT TTG ATC CTG GCT CAG AAC G-3'	*Repetition 1 cycle* 1- 95°C for 2 minutes
Reverse: 5'-GAG TTT GCC GGG ACT TCT TCT GTA-3'	*Repetition 45 cycles* 2- 94°C for 30 seconds 3- 55°C for 30 seconds 4- 72°C for 1minutes *Repetition 1 cycle* 5- 72°C for 7 minutes 6- 4°C hold

Step 2: Purification of Positive Amplicons

Positive amplicons were excised from the gel using sterile Xtracta tools (Sigma Aldrich) and purified using the Wizard® SV Gel and PCR Clean-Up Kit (Promega, Inc), following manufacturer's recommendations. Briefly, excised DNA band was placed in a 1.5ml microcentrifuge tube and 10µl of membrane binding solution per 10mg of gel slice was added. The tube was then vortex and incubated at 64°C until the gel was dissolved. Once the gel was dissolved 460µl of additional membrane binding solution was added to the PCR amplification. The gel was transferred to a minicolumn/collection tube assembly and centrifuged at 16,000 × *g* for 1 minute. The flow-through was discarded and the minicolumn was placed in another collection tube and washed with 700µl of membrane wash solution and centrifuged at 16,000 × *g* for 1 minute. After the first initial wash, the minicolumn was washed again with 500µl of membrane wash solution for 5 minutes at 16,000 × *g*. All liquid that was filtered through was discarded. The minicolumn was then centrifuged at 16,000 × *g* for 1 minute with the lid off to allow for evaporation of residual ethanol. The final step of the Wizard® SV Gel and PCR Clean-Up Kit (Promega, Inc) required adding 45µl of Nuclease-free water to mini column/collection tube assembly and centrifuge the assembly at 16,000 × *g* for 1 minute. The product that was collected in the 1.5ml microcentrifuge tube was then sent to Eurofin Genomics, LLC for sequencing using specific primers provided.

Step 3: Sequencing and Analysis

Clean amplicons were submitted to Eurofin Genomics, LLC, for Sanger sequencing. We provided with the forward and reverse primers for the company to sequence each amplicon in both directions. Once the sequences were received, our team used the MacVector Version 13.0.7 (MacVextor Inc. North Carolina) to clean the sequences individually. First, forward and reverse

sequences were prepared by removing unclean and noisy sections from the 5'- and 3'- end. After the forward and reverse sequences of each sample were aligned. Once the consensus of each sequence was generated, nucleotide accuracy was checked by looking at a chromatogram, to detect any contractions or miss-readings. The final cleaned sequence was then compared to other sequences found in the Basic Local Alignment Search Tool (BLAST®). Using the international database, BLAST®, our positive amplicons were then identified to the species level.

Step 4: Phylogenetic Tree

A phylogenetic tree was created using MacVector Version 13.0.7 (MacVextor Inc. North Carolina). The tree was inferred using the UPGMA method, while support from each node was assessed using 1,000 bootstrap resampling. All gaps were distributed proportionally. The phylogenetic tree was compiled using all positive samples as well as comparison samples acquired from GenBank®. All accessions numbers are provided (Table 3).

