USE OF THE REAL TIME TAQMAN®-PCR FOR DETECTION OF *BABESIA BOVIS* AND *BABESIA BIGEMINA* INFECTION IN DAIRY FARMS OF PUERTO RICO

by
Natalia A. Rivera Diaz

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Approved by:

_____ Alexander Mesonero, M.S/D.V.M. President, Graduate Committee	_____ Date
_____ Esbal Jimenez, Ph.D. Co- President, Graduate Committee	_____ Date
_____ Héctor Sánchez, Ph.D. Member, Graduate Committee	_____ Date
_____ Guilherme Klafke, D.V.M/Ph.D. Member, Graduate Committee	_____ Date
_____ Pedro J. Resto Irizarry, Ph D. Representative of Graduate Studies	_____ Date
_____ José R. Latorre, Ph.D. Chairperson of the Department	_____ Date

ABSTRACT

The dairy industry constitutes an important resource to the socioeconomically development of Puerto Rico. It is the main agricultural enterprise supporting more than 25,000 employees and producing nearly $215 million during the fiscal year 2010 (26% of the total agriculture revenue. However, the production and stability of our dairy industry is challenged by hemoparasitic diseases like babesiosis, a vector borne disease transmitted by the cattle fever tick (*Rhipicephalus microplus*), that result in economical and health repercussions to the dairy farms. Fast and efficient identification of cattle babesiosis can prevent or decrease the dissemination of the disease, and consequently allow for early control and proper treatment. The present study evaluates the efficiency of a multiplex Real Time Taqman®- PCR technique as a laboratory diagnostic test for cattle babesiosis, compared with conventional detection techniques currently used (immunoassays-ELISA; conventional end point PCR). The prevalence of *Babesia bovis* and *B.bigemina* in different cattle ranches was analyzed taking into account the results obtained with nested-PCR and the suggested real time Taqman® PCR. Sample collection was performed in four commercial dairy cattle farms located in the northwest and southwest part of Puerto Rico. A total of 158 cows were tested for identification of *Babesia bovis* and *B*abesia *bigemina* using nPCR and real time Taqman®-PCR. A McNemar's Chi-square test performed with the SAS/STAT program indicated that both detection methods resulted with 100% of specificity with some variation of sensitivity for detection of *B.bovis* and

B.bigemina. Statistical analysis revealed no difference between tests, with pvalue 0.0833 for *B.bovis* and pvalue 0.3173 for *B.bigemina* (p value>0.05). Despite the lack of significant differences in the results obtained between the two modalities employed, there are clear advantages of using Real Time Taqman® PCR over nPCR. Time consumption in preparation and turnaround time using Real Time Taqman®-PCR, especially if a large amount of samples are to be processed, demonstrated to be much lower than nPCR. Results obtained in the study indicated the prevalence of *Babesia bovis* in the premises under evaluation, a total of 15 positive results. San Sebastian farm being the main premise infected with a total of 12 positive results for *Babesia bovis.*

RESUMEN

El ganado lechero constituye un recurso importante para el desarrollo socioeconómico de Puerto Rico. Esta es la principal empresa agrícola generando alrededor de 25,000 empleos y cerca de $215 millones para el año fiscal 2010 (26% del total ingreso agrícola). Sin embargo, la productividad y estabilidad de esta industria, son desafiados por enfermedades hemoparasitarias como babesiosis, enfermedad transmitida por vectores como la garrapata de la fiebre de ganado (*Rhipicephalus microplus*), las cuales afectan el hato lechero teniendo repercusiones económicas y de salud animal. Una prueba de detección rápida y eficiente para la babesiosis bovina puede prevenir la diseminación de la enfermedad, y consecuentemente permitiendo su control y tratamiento apropiado. El presente estudio evaluó la eficiencia de una técnica diagnóstica de laboratorio multiplex de Real Time Taqman®-PCR para detección de la babesiosis bovina, comparada con métodos convencionales actualmente utilizados (inmunoensayos-ELISA; PCR convencional de punto final). La prevalencia de *Babesia bovis* y *Babesia bigemina* en las premisas bajo estudio, fue analizada teniendo en consideración los resultados obtenidos con el nested PCR y el Real Time Taqman® PCR sugerido. Este estudio se llevó a cabo en cuatro vaquerías comerciales localizadas en las regiones noroeste y sureste de Puerto Rico. Un total de 158 vacas fueron muestreadas para la identificación de *Babesia bovis* y *B.bigemina* con el método de detección de Real Time Taqman®-PCR y nPCR. Una prueba McNemar de Chi-cuadrado fue realizada con el programa SAS/STAT, indicando que ambas técnicas de detección resultaron en un 100%

de especificidad con alguna variación en sensibilidad para la identificación de *B. bovis* y *B.bigemina*. Los análisis estadísticos demostraron que no hay diferencias entre ambos métodos de detección, pvalue 0.0833 para *B.bovis* y pvalue 0.3173 para *B.bigemina* ($p>0.05$). Sin embargo, Real Time Taqman®-PCR resultó ser numéricamente más sensitiva para la detección de ambas enfermedades hemoparasitarias. Además, el tiempo consumido durante el proceso de preparación y ejecución del Real Time Taqman®-PCR con una alta cantidad de muestras, demostró ser mucho menor en comparación con el nPCR. Los resultados obtenidos indicaron la prevalencia de la especie *Babesia bovis* en las premisas evaluadas en este proyecto, un total de 15 resultados positivos. La finca de San Sebastián fue la principal premisa infectada con un total de 12 resultados positivos para *Babesia bovis.*

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GLOSSARY OF TERMS

ARS	Agriculture Research Services
CFTRL	Cattle Fever Tick Research Laboratory
DNA	Deoxyribonucleic acid
nPCR	Nested Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
USDA	United Sates Department of Agriculture

INTRODUCTION

The dairy industry is an important economic resource in the agriculture of Puerto Rico, hemoparasitic diseases like Babesiosis affect production goals because of economical losses. After the *Rhipicephalus microplus* eradication program ended in 2010, tick-borne diseases like *Babesia bovis* and *bigemina* have re-emerged and no prevalence studies have been performed in the island.

Even though there are several detection techniques like PCR that have demonstrated the ability to detect and differentiate species of Babesia in carrier infections, PCR assays generally do not lend themselves well to large-scale testing. Immunological assays like ELISA are useful as a large-scale confirmatory test in chronic animals when high levels of parasitemia are detectable, but it's not useful for detection at the initial and intermediate stages of the disease. However, none of these tests appears to have been adopted for routine diagnostic use in laboratories other than those in which the original development and validation took place.

