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Epidemiological study of tick-borne diseases in cattle in Minas Gerais, Brazil

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Dedico aos animais,

mais nobres habitantes deste planeta

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I. INTRODUCTION

The complex of the three pathogens, the bacteria *Anaplasma marginale* and the protozoan parasites *Babesia bovis* and *Babesia bigemina,* is a tick-borne disease responsible for important economic losses in the cattle industry in tropical and subtropical areas of the world (Barros et al., 2005). In Brazil in many cases anaplasmosis and babesiosis are considered as one complex of disease popularly known as "Tristeza Parasitária Bovina" (TPB). It is agreed upon that the economic impact of TPB consists not only in direct losses like abortion, mortality and reduction in milk and meat yield but also in costs for treatment, control measures and prevention (Ribeiro and Passos, 2002). In Latin America recent studies estimated that bovine anaplasmosis and babesiosis are responsible for an annual economic losses of $800 to $875 million (Kocan et al., 2003). In South and Central America the main vector of the three agents is the tick *Rhipicephalus (Boophilus) microplus,* in addition, *A. marginale* can be transmitted mechanically by other arthropods or blood-contaminated fomites and congenitally (Dikmans, 1950; Ewing, 1981; Guglielmone, 1995). In most areas of Brazil the vector *R. (B.) mircoplus* is present throughout the year implicating an infection with TPB in almost all cattle resulting in a natural endemic stability (Kessler and Schenk, 1998). The animals already develop an immunity in the first months of life thus clinical cases and mortality are very low in areas with endemic stability (Soares et al., 2000; Souza et al., 2000a; Souza et al., 2000b). However, in Brazil epidemiological studies indicated outbreaks with high incidences of clinical cases, leading to the adverse effect of areas of endemic instability (Araújo et al., 1998; Ribeiro and Reis, 1981). According to Madruga et al. (1983) recent adoptions of sanitary management such as frequent use of acaricide products as well new techniques in handling, feeding and breeding lead to less and delayed contact of the cattle with the vectors of *A. marginale*, *B. bovis* and *B. bigemina* facilitating the appearance of areas of endemic instability.

Another problem related to TPB is the restriction to import cattle from temperate areas into tropical areas with the intention to improve the production of milk and meat. The importation of animals without immunity to tick-borne-diseases like TPB, leads to significant losses if prevention measures like vaccination were not taken (Lima, 1991). The livestock population of Brazil is estimated to include 209 million head of cattle and the state of Minas Gerais is one of the biggest producer of milk in the country

contributing with an annual production of 27.3% (IBGE, 2010). Even though in nowadays TPB is still a tropical occurrence, in future the distribution of vector-borne diseases may be expected to change in part as a result of climate changes and by this expand diseases more over the world. Therefore the research of the disease does not only contribute to a wide knowledge but also a better understanding for the control and prevention of the disease. Although several studies have been done regarding the epidemiological situation of anaplasmosis and babesiosis in determinate regions of Brazil (Barros et al., 2005; D´Andrea et al., 2006; Folly et al., 2009; Guglielmone, 1995; Madruga et al., 1983; Soares et al., 2000; Souza et al., 2000b), little is known about specific diversity and occurrence of TPB within an individual herd. Therefore this study aimed to characterize the occurrence of tick-borne diseases within a herd in an endemic region in Brazil, focusing on two different aspects:

(a) to determine the occurrence of the three pathogens *A. marginale*, *B. bovis* and *B. bigemina* using direct and indirect detection methods and

(b) to analyze the incidence of genetic diversity among *A. marginale* isolates based on a Major Surface Protein (MSP) as a marker of variation within the farm.

The understanding of the epidemiology of TPB including the characterization of the genetic diversity of strains of *A. marginale* provides knowledge for the development and implementation of control and prevention measures and will contribute to a better understanding of the dynamic and epidemiological features of the studied pathogens.

II. LITERATURE REVIEW

1. *Anaplasma marginale*

1.1. Taxonomy and morphology

One of the three agents of TPB is *A. marginale*, which causes the infectious but not contagious disease bovine anaplasmosis (Theiler, 1910). The classification of this agent remained undefined and contested for a long time. In 1893 Smith and Kilborne, while studying the biological cycle of *B. bigemina,* described bodies with a coccoid structure at the periphery of the erythrocytes, which they thought to be a phase of the *B. bigemina* life cycle. Foote et al. (1958) suggested that the inclusion bodies of *A. marginale* were the product of a cellular reaction in the erythrocytes caused by a virus. After studies with phase contrast and electron microscopy (Espana et al., 1959) *Anaplasma* was described as a protozoan, and it was only in 1961 that Ristic and Watrach discovered the *Anaplasma* initial bodies belonged to a rickettsia due to their binary division and coloration.