Table 3: Samples and GenBank® Accession numbers

Isolate	Species	Accession number
PRA11	*Anaplasma phagocytophilum*	MK736728
PRA1	*Anaplasma platys*	MK736870
PRA5	*Anaplasma platys*	MK736871
PRA8	*Anaplasma platys*	MK736872
PRA9	*Anaplasma platys*	MK736873
PRA10	*Anaplasma platys*	MK736874
PRA12	*Anaplasma platys*	MK736875
2PRA1	*Anaplasma platys*	MK736876
2PRA8	*Anaplasma platys*	MK736877
2PRA13	*Anaplasma platys*	MK736878
2PRA14	*Anaplasma platys*	MK736879
2PRA15	*Anaplasma platys*	MK736880
2PRA16	*Anaplasma platys*	MK736881
2PRA19	*Anaplasma platys*	MK736882
2PRA21	*Anaplasma platys*	MK736883
2PRA26	*Anaplasma platys*	MK736884
2PRA27	*Anaplasma platys*	MK736885
2PRA36	*Anaplasma platys*	MK736886
2PRA38	*Anaplasma platys*	MK736887
PRA2	*Anaplasma marginale*	MK737006
PRA3	*Anaplasma marginale*	MK737007
PRA4	*Anaplasma marginale*	MK737008
PRA6	*Anaplasma marginale*	MK737009
PRA7	*Anaplasma marginale*	MK737010
2PRA2	*Anaplasma marginale*	MK737011
2PRA3	*Anaplasma marginale*	MK737012
2PRA4	*Anaplasma marginale*	MK737013
2PRA5	*Anaplasma marginale*	MK737014
2PRA6	*Anaplasma marginale*	MK737015
2PRA7	*Anaplasma marginale*	MK737016
2PRA9	*Anaplasma marginale*	MK737017
2PRA10	*Anaplasma marginale*	MK737018
2PRA11	*Anaplasma marginale*	MK737019
2PRA20	*Anaplasma marginale*	MK737020
2PRA23	*Anaplasma marginale*	MK737021
2PRA24	*Anaplasma marginale*	MK737022
2PRA25	*Anaplasma marginale*	MK737023
2PRA28	*Anaplasma marginale*	MK737024
2PRA29	*Anaplasma marginale*	MK737025
2PRA31	*Anaplasma marginale*	MK737026
2PRA32	*Anaplasma marginale*	MK737027

CHAPTER III

RESULTS

A total of 198 samples were used in this study. Of the 198 samples, 21.71% (43/198) of the samples tested positive for *Anaplasma* at a genus level. Out of those positive samples 53.49% (23/43) were identified as either *Anaplasma centrale /Anaplasma marginale*. The two species of *Anaplasma* are grouped together due to the difficulty of differentiating them based on the sequences obtained. Interestingly enough, other species identified in this study are *A. platys*-like strains 44.19% (19/43) and 2.32% (1/43) *A. phagocytophilum* (Figure 3).