Therefore, the present study was executed with the use of a Real Time Taqman®-PCR as a detection method for the prevalence of *Babesia bovis* and *Babesia bigemina* in Puerto Rico. It intends to demonstrate the effectiveness of the technique to provide adaptability as a routine diagnostic test for a large number of samples.

CHAPTER 1- LITERATURE REVIEW

1.1 Bovine Babesiosis and its socioeconomical impact in the dairy industry in Puerto Rico

The dairy industry is the main agriculture enterprise in Puerto Rico, supporting more than 25,000 employees and producing nearly $215 million during fiscal year 2010 (26% of the total agriculture revenue) (RODI, 2010). Despite its prominent place in the local economy, the long run stability of the dairy industry is challenged by increasing costs of production (i.e. feed ingredients, labor, fuel, electricity), and the incidence of diseases that cause economic losses and limit milk production efficiency.

Raising a heifer calf to reproductive age represents an enormous cost to the producer. Poor neonatal growth delays the age at which calves reach puberty (Graham et al., 2010). A delayed calving age significantly impacts the economic efficiency of raising dairy heifers, which accounts for 33.3% of the expenses in dairy operations (Pantoja-López, 2008). Even though, the incidence of diseases affects the calving rate in dairy heifers, it has been reported that calves infected with *Babesia sp.* obtained a degree of immunity, through colostral derived antibodies which persists for about 6 months. At high levels of tick transmission, newborn calves will become infected with *Babesia sp*. by 6 months of age, show few if any clinical signs, and subsequently develop immunity (Chigozie et al., 2014). The immune response of cattle to *B. bovis* and *B. bigemina* infection involves both innate and acquired immune mechanisms. Innate immunity is

non-specific and includes factors such as host-parasite specificity, genetic factors, age of the host and the response of host cells. Goff et al. (,2001) stated that young calves exhibit a strong innate immunity compared to adult cattle. However, the metabolic energy directed to growth and development will become compromised as a result of the infection causing a delayed growth in calves and eventually, this will become an economical issue to the farmer.

Different levels of parasitemia will induce a reaction from the host immune system. The clinical expression of babesiosis is highly linked to the characteristics of the animal (breed, age, etc.) (Chauvin et al., 2009). For this reason, three different phenotypes have been described in the *Bos taurus/B.bovis, B.bigemina* relationship: ‘‘susceptible’’ animals with severe clinical signs which may lead to death; the ‘‘intermediate’’ animals, with mild clinical signs; and the ‘‘resistant’’ animals, with no clinical signs of the disease (Benavides and Sacco., 2007). Dairy production will be negatively impacted due to expenses in veterinary care for susceptible animals, lower milk production, fertility issues and less weigh gain as a result of intermediate sick animals and sudden death (Benavides and Sacco., 2007). Ultimately, the babesiosis life cycle will establish in the herd with the help of carrier animals.

Currently, detection for bovine babesiosis in Puerto Rico is limited to blood smears or ELISA tests performed by the APHIS laboratory services, provided by the National Veterinary Services Laboratories (NVSL). Both methods of detection can identify positive results with certain levels of parasitemia during acute infections, but are not suitable for detection of persistently infected animals (Schnittger et al., 2012). Even

though ELISA has good sensitivity, serological cross-reactions complicate differentiation of *Babesia* organism and lead to equivocal results (Mosqueda et al., 2012).

Diagnosis of bovine babesiosis is an important tool for the control, prevention and avoiding dissemination of the disease. However, in chronically infected animals (intermediate) where a subclinical form of the disease occurs, more sensitive methods must be employed. In the present study, we analyze the efficiency of the Real Time Taqman®-PCR as a detection method for babesiosis, in order to establish its availability to the farmers in Puerto Rico and determine the frequency of the disease in the island.

1.2 *Babesia bovis* and *Babesia bigemina* taxonomy, life cycles and consequences

The genus *Babesia* belongs to the phylum Apicomplexa, class Sporozoasida, order Eucoccidiorida, suborder Piroplasmorina and family Babesiidae (Bock et al., 2004). Apicomplexan parasites exhibit a variety of morphological features that are considered diagnostic for this phylum. These protists have an elongated shape and a conspicuous specialization of the apical region that constitutes a collection of unique organelles termed the apical complex (Laha et al., 2015). These organelles include the rhoptries, the micronemes, dense granules, the conoid and the apical polar ring (Figure 1.1). Rhoptries, dense granules and micronemes are unique secretory organelles that contain products required for motility, adhesion to host cells, invasion of host cells, and establishment of the parasitophorous vacuole (Morrissette and Sibley, 2002). The conoid is a small cone-

shaped structure composed of a spiral of unidentified filaments that play a mechanical role in invasion of host cells (Morrissette and Sibley, 2002). The apical polar ring is a hallmark organelle of all members of the Apicomplexa, and serves as one of the three microtubule-organizing centers (MTOCs) in these parasites; spindle pole plaques and centrioles/basal bodies are the other MTOCs (Morrissette and Sibley, 2002). These spirally arranged microtubules are responsible for the serpentine body shape of apicomplexans. Subpellicular microtubules confer both elongated shape and apical polarity (Morrissette and Sibley, 2002).

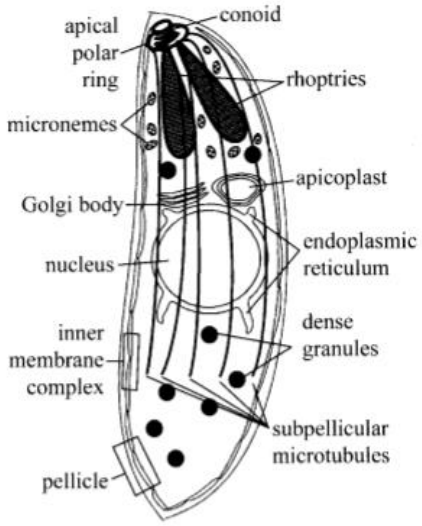


Figure 1-1: The morphology of apicomplexan parasites. (Morrissette and Sibley, 2002) Representation of the characteristical organelles of apicomplexan parasites.

In terms of external morphology, babesias are divided into two groups: the small babesias (1.0–2.5 μm long), which include *B. bovis* and the large babesias (2.5–5.0 μm long), which includes *B. bigemina* (Laha et al., 2015). More than 100 species of *Babesia* have been identified that are infecting many mammalian and some avian species, but *B. bovis* and *B. bigemina* are predominantly species affecting the cattle industry (Schnittger et al., 2012).