According to the emended description of Dumler et al. (2001) *A. marginale* belongs to the domain of Bacteria and after the recent reclassification of the order Rickettsiales, to the family Anaplasmataceae. The organisms were reclassified based upon genetic analyses of 16S rRNA, groESL and surface protein genes and assigned to one of two families: Anaplasmataceae or Rickettsiaceae. Among the Anaplasmataceae genetic analyses supported the formation of four distinct genetic groups: *Anaplasma, Ehrlichia, Wolbachia,* and *Neorickettsia*. Organisms placed in the family Anaplasmataceae are obligate intracellular bacteria that replicate while enclosed in a eukaryotic host cell membrane-derived vacuole. Moreover, for each species of these genera for which sufficient study has been accomplished, an invertebrate vector host has been identified, predominantly ticks or tremadotes, except for *Wolbachia*, which are also found in a variety of helminths (Dumler et al., 2001; Rikihisa, 1991).

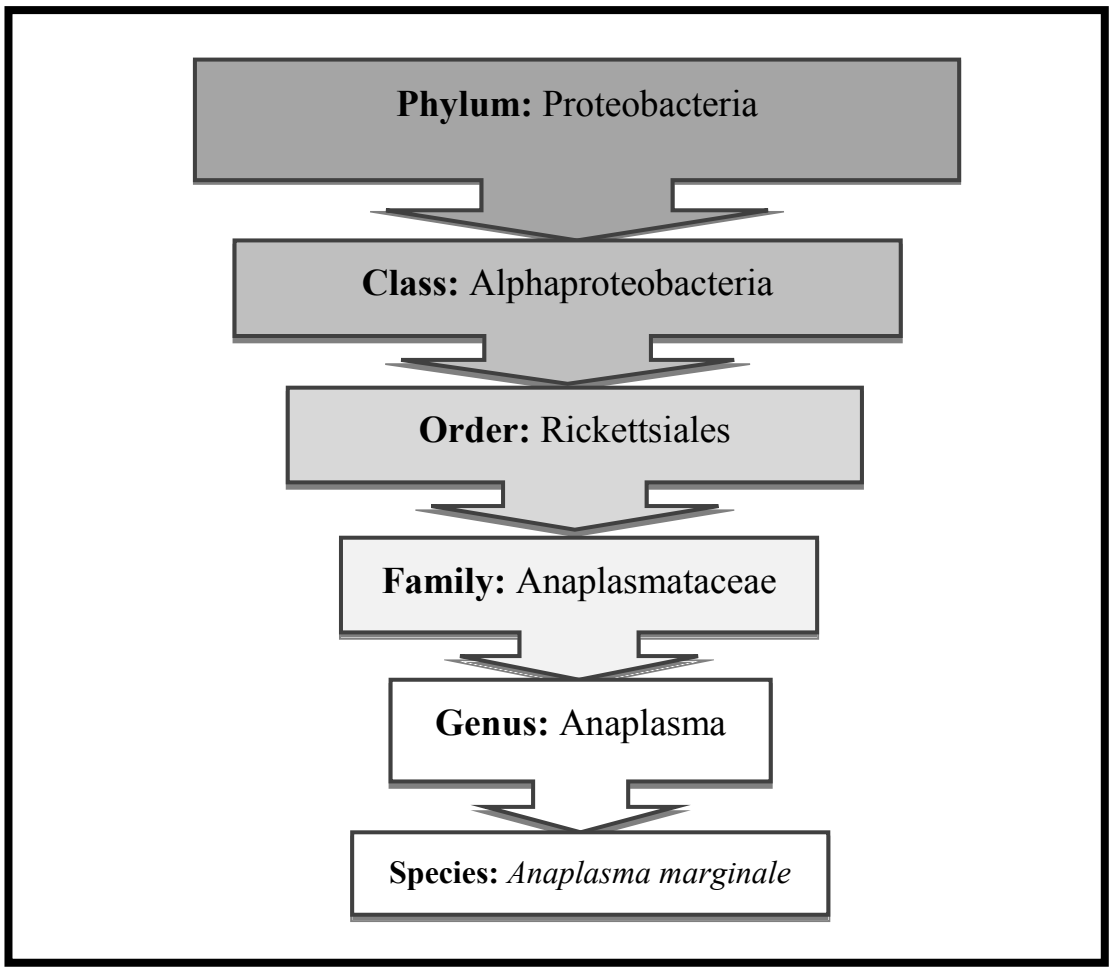

Figure 1: Taxonomy of the genus *Anaplasma* (adapted from Dumler et al., 2001)