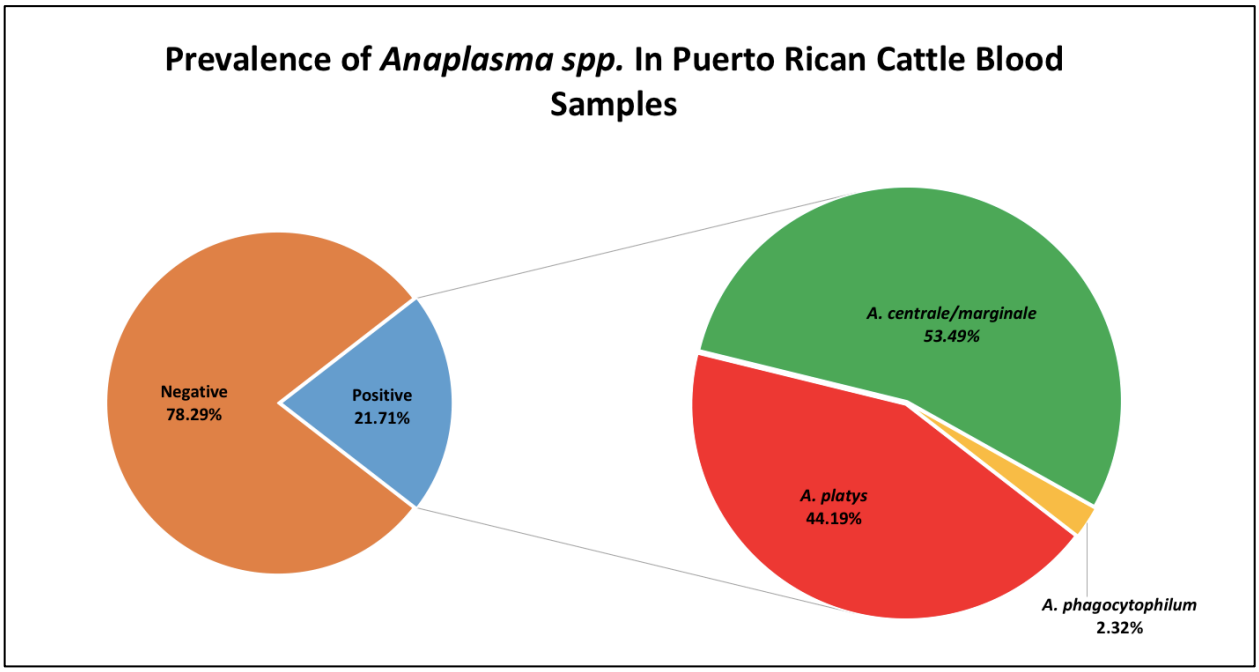

Figure 3. Prevalence of *Anaplasma* spp. in Puerto Rican Cattle Blood Samples

A phylogenetic tree was also generated to compare samples used in this study to other samples around the globe (Figure 4).