Naturally, all species of *Babesia* are transmitted through the bite of an infected tick (Uilenberg, G., 2006). Ticks are widely distributed throughout the world particularly in tropical and subtropical countries, and 80% of the world cattle are affected with ticks and ticks borne diseases (Ghosh et al., 2007). The ticks of genera *Boophilus*, *Rhipicephalus*, *Hemaphysalis*, *Hyalomma*, and *Ixodes* acts as vectors for the transmission of *B. bigemina* and ticks of genera *Boophilus*, *Rhipicephalus*, and *Ixodes* are responsible for transmission of *B. bovis* (Laha et al., 2015).

The development of *Babesia bovis* and *bigemina* follow similar patterns within the adult tick (Figure 1-2). It is known that the first evolutionary cycle starts when a tick ingest babesia- infected erythrocytes, most of the parasites degenerate and are destroyed but a specific stage of development named pre-gametocyte survives and evolves into gametocytes (Chauvin et al., 2009). After a few hours of ingestion, those gametocytes turn into elongated bodies with arrowhead shaped ray, those are now called gamonts or ray bodies that will fuse in the lumen of the tick's digestive tract to form an elongated zygote of 8-10μm in length (Chauvin et al., 2009). The growth of a spike-like arrowhead organelle facilitates cell penetration across the midgut cell membrane, it invaginates

around this organelle at the point of contact and lysed at the point of entry due to the action of enzymes released from a coiled structure in the invading parasite (Chauvin et al., 2009). Once the babesia zygote has been internalized, the arrowhead structure disintegrates and the zygote is transformed into a motile stage called the ookinete, which appears to be an haploid structure (Chauvin et al., 2009). Taking place the beginning of sporogony (meiosis), the ookinete escapes from the midgut epithelium and invades the tick's body tissues which will undergo transovarial transmission (in the ovaries of female ticks) where asexual multiplication will take place and the development of numerous kinetes (sporokinetes) will subsequently continue and remain dormant in eggs until larval stages where it will continue differentiation (Bock et al., 2004). At the same time, transtradial transmission will develop with the invasion of kinetes in the salivary glands of ticks, and the final cycle of development concludes with the production of sporozoites (Hollew et al., 2007). Sporozoites represents the infectious stage of the parasite and delineates the transmission between host and vector (Schnittger et al., 2012).

Once the vertebrate host is injected with the sporozoites found in the saliva of the infected tick, they will penetrate directly into the erythrocytes and all the parasitic stages will develop (Schnittger et al., 2012). The parasite produces two merozoites by binary fission, presented in figure 1.2. After erythrocyte lysis, each merozoite invades a new erythrocyte and successive morogonies (asexual reproduction) occurs (Chauvin et al., 2009).

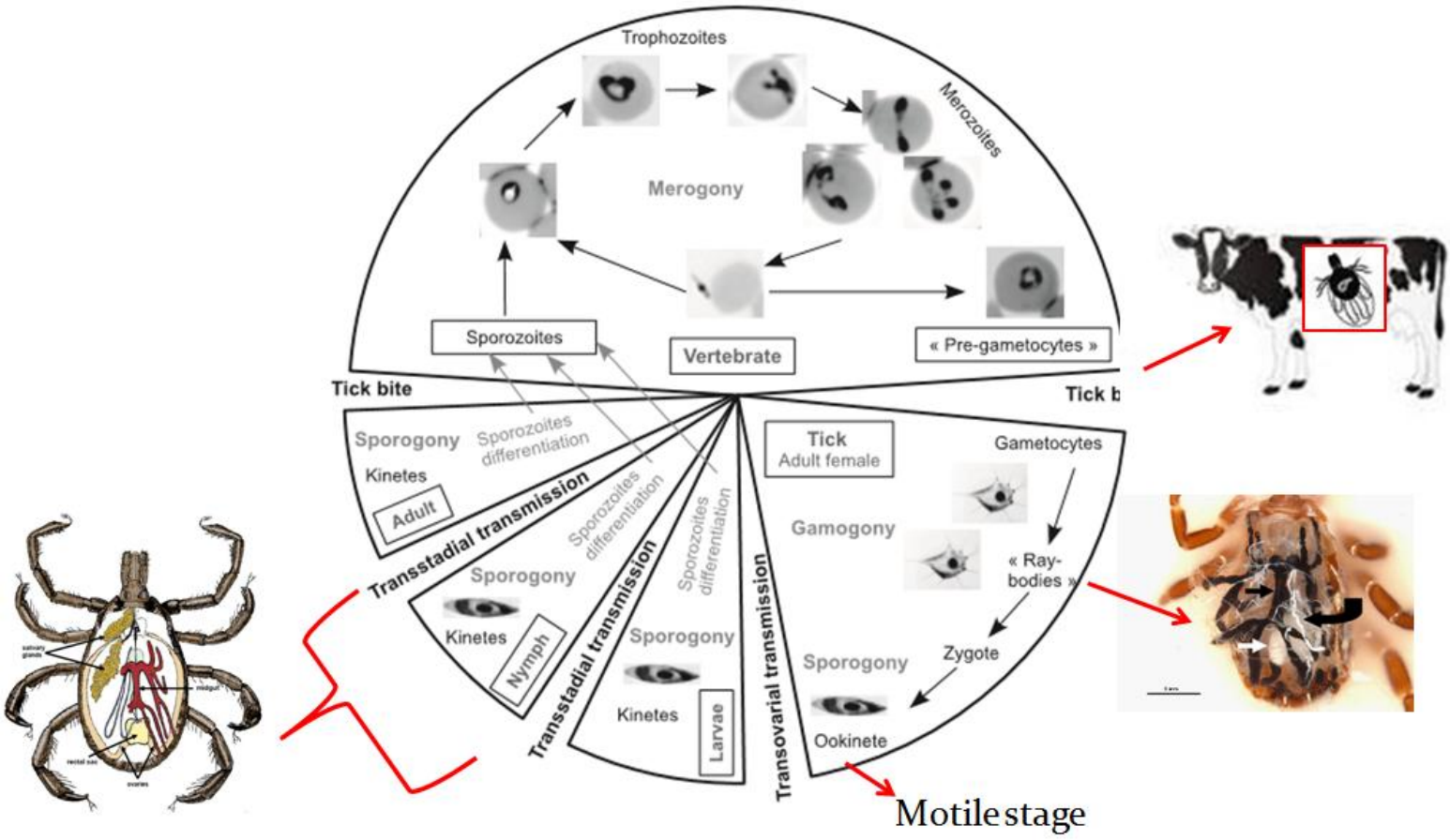


Figure 1-2: *Babesia spp*. Life cycle. Taken from Chauvin et al. (2009). Adapted by Natalia A. Rivera Diaz. Schematic representation of the life cycle of *Babesia spp.* hemoprotozoan parasite.