In addition to *A. marginale,* another *Anaplasma* species found in cattle is *Anaplasma centrale* (Theiler, 1911). *A. centrale* appears to be less pathogenic than *A. marginale,* and its inclusions are often found in the centre of erythrocytes, as opposed to at the margin for *A. marginale*. After infection with *A. centrale,* cattle show partial protection against infection with *A. marginale*, due to cross immunity between the two species. *A. centrale* has been used as a live vaccine in many countries such as Israel, South-Africa, Australia, and parts of South America (de la Fuente et al., 2005; Ribeiro and Passos, 2002). Morphologically the genus *Anaplasma* can be described as a gram-negative, small, often pleomorphic coccoid to ellipsoidal organisms that reside within cytoplasmic vacuoles, either singly and more often in compact inclusions (morulae). In mammalian hosts they are present in mature or immature haematopoietic cells, particularly myeloid cells and erythrocytes, in peripheral blood or tissues as well as organs of the mononuclear phagocyte system (Dumler et al., 2001).

1.2. Geographic distribution

Anaplasmosis is distributed worldwide throughout tropical and subtropical areas of South, Central and Nord America, Australia, Asia and Europe (Aubry and Geale, 2011). In the U.S.A., anaplasmosis has been reported in almost every state; this widening distribution may result from transport of carrier cattle and subsequent mechanical or biological transmission to susceptible cattle (Kocan et al., 2010). In all Latin America countries, as well as in the Caribbean Islands, anaplasmosis is enzootic with the exception of desert areas and certain mountain ranges (Andes) (Guglielmone, 1995). Temperature is one predominant factor for the development of the ticks and consequently for the epidemiology of *Anaplasma* (Kessler and Schenk, 1998). Global warming may influence the movement of the tick vectors and accordingly the distribution of anaplasmosis cases (Jonsson and Reid, 2000).

1.3. Transmission and life cycle

A. marginale can be transmitted to a different number of domestic and wild ruminants such as cattle, water buffalo, bison, African antelopes, deer, sheep and goats; however, clinical disease is only notable in cattle (Kuttler, 1984). Cattle of any ages can become infected and remain persistently infected for life (Aubry and Geale, 2011).

Transmission can be biologically by ticks, mechanically by biting flies or blood-contaminated fomites, and transplacentally from cow to calf (Dikmans, 1950; Ewing, 1981; Ribeiro et al., 1995). Worldwide, biological transmission is considered the most frequent mechanism, with approximately 20 species of ticks acting as vectors (Kocan et al., 1981). However, South America is an exception, with mechanical transmission considered to be more important there (de Rios et al., 1990; Ruiz et al., 2005). The one-host-tick *R. (B.) microplus* is estimated to be the main vector of *A. marginale* in Brazil (Kessler and Schenk, 1998; Ribeiro and Passos, 2002).

Tick transmission can occur within a stage (intrastadial) or from stage to stage (transstadial), while transovarial transmission does not appear frequently under natural conditions (Stich et al., 1989). Mechanical transfer frequently occurs via fresh erythrocytes from needles, dehorning-saws, nose-tongs, tattooing equipment, ear-tagging devices and castration instruments. Mechanical transmission by arthropods has also been reported, for bloodsucking diptera of the genera *Tabanus, Stomoxys, Culex* and *Aedes* (Ewing, 1981; Foil, 1989; Potgieter et al., 1981). Furthermore *A. marginale* can be transmitted from cow to calf transplacentally during gestation

(Norton et al., 1983; Zaugg, 1985; Zaugg and Kuttler, 1984). A South African study reported a 15.6% incidence of *in utero* transmission of *A. marginale* or *A. centrale* during the first, second or third trimester of gestation (Potgieter and Van Rensburg, 1987). Transplacental transmission of *A. marginale* may therefore contribute to the epidemiology of this disease in some regions (Kocan et al., 2003).