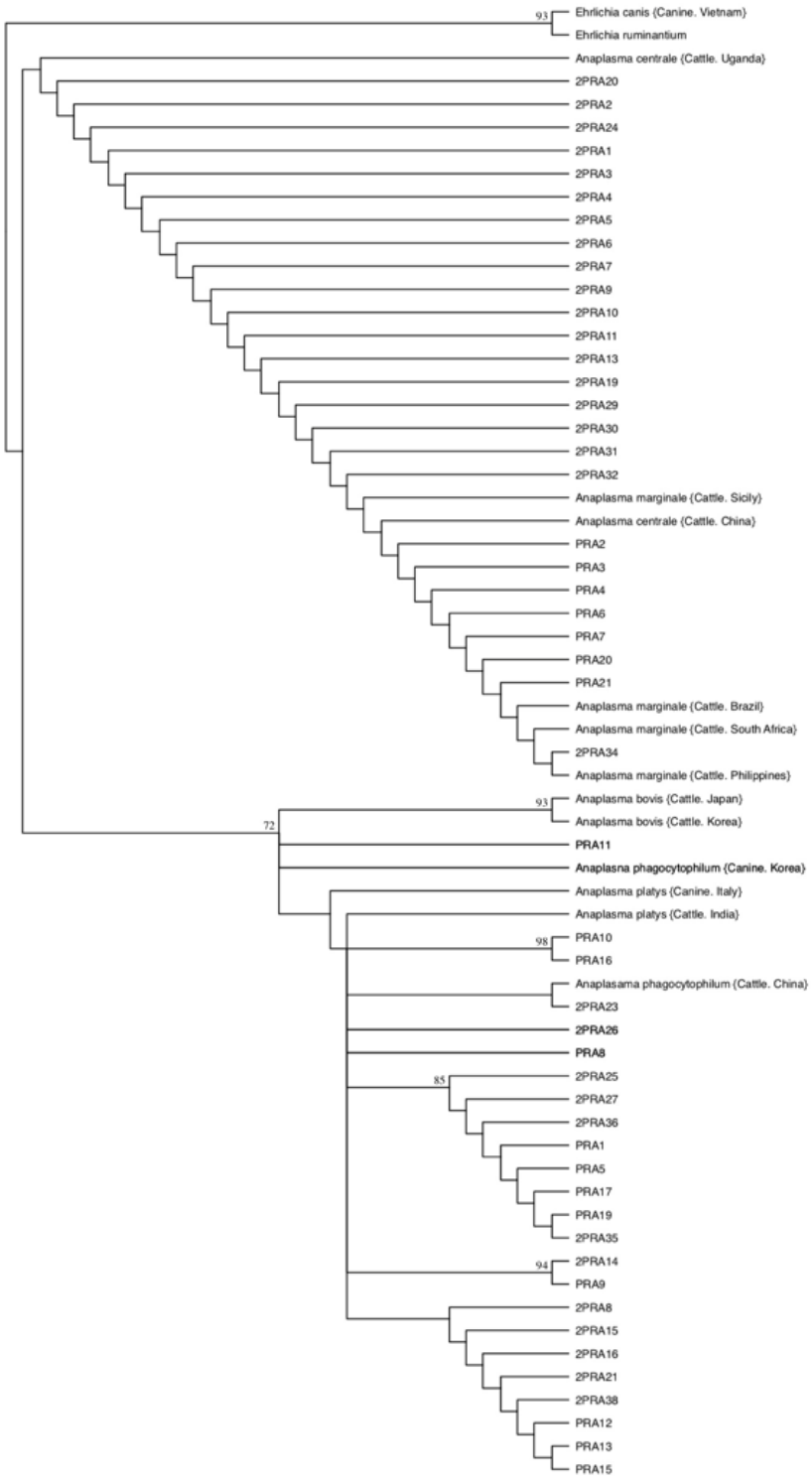

Figure 4: Phylogenetic Tree of *Anaplasma* Samples

As referenced in Table 1, *A. platys* is a specie of *Anaplasma* normally found only in canine hosts. However, there has been a few cases in which *A. platys*-like samples have been found in cattle.[16,27,28] The *A. platys*-like samples detected in this study were compared to other *A. platys*-like samples in GenBank®. The phylogenetic tree indicates that the *A. platys*-like samples in this study were more similar to those found in cattle than those found in canine samples.

CHAPTER IV

CONCLUSION

Information on the prevalence of *Anaplasma spp.* in cattle is geographical regions such as the United States and Puerto Rico is very limited. Further research is necessary to better understand the prevalence of anaplasmosis as well as the distribution of various species of Anaplasma. This study was done to comprehend the distribution of *Anaplasma spp.* in Puerto Rico cattle production. A total of 198 blood samples from Puerto Rican cattle were tested in this study showing a 21.71% infection status of *Anaplasma spp.* Out of those positive samples 53.49% were infected with either *A. marginale* or *A. centrale;* 44.19% were infected with *A. platys* -like strains and 2.32% were infected with *A. phagocytophilum*.

There are no studies that look at the prevalence of *Anaplasma spp.* at a genus level in the island of Puerto Rico, and most studies focus on the prevalence of *A. marginale* as a determinant of anaplasmosis. In this study, the majority of positive samples were infected with either *A. marginale* or *A. centrale*. The two species of *Anaplasma* are grouped together because they were unable to be differentiated due to the similarities between them. In previous studies conducted in Puerto Rico, researchers have been able to distinguish the two species using MSP-5 cElisa. The overall prevalence of *A. marginale* detected in Puerto Rico has range from 27.4% to 30.8%.[28,29] Because this study was unable to differentiate between *A. marginale* and *A. centrale* it is inconclusive what percent of the samples were *A. marginale* and if the observation is in accordance with previous studies. However, the high prevalence of the combination of the two species in this study is no surprise due to Puerto Rico tropical humid climates that allows for sufficient survival of tick vectors.

In this study, one sample was identified as *A. phagocytophilum*. There are no other studies that has looked at the prevalence of *A. phagocytophilum* in Puerto Rican cattle for comparison. Studies that have looked *A. phagocytophilum* in cattle have been conducted in France and Tunisia amongst others. Most of the studies have indicated a prevalence ≤1% of *A. phagocytophilum*. [16,31] Though the studies were conducted in different geographical regions the results that are accordance to this study. One possible reason for the low detection of *A. phagocytophilum* in bovine species could be due to the vector preference for other animal reservoirs. In the United States and Europe, *A. phagocytophilum* is more linked to human granulitic anaplasmosis. While in Puerto Rico, *A. phagocytophilum* is also associated with canines with one study indicated a 6% prevalence of *A. phagocytophilum* in dogs.[32]