Babesiosis resembles other conditions that cause fever, and hemolytic anemia. The differential diagnosis includes anaplasmosis, trypanosomiasis, theileriosis, bacillary hemoglobinuria, leptospirosis, eperythro-zoonosis, rapeseed poisoning and chronic copper poisoning (Food Security and Public Health, 2008). Rabies and other encephalitis may also be considered in cattle with central nervous system clinical signs.

The clinical signs associated with babesiosis vary with the age of the animal, breed and strain of the parasite (Ukwueze and Orajaka, 2014) Most cases are seen in adults, animals younger than 9 months usually remain asymptomatic (Benavides and Sacco, 2007). The clinical signs attributed to *B.bovis* and *B.bigemina* are similar. Infected animals become inappetent, weak, lethargic and may separate from the rest of the herd. (Suarez and Noh, 2011). The characteristic signs are caused by hemolytic anemia, which develops rapidly and is frequently accompanied by hemoglobinemia and hemoglobinuria (Suarez and Noh, 2011). Consequently, the mucous membranes become pale, and respiration and heart rate increases (Shebish et al., 2012). As a result from the parasitic response, fever may cause decreased milk production, abortion in pregnant cows, and bulls present a temporary decrease in fertility (Uilenberg, 2006).

Typically, animals infected with *B. bigemina* develop anorexia and a high fever that may be present before other clinical signs appear (Ukwueze and Orajaka, 2014). Jaundice occurs mainly in subacute cases (Bock et al., 2004). Diarrhea or constipation may also been seen, and a respiratory distress syndrome with dyspnea can develop in severely affected animals (Brown et al., 2006). Usually some cattle die, but the animals

that survive the infection appear to be weak and in reduced condition, although they usually recover fully (Uilenberg, 2006).

It's been demonstrated that *Babesia bovis* is more pathogenic than *Babesia bigemina* due to the sequestration of infected erythrocytes to microcapillary endothelia of vital organs and a hypotense shock syndrome (Brown et al., 2006). Characteristically, the infected erythrocytes sequestered in brain capillaries, results in neurologic signs such as incoordination, teeth grinding and mania, and some cattle may be found on the ground with the involuntary movements of the legs. Most animals with central nervous system clinical signs die (Brown and Palmer, 1999; Ahmed, 2002).

1.3 Control, treatment and detection

The infectious agent for Babesiosis does not survive outside its hosts and can only be transmitted through a tick vector (Carter and Rolls, 2016). Therefore, the only control method to prevent propagation of the transmission is the eradication of the tick host in the herd. Farm management factors will determine the efficiency of disease control. This has been an issue debated in the agriculture industry of Puerto Rico for years. Options for tick control range from: proper fencing, sanitation, cattle movement restrictions and usage of acaricides. Since 2000, there has been no specific federal funding dedicated to a *R. microplus* re-eradication program for Puerto Rico; therefore, in recent years, funding for the eradication program was provided by the State (until 2010) (Urdaz et al., 2012).

The success of the treatment depends on early diagnosis and the prompt administration of effective drugs (Mosqueda et al., 2012). Many drugs have been reported to be effective against bovine babesia parasites, some of them being very specific and effective but eventually the pathogen developed resistance (Klafke et al., 2017). Also, many drugs have been withdrawn from use because studies demonstrated chemical residues in milk and muscle tissues (Vial and Gorenflot, 2006).

The first specific drug used against bovine babesiosis was trypan blue, which is a very effective compound against *B. bigemina* infections (Kuttler et al.,1981). However, it did not have any effect on *B. bovis* and it had the disadvantage of producing discoloration of animal's flesh, (Kuttler et al.,1981). For many years, the babesiacides: Quinuronium sulfate, Amicarbalide, Diminazene aceturate and Imidocarb diproprionate were used in most of Europe; however, Quinuronium sulfate and Amicarbilide were withdrawn because of manufacturing safety issues, and Diminazene, which is widely used in the tropics, was withdrawn from Europe for marketing reasons (Vial and Gorenflot, 2006). In addition, the product was also withdrawn from the market in Japan and its not approved by the Food and Drug Administration in U.S.A. (Goo et al., 2010). In the USA, Imidocarb is labeled for use only in dogs due to concerns of residues in food products (Urdaz, 2007).

Table 1.1 summarizes the drugs used for the treatment of bovine babesiosis. Most of these drugs are associated with residue problems. Several studies have demonstrated that chemicals are retained in edible tissues of ruminants for long periods after treatment which is related to resistance of the drug to biotransformation processes due to a strong

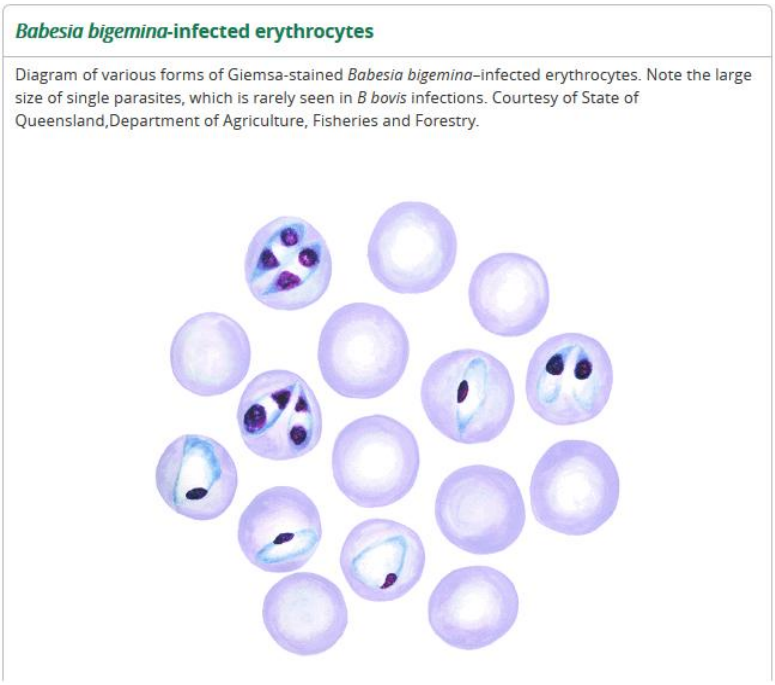
binding of the drug to nuclear components, causing the formation of large deposits principally in hepatocytes (Belloli et al., 2006). Other studies reported high concentrations in the milk, reflecting efficient passage through the blood-milk barrier (Mosqueda et al., 2012). Belloli et al. (2006) demonstrated a low mammary elimination of the drug in goats, probably associated with strong binding to mammary tissue.

Table 1-1: **Chemical Drugs Used to Treat Babesiosis.** Taken from Mosqueda et al., 2012. Adapted by Natalia A. Rivera Diaz

Compound	***Babesia spp.***
Imidocarb	*B. bovis* *B. bigemina* *B. divergens* *B. caballi*
Diminazene aceturate	*B. bovis* *B. bigemina* *B. divergens* *B. caballi*
Nerolidol	*B. bovis* *B. bigemina* *B. ovata* *B. caballi*
Artesunate	*B. bovis* *B. gibsoni* *B. caballi* *B. microti*
Triclosan	*B. bovis* *B. bigemina* *B. caballi*
Epoxomicin	*B. bovis* *B. bigemina* *B. ovata* *B. caballi* *B. microti*
Gossypol	*B. bovis*
Atovaquone	*B. divergens*

The necessity to identify diseases like babesiosis has become a primordial focus in the research of many disciplines in order to improve production in the dairy industry. There are many detection methods and analysis for the identification of hemoparasitic diseases, among these are direct and indirect methods.

As a direct method, a blood smear can be performed and the parasite inside the erythrocyte can be observed in a light microscope when cells are stained with Giemsa stain or acridine orange (with a fluorescent microscope) as shown in figure 1-3 (Urdaz, 2007). Even though, this test is easy to perform and inexpensive, it is most sensitive during the acute form when parasitemia levels can be detected for up to one infected erythrocyte per ten thousand cells, requiring the analysis of 100-200 fields, the equivalent to 0.5 μl of blood (Morzaria et al.,1992; Potgieter and Els, 1979). It additionally requires an experienced microscopist to differentiate species, this technique is also labor-intensive for large numbers of samples.



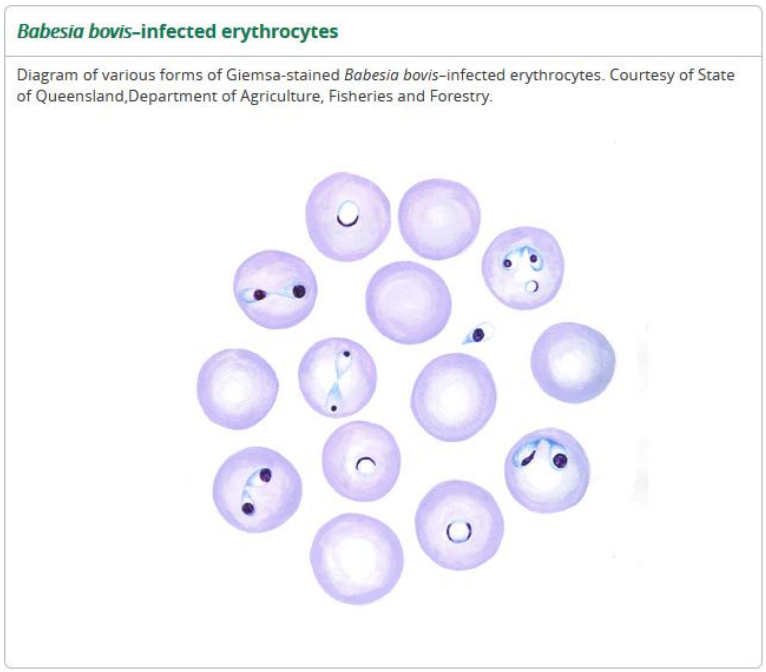


Figure 1-3 Diagrams of various forms of *Babesia bigemina* and *bovis*. (Carter and Rolls, 2016). Demonstrates different stages of babesiosis on a blood smear.

Special attention must be paid to the source of blood collected in the animal, because peripheral blood is useful for the identification *B. bigemina* species which do not adhere to the vascular endothelium (Aikawa et al.,1985). Species like *B. bovis* adhere to endothelial cells and the diagnosis provide the best results if blood collection is obtained from making thick blood smears from capillaries, obtained after pricking the tip or skin of the tail or margin of an ear (Bock et al., 2004; Mosqueda et al.,2012). Since capillary blood contains a higher percentage of infected erythrocytes, determination of *B. bovis* infections in thick smears is 10 times more sensitive than in thin smears because babesia-infected erythrocytes are more tightly packed together (Urdaz, 2007). As in figure 1-3, the observation of paired intraerythrocytic merozoites is indicative of infection, but there are other stages of the parasite like the trophozoites, which present different forms and sizes depending on the species, and make their detection difficult and time-consuming (Mosqueda et al.,2012). For all of those reasons, blood smears are not a reliable detection method for bovine babesiosis and more sensitive molecular techniques are available for disease detection.

Another detection method used in this category would be *in vitro* cultures hat consist of applying a tissue sample to identify the presence of the parasite when colonies are obtained in a culture plate (Bock et al., 2004). Detectable parasitemia will depend on the infrastructures that will allow efficient growth, and the investigators abilities to implement good protocol techniques to prepare the test (Roger and Trees, 1994).

Polymerase Chain Reaction (PCR) is another method that falls under the direct detection techniques, and can identify and differentiate the babesia species. The efficiency of the test has been demonstrated due to the sensitivity to provide identification with low levels of parasitemia with a small amount of sample (Fahrimal et al., 1992). The PCR consists of a combination of DNA sample with initial oligonucleotides, deoxyribonucleotide triphosphates and a thermostable DNA polymerase in a Thermal Cyler for the amplification of a target region in the DNA sample in order to indicate the presence of the parasite and quantify how much concentration is in the animal under study (Solorio and Rodríguez, 1997). In gel analysis of ethidium bromide-stained DNA is required to visualize a result. Even though is a great method, DNA-binding dyes, bind to all double stranded DNA. As a result, the presence of nonspecific products in the reaction, such as PCR primer-dimers, contributes to the overall fluorescence and reduces the accuracy of quantification (Lind et al., 2006).

An adaptation of the technique is the nested polymerase chain reaction (nPCR), in which two pairs of primers are used in two successive PCR amplifications where the second pair is intended to amplify a secondary target within the first amplified product (Mosqueda et al., 2012). A nested PCR has been effective for the detection of carrier animals infected with *B. bigemina* and the sensitivity has been reported to be as low as one infected erythrocytes (Figueroa et al.,1992). The nPCR is a very sensitive tool, but it is more expensive, takes more time and the risk of self contamination is higher because

detection of the product at the end of the reaction requires electrophoretic analysis (Mosqueda et al., 2012).