The second most prevalent *Anaplasma* species found in this study is *A. platys*-like. *A. platys* is not normally found it cattle and is a specie of *Anaplasma* that is only thought to infect dogs. Only a handful of cases of *A. platys*-like strains that have been detected in cattle in the following countries: Vietnam, Algeria, and Tunisia. There are currently no reports of *A. platys*-like strains in Puerto Rican cattle. In the study conducted in Vietnam ≤1% cattle blood samples were positive for *A. platys*.[27] In Algeria 4.8% of cattle blood samples came back positive for *A. platys* and in Tunisia *A. platys* was prevalent in 22.8% goat samples, 11% sheep samples, and 3.5% cattle samples.[16,27] In all three countries the prevalence of *A. platys* were significantly lower than those found in our study (9.60%; 19/198); however *A. platys* was detected in cattle when it was previously thought unable to do so. Furthermore, when the *A. platys*-like samples in this study was compared to other *A. platys*-like sample in cattle and *A. platys* in dogs, the results showed more similarities with the *A. platys*-like strains found in cattle. Alternatively, another reason for the detection of this species in cattle could be due to cross-reacting strains of different species of *Anaplasma* that creates an

appearance of *A. platys*. The discovery of *A. platys*-like strains is very concerning due to the unique ability of *A. platys* to infect platelets instead of erythrocytes. As a result, anaplasmosis infection caused by *A. platys* can lead to decrease in blood clotting resulting in thrombocytopenia. It is unsure if the pathogenicity of *A. platys* in cattle is similar to that observed in domestic dogs; nevertheless, more research is needed in this front to elucidate the virulence of A. platys affecting cattle to better recognize its negative impact in the cattle production. Another concern of *A. platys*-like strains discovery in cattle is the change of vector-host interaction. *A. platys* has been traditionally associated with infections in dogs due to its primary vector *Rhipicephalus sanguineus,* more commonly known as the Brown Dog Tick.[1] Though this tick species prefers to feed on dogs, there are some instances where it has been seen feeding on other mammals including humans. Hence, it could be possible that environmental pressure placed on the tick vector could have influenced the host preferences. If that is the case, concerns for human health comes into question. For instance, *A. platys* has already been detected in several cases of humans in countries such as Venezuela and Grenada, highlighting its potential as a zoonotic pathogen.[33]

In conclusion this study has provided molecular identification of *Anaplasma* species in Puerto Rican Cattle. This study indicates the presence of *A. platys*-like species in Puerto Rican Cattle for the first time. The presence of this species indicates the need for better molecular tools to help confirm the *A. platys*-like status as well as a need to update epidemiological data of *Anaplasma spp.* in the Puerto Rico. Further studies are also needed to better understand the impact of disease transmission with possible differences in vector-host interaction.

REFERENCES

1. Aubry, P., and D. W. Geale. A Review of Bovine Anaplasmosis. *Transboundary and Emerging Diseases*, vol. 58, no. 1, Dec. 2010, pp. 1–30

2. Kocan KM, de la Fuente J, Blouin EF, Coetzee JF, Ewing SA. The natural history of *Anaplasma marginale*. *Veterinary parasitology*. https://www.ncbi.nlm.nih.gov/pubmed/19811876. Published February 10, 2010. Accessed April 7, 2019.

3. Rymaszewska A, Grenda S. Bacteria of the genus *Anaplasma*– characteristics of *Anaplasma* and their vectors: a review. *Veterinární Medicína*. 2008;53(No. 11):573-584. doi:10.17221/1861-vetmed.

4. Kocan KM, Fuente JDL, Guglielmone AA, Melendez RD. Antigens and Alternatives for Control of *Anaplasma marginale* Infection in Cattle. *Clinical Microbiology Reviews*. 2003;16(4):698-712. doi:10.1128/cmr.16.4.698-712.2003.