In this study we focused on the latest adaptation of the PCR technique, named Real Time Taqman® -PCR. It is a single step test where the amplified DNA is quantified as it is being generated in "real time", therefore it determines whether a specific sequence is present in the sample, and determines the number of copies of that sequence (Bio-Rad Laboratories, 2017). The Real Time Taqman®-PCR does this by detecting a fluorescent signal emitted during the reaction as an indicator of the production of the sequence being generated in each cycle (Mosqueda et al., 2012). In our case, we used a probe as a target-specific fluorescent indicator, resulting in increased specificity and sensitivity. The mechanism of the probe consists of a reporter fluorophore where its fluorescence is quenched when the specific target DNA sequence is not present (Bio-Rad Laboratories, 2017). Usually, this quenching is accomplished by covalently attaching a quencher molecule to the DNA primer or probe in combination with some mechanism by which the reporter and quencher are separated when the primer or probe binds to its specific target sequence, resulting in a fluorescence signal that is proportional to the amount of amplified product in the sample (Bio-Rad Laboratories, 2017).

Detecting specific antibodies is an indirect way to identify the parasite, although recent studies have demonstrated to be an effective detection technique (Goff et al., 2006), it has the disadvantage of relying on the presence of specific antibodies against those parasites which may take days or weeks to develop in an infected animal. In the same way, the antibodies might be present for months after the infection has disappeared, but are not detected by this method, making their usefulness very limited in acute disease cases because they tend to generate false-negative results (Mosqueda et al., 2012). Another drawback to serologic testing is that other protozoal parasites or infections might elicit cross-reactivity, generating false-positive results (Homer et al., 2000).

Urdaz (2007) described the previously used immunoassays for the detection of bovine babesiosis, including the following: Complement fixation (CF), Rapid card agglutination (CAT), Indirect fluorescent antibody (IFAT) and Enzyme-linked immunosorbent assays (ELISA). Adaptations of the mentioned tests are available, but they all rely on the same principle, a biochemical test that measures the presence or concentration of a molecule (antibody in the case of disease detection) when recognized or binded to a specific antigen in a solution (Ford, 2010). The molecule detected by the immunoassay is referred to as an analyte, which in many cases it's a protein, although it may be other kinds of molecules of different size and types, as long as it can de detectable. In some immunoassays, the analyte may be an antigen rather than an antibody (Goff et al., 2006). In addition, all immunoassays produce a measurable signal in response to the binding of molecules, commonly involving chemically linking antibodies

or antigens with a detectable label (Mosqueda et al., 2012). A large number of labels exist in modern immunoassays, and they allow for detection through different means. Many labels are detectable because they either emit radiation, produce a color change in a solution, fluoresce under light, or because they can be induced to emit light (Yolken, 1990).

CHAPTER 2 – METHODOLOGY

2.1 Animal Selection

The study was conducted in four commercial dairy cattle farms, located in the northeast and southeast part of Puerto Rico. A hundred and fifty eight cows over one year old were randomly selected for the collection of samples. For each animal, we collected whole blood samples.

Figure 2-1. Localization of farms under study. (In yellow diamonds).

The present study was supported by the Agricultural Research Service (ARS), and data collection was performed simultaneously with USDA research project that proposes to control tick infestation and propagation of bovine babesiosis using nPCR and ELISA as a detection method. As a result, comparative analysis of molecular techniques will evaluate their efficiency for detection of hemoparasitic diseases like babesiosis.

2.2 Blood sample collection and DNA extraction

Because this was a collaborative study with a federal agency, information about the farmers or methodology details for the Real Time Taqman®-PCR protocol, will not be disseminated. A confidential agreement has been signed with each farm under study except the Agricultural Dairy Station in Lajas. A patent for the Real Time Taqman® -PCR protocol is pending under the property and protection of the ARS as a detection method for bovine babesiosis in their Cattle Fever Tick Research Laboratory in Texas.

From each animal under study, 5 ml of blood sample were collected aseptically via coccygeal venipuncture into a 6 ml EDTA purple top tube (Plastic Vacutainer, Fisher Scientific™) using disposable needles of 20 gauge x 1 inch. Samples were labeled and transported on ice to the laboratory facilities at the University of Puerto Rico Mayagüez Campus, they were stored in a -30 °C freezer until further analysis.

From each sample, 200µl of whole blood was used to extract DNA using E.Z.N.A.® Blood DNA Mini Kit (Omega Bio-tek). DNA concentration analysis was performed by spectrophotometry using a NanoDrop 2000c (Thermo Scientific ™). The extracted DNA was stored at -30°C and shipped in a cooler with icepacks, to ARS Cattle Fever Tick Laboratory in Texas (CFTRL). Once in the CFTRL, the samples were stored at -20°C until use for Real Time Taqman®-PCR analysis.

Table 2-1. Overall amount of blood samples collected in premises.

Sample Collection	
Premises	**Total Samples**
San Sebastian	44
Isabela	40
Moca	35
Lajas-UPRM	39
n=158	

2.3 Selection of Primers for Real Time Taqman®-PCR

Based on earlier studies about detection of *Babesia bovis* and *Babesia bigemina*, selection of primers was based on the study conducted by Kim et al. (2007). As shown in Table 2-2, to detect the presence of Babesia parasites, amplification with the selected sequence of primers and probes for the 18S ribosomal RNA (rRNA) gene was performed.

Table 2-2. Primers and probes for detection of *Babesia bovis* and *Babesia bigemina*

Taken from Kim et al. (2007).

Primers and probes selected for TaqMan PCR of 18S rRNA gene of *Babesia* parasites

Species	Primer or probe	Sequence (5′-3′)	Nucleotide position*
B. bovis	BoF	AGCAGGTTTCGCCTGTATAATG	651–673
	BoR	AGTCGTGCGTCATCGACAAA	786–805
	BoP probe (FAM)	CCTTGTATGACCCTGTCGTACCGTTGG	685–712
B. bigemina	BiF	AATAACAATACAGGGCTTTCGTCT	436–459
	BiR	AACGCGAGGCTGAAATACAACT	588–609
	BiP probe (VIC)	TTGGAATGATGGTGATGTACAACCTCA	465–491

* The nucleotide positions are those reported in GenBank (accession numbers, AY603398 for *B. bovis* and AY603402 for *B. bigemina*).

2.4 Optimization of Real Time Taqman®-PCR as a detection method for *Babesia bovis* and *Babesia bigemina*

Because previous experiments using Real Time PCR to detect *Babesia bovis* and *Babesia bigemina* are not commercially available, they have only been validated in the laboratory of study. For that reason, a protocol for optimization and validation of the Real Time Taqman®-PCR, was conducted in the CFTRL in Texas.

To completely optimize Real Time Taqman®-PCR protocol for detection of cattle babesiosis in this project, positive controls were designed by in vitro cultures of nPCR products of positive infected animals, provided by the ARS. After acquired growth, plasmids extraction and purification was obtained with the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) and DNA concentration was quantified. The Real Time Taqman®-PCR Melt Curve analysis was performed with the previously mentioned primers and probes sequences. As shown in figure 2-2, different dilutions of the positive controls were analyzed with the Melt Curve graph. The best consistent result of amplification with a given temperature was selected for use in this study.