5. Fuente JDL, Bussche RAVD, Garcia-Garcia JC, et al. Phylogeography of New World isolates of *Anaplasma marginale* based on major surface protein sequences. *Veterinary Microbiology*. 2002;88(3):275-285. doi:10.1016/s0378-1135(02)00122-0.

6. Eriks, I S, et al. Impact of Persistent *Anaplasma marginale* Rickettsemia on Tick Infection and Transmission. *Journal of Clinical Microbiology*, 1993.

7. Torioni de Echaide S, Knowles DP, McGuire TC, Palmer GH, Suarez CE, McElwain TF. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. Journal of clinical microbiology. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC104624/. Published March 1998. Accessed April 8, 2019.

8. Irsik, Max. Treatment for *Anaplasma marginale* in Beef Cows. *Beef Cattle Extension Veterinarian University of Florida College of Veterinary Medicine*,extension.vetmed.ufl.edu/files/2012/02/Treatment-of-the-Carrier-State-of-Anaplasmosis-in-Beef-Cattle-sent-for-the-august-issue-of-FCA-magazine.pdf.

9. Fuente JDL, Garcia-Garcia JC, Blouin EF, Kocan KM. Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. *Veterinary Microbiology*. 2003;91(2-3):265-283. doi:10.1016/s0378-1135(02)00309-7.

10. Whittier, Dee, et al. Anaplasmosis in Beef Cattle. *Virginia Cooperative Extension*, vol. 400, no. 465

11. Zaugg, J. L., 1985: Bovine anaplasmosis: transplacental transmission as it relates to stage of gestation. Am. J. Vet. Res. 46, 570–572.

12. Woldehiwet Z. *Anaplasma phagocytophilum* in ruminants in Europe. Annals of the New York Academy of Sciences. https://www.ncbi.nlm.nih.gov/pubmed/17114753. Published October 2006. Accessed April 8, 2019.

13. Brown, D. C. G. "Dynamic and impact of tick-borne diseases of cattle." Trop. Anim. Health Prod. 29:1S-3S, Nov. 1997

14. Knowles D, Torioni de Echaide S, Palmer G, McGuire T, Stiller D, McElwain T. Antibody against an *Anaplasma marginale* MSP5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. Journal of clinical microbiology. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC229221/. Published September 1996. Accessed April 8, 2019.

15. Rikihisa Y. Mechanisms of obligatory intracellular infection with *Anaplasma phagocytophilum*. *Clin Microbiol Rev*. 2011;24(3):469–489. doi:10.1128/CMR.00064-10

16. M'ghirbi Y, Bèji M, Oporto B, Khrouf F, Hurtado A, Bouattour A. *Anaplasma marginale* and *A. phagocytophilum* in cattle in Tunisia. *Parasit Vectors*. 2016;9(1):556. doi:10.1186/s13071-016-1840-7

17. Atif, Farhan Ahmad. *Anaplasma marginale* and *Anaplasma phagocytophilum*: Rickettsiales Pathogens of Veterinary and Public Health Significance. *Parasitology Research*, 2015; 114(11): 3941–3957.

18. Deringer JR, Forero-Becerra EG, Ueti MW, et al. Identification of a T-Cell Epitope That Is Globally Conserved among Outer Membrane Proteins (OMPs) OMP7, OMP8, and OMP9 of *Anaplasma marginale* Strains and with OMP7 from the *A. marginale* subsp. *centrale* Vaccine Strain. *Clin Vaccine Immunol*. 2017;24(1):e00406-16. Published 2017 Jan 5. doi:10.1128/CVI.00406-16

19. Rosa Estela Quiroz-Castañeda, Itzel Amaro-Estrada, and Sergio Darío Rodríguez-Camarillo, "Anaplasma marginale: Diversity, Virulence, and Vaccine Landscape through a Genomics Approach," BioMed Research International, vol. 2016, Article ID 9032085, 18 pages, 2016.

20. Kocan, K. M., E. F. Blouin, and A. F. Barbet,: Anaplasmosis control. Past, present, and future. 2000 http://onlinelibrary.wiley.com/doi/10.1111/j.1749-6632.2000.tb05329.x/abstract

21. Jones, E.W., 1968. Patterns of Resistance in Anaplasmosis. J Am Vet Med Assoc 153, 202.

22. Jones, E.W., Brock, W.E., 1966. Bovine Anaplasmosis - Its Diagnosis Treatment and Control. J Am Vet Med Assoc 149, 1624-1633

23. Richey EJ. Anaplasmosis in Beef Cattle. AgriLife Extension Texas A&M System.

24. Thompson BS, Goodrich EL. Miscellaneous Infectious Diseases. *Rebhuns Diseases of Dairy Cattle*. 2018:737-783. doi:10.1016/b978-0-323-39055-2.00016-4.

25. Losos, G. J. 1986. Anaplasmosis, p. 742–795. *In* G. J. Losos (ed.), Infectious tropical diseases of domestic animals. Longman Press, Essex, United King- dom.

26. VMRD Inc. 1998: *Anaplasma* Antibody Test Kit, cELISA. Available at http://www.vmrd.com/docs/tk/*Anaplasma*/ *Anaplasma*_Flyer_050113.pdf (accessed November 3, 2009).

27. Chien, N., Nguyen, T., Bui, K., Nguyen, T. and Le, T. *Anaplasma marginale* and *A. platys* Characterized from Dairy and Indigenous Cattle and Dogs in Northern Vietnam. *The Korean Journal of Parasitology*, 2019;57(1), pp.43-47.

28. Dahmani, M., Davoust, B., Benterki, M., Fenollar, F., Raoult, D. and Mediannikov, O. (2015). Development of a new PCR-based assay to detect Anaplasmataceae and the first report of *Anaplasma phagocytophilum* and *Anaplasma platys* in cattle from Algeria. *Comparative Immunology, Microbiology and Infectious Diseases*, 39, pp.39-45.

29. Urdaz-Rodríguez, J., Fosgate, G., Alleman, A., Rae, D., Donovan, G. and Melendez, P. Seroprevalence estimation and management factors associated with high herd seropositivity for *Anaplasma marginale* in commercial dairy farms of Puerto Rico. *Tropical Animal Health and Production*, 2009;41(7), pp.1439-1448.

30. Rodríguez, J. Epidemiology of Bovine Anaplasmosis and Babesiosis in Commercial Dairy Frams of Puerto Rico. 2007. Ufdcimages.uflib.ufl.edu.

31. Dugat T, Leblond A, Keck N, et al. One particular *Anaplasma phagocytophilum* ecotype infects cattle in the Camargue, France. *Parasites & Vectors*. 2017;10(1). doi:10.1186/s13071-017-2305-3.

32. Mccown, Michael & Opel, Taylor & Grzeszak, Benjamin. Vector-borne disease surveillance in Puerto Rico: pathogen prevalence rates in canines- Implications for public health and the U. S. Military- Applying the one health concept. *Journal of special operations medicine*. 2013; 13. 59-63.

33. Arraga-Alvarado CM, Qurollo BA, Parra OC, Berrueta MA, Hegarty BC, Breitschwerdt EB. Case report: Molecular evidence of *Anaplasma platys* infection in two women from Venezuela. *Am J Trop Med Hyg*. 2014;91(6):1161–1165. doi:10.4269/ajtmh.14-0372

34. Silaghi C, Santos AS, Gomes J, et al. Guidelines for the Direct Detection of Anaplasma spp. in Diagnosis and Epidemiological Studies. *Vector-Borne and Zoonotic Diseases*. 2017;17(1):12-22. doi:10.1089/vbz.2016.1960.