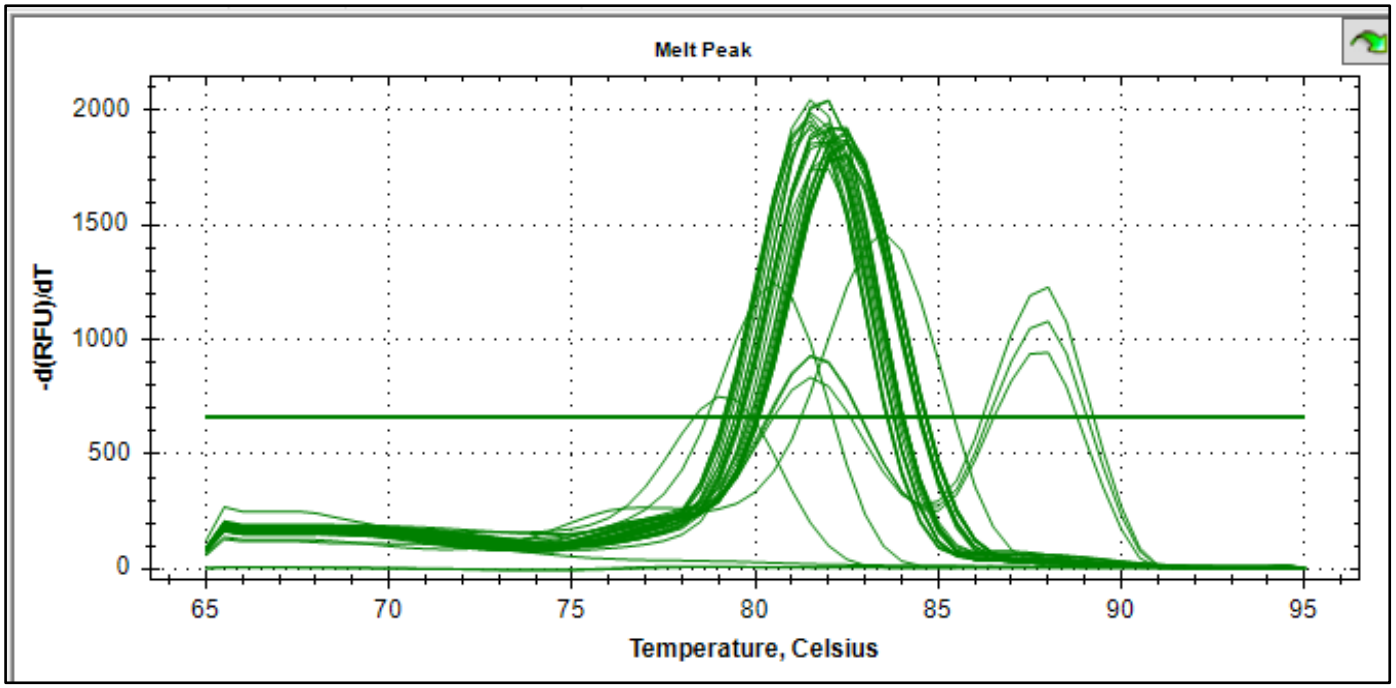


Figure 2-2. Real Time Taqman®-PCR Melt Curve for *Babesia bovis* and *Babesia bigemina.*

Electrophoresis gel (The FlashGel™, Lonza) confirmed amplification of correct sequence fragments obtained from the Real Time Taqman®-PCR Melt Curve products (figure 2-3,) 154bp for *B. bovis* and 173bp for *B.bigemina*.

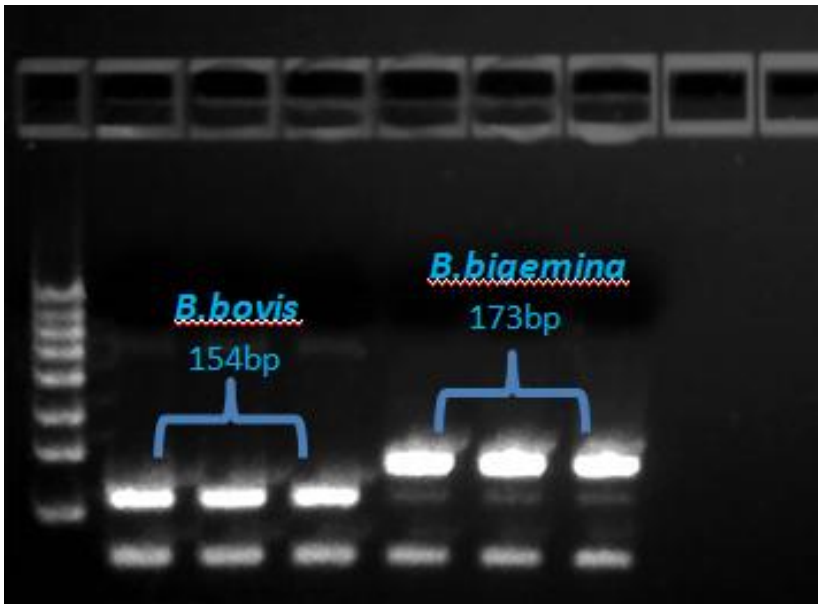


Figure 2-3. Electrophoresis gel of Melt Curve products for *Babesia bovis* and *Babesia bigemina*

2.5 Real Time Taqman®-PCR Performance

Intellectual property protects the dissemination of information until patent approval under the property and protection of the Cattle Fever Tick Research Laboratory in Texas. Information for the specifications in the protocol of the Real Time Taqman®-PCR for detection of *Babesia bovis* and *Babesia bigemina*, will not be disclosed.

2.6 Statistical Analysis

Statistically, an observational study with sample design was conducted with a Multistage Stratified model. Three stages we divided in: Strata (PR regions), Conglomerates (municipality/dairy farms) and Units of interest (cattle).

A McNemar's Chi-square test was performed for the paired data results and methods that accounted for the correlated binary outcomes. Contingency 2x2 tables were constructed with the paired observations of the nPCR and Real Time Taqman®-PCR results. Sensitivity and specificity were evaluated for the two diagnostic test used in this study for detection of *B.bovis* and *B.bigemina*, with the SAS/STAT, university edition version. In addition, a PROC FREQ determined the significance of the test methods under study, using an α=0.05.

CHAPTER 3 – RESULTS

3.1 Contingency tables

The overall results for the nPCR and Real Time Taqman®-PCR from all premises are resumed in table 3-1. Both methods were performed in triplicates with a cut off score, 2 out of 3 that determined true positives.

Table 3-1. Results summary of nPCR and Real Time Taqman®-PCR results from all premises

		Data provided by ARS-CFTRL		**Data found in the present study**	
Premises	**Total Samples**	**nPCR *B.bovis***	**nPCR *B.bigemina***	**RTT-PCR *B.bovis***	**RTT-PCR *B.bigemina***
San Sebastian	44	10	3	12	4
Isabela	40	0	0	0	0
Moca	35	0	0	1	0
Lajas-UPRM	39	1	0	2	0

Contingency tables were constructed from the results in table 3-1 and arranged in the following manner as demonstrated in table 3-2.

Table 3-2. Contingency Tables

Babesia bovis			***Babesia bigemina***		
nPCR	**RTT -PCR**	**Count**	**nPCR**	**RTT -PCR**	**Count**
1	1	11	1	1	3
1	2	0	1	2	0
2	1	3	2	1	1
2	2	144	2	2	154

The number 1 corresponds to the results that were positive, and the number 2 for the negative results. RTT-PCR is an abbreviation for Real Time Taqman®-PCR.

3.2 McNemar's test for *B. bovis* and *B.bigemina*

The SAS/STAT PROC FREQ analysis with the McNemar's Chi-square test, indicate the following results as shown in the tables.

Table 3-3. McNemar's Chi-square results for B. bovis

The FREQ Procedure

Table of test1 by test2			
test1	**test2**		
Frequency Percent Row Pct Col Pct	**positive**	**negative**	**Total**
positive	11 6.96 100.00 78.57	0 0.00 0.00 0.00	11 6.96
negative	3 1.90 2.04 21.43	144 91.14 97.96 100.00	147 93.04
Total	14 8.86	144 91.14	158 100.00

Statistics for Table of test1 by test2

McNemar's Test	
Statistic (S)	3.0000
DF	1
Asymptotic Pr > S	0.0833
Exact Pr >= S	0.2500

Sample Size = 158

The value of specificity obtained from the results provided indicate a 100 percentage of healthy individuals who correctly received a negative result for detection of *Babesia bovis* from test 1 over test 2. Due to this high percentage of specificity, the false positive probability resulted as zero with a positive predictive value of 100. Overall, there are no false positives from test 1 over test 2, both methods are highly specific for detection of *Babesia bovis*.

However, the value of sensitivity resulted in 78.57 %, indicating the infected animals who correctly received a positive result from test 1 over test 2. The negative predictive value was 97.96 %, with a false negative probability of 21.43 % for the 14 observations obtained. This means that 3 out of the total 14 positive results were false negatives for *Babesia bovis* detected with nPCR when tested with Real Time Taqman® PCR , demonstrating that Real Time Taqman® PCR is slightly more sensitive for detection.

The McNemar's Chi-square p value resulted 0.0833, demonstrating no significant difference between test 1 over test 2 for detection of *Babesia bovis* in dairy cattle.

Table 3-4. McNemar's Chi-square results for *B. bigemina*

The FREQ Procedure

Table of test1 by test2			
test1	**test2**		
Frequency Percent Row Pct Col Pct	**positive**	**negative**	**Total**
positive	3 1.90 100.00 75.00	0 0.00 0.00 0.00	3 1.90
negative	1 0.63 0.65 25.00	154 97.47 99.35 100.00	155 98.10
Total	4 2.53	154 97.47	158 100.00

Statistics for Table of test1 by test2

McNemar's Test	
Statistic (S)	1.0000
DF	1
Asymptotic Pr > S	0.3173
Exact Pr >= S	1.0000

Sample Size = 158

The value of specificity indicates a 100 percentage of healthy individuals who correctly received a negative result for detection of *Babesia bigemina* from test 1 over test 2. Due to this high percentage of specificity, the false positive probability resulted as cero with a positive predictive value of 100. Overall, there are no false positives from test 1 over test 2, both methods are highly specific for detection of *B. bigemina*.

However, the value of sensitivity resulted in 75.00 %, indicating the infected animals who correctly received a positive result from test 1 over test 2. The negative predictive value was 99.35 %, with a false negative probability of 25.00 % for the 4 observations obtained. This means that 1 out of the total 4 positive results were false negatives for *Babesia bigemina* detected with nPCR over Real Time Taqman® PCR , demonstrating that Real Time Taqman® PCR is slightly more sensitive for detection.

The McNemar's Chi-square p value was 0.317, demonstrating no significant difference between test 1 over test 2 for detection of *Babesia bigemina* in dairy cattle.

3.3 Discussion

Both detection methods resulted with 100% of specificity with discrepancies of sensitivity for detection of *B.bovis* and *B.bigemina*. Statistical analysis demonstrate there is no significant difference between tests, this could be as a result of a low amount of positives in the total quantity of samples analyzed by the McNemar's test model. However Real Time Taqman® -PCR resulted to be slightly more sensitive for detection of both hemoparasitic diseases.

Even though statistically there is no significant difference, the time consumed in the process of preparation and performance of the Real Time Taqman®-PCR, is far shorter than the nPCR. Real Time Taqman®-PCR takes approximately 2 hours from the moment of preparation to the submission of results. On the contrary, nPCR takes approximately 5.5 hours to do the same. Additionally, the risk of contamination is higher with the nPCR because it is a two step protocol that also requires electrophoretic analysis and photo-documentation (Mosqueda et al., 2012).

In terms of prevalence, tests results indicated a higher estimate of animals carrying *Babesia bovis* hemoparasite. As demonstrated in table 3-1, San Sebastian premise have the highest frequency of cattle with the pathogen infection. Even though this farm is relatively near other premises, such as Moca and Isabela, the presence of Babesia can be attributed to management, tick control program in the farm and ability to receive proper veterinary assistance for disease control (Urdaz, 2006).

CHAPTER 4 - CONCLUSION

Conventional techniques for detection of cattle babesiosis have been substituted by molecular methods that aimed to detect a specific sequence of nucleic acids, providing higher sensitivity and specificity. Different approaches of molecular methods for detection were performed to improve the delivery of a highly efficient result in less time. Being a one step protocol that detects a positive fluorescent signal in the thermocycler, Real Time Taqman®-PCR is an improved technique that provides results with large quantities of samples, this have been a tedious process when nPCR was used for disease identification.

In this study, the identification of *Babesia bovis* and *Babesia bigemina* was efficiently determined by the use of a multiplex Real Time Taqman® -PCR as a detection method for a large amount of samples in less time. It successfully detected the frequency of the *Babesia bovis* species in the premises evaluated throughout the study.

For future research, the analysis of a larger quantity of subjects is recommended in order to establish statistical difference between test methods. Furthermore, a wider selection of premises that would cover the four cardinal points of the island could provide a better understanding of the status of cattle babesiosis in Puerto Rico.

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