The life cycle of *A. marginale* begins with the invasion of erythrocytes, which are the most common site of infection in cattle and other vertebrate hosts (Kocan et al., 2003). Initial bodies adhere to the surface of the erythrocytes and penetrate them by invaginating the cytoplasmatic membrane, resulting in a parasitic vacuole. Inside the vacuole, the initial bodies reproduce by binary fission to form four to eight rickettsiae. Subsequently the rickettsiae leave the membrane bound-inclusions by reverse phagocytosis, without disrupting the erythrocyte membrane, to start a new cycle of infection (Schnieder, 2006).

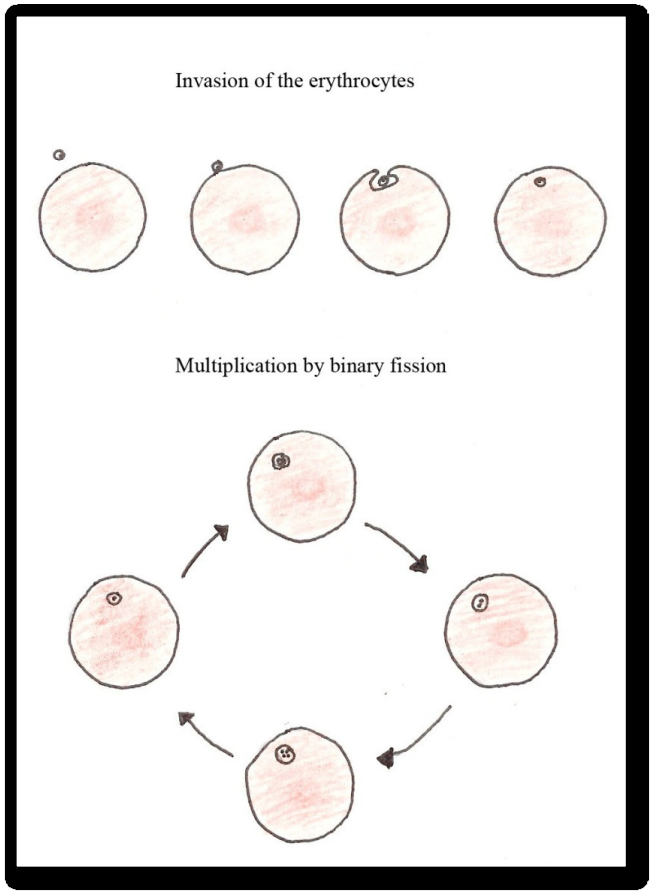

Figure 2: Life cycle of *Anaplasma marginale* (A. Pohl)

In the tick the developmental cycle of *A. marginale* is complex and coordinated with the ticks feeding cycle (Kocan et al., 1992a; Kocan et al., 1992b). Ticks ingest infected erythrocytes with the blood meal, resulting in a first site of infection in the gut cell; subsequently many other tick tissues become infected, including the salivary glands. From the salivary glands the rickettsia can be transmitted to ruminants as the tick feed (Ge et al., 1996; Kocan et al., 1992a). At each site of infection in ticks, *A. marginale* develops within membrane-bounds colonies or vacuoles into two forms. The first form within the colony is the reticulated (vegetative) form which divides by binary fusion to form hundred of organisms. The reticulated form changes into the dense form, which can survive for a limited period outside the host cell, and is the only infective form, passed from tick salivary glands to the vertebrate host during tick feeding (Kocan et al., 1992a).

The transovarial transmission of *A. marginale* by the tick *R.* (*B.) microplus* has been investigated in different studies, but no evidence were found confirming that the mechanism occurs in natural conditions (Kocan et al., 1992b; Leatch, 1973; Ribeiro and Lima, 1996) .

However, research has related the occurrence of transstadial and intrastadial transmission of *A. marginale* for the monogenetic tick *R. (B.) microplu*s (Connell and Hall, 1972).

1.4. Clinical symptoms

According to Kocan et al. (2003) the incubation period depends on the number of infective organism and ranges from seven to 60 days. After infection *A. marginale* invades erythrocytes, undergoes cycles of replication and is principally cleared from the body by macrophages of the reticuloendothelial system in the spleen. The removal of these infected erythrocytes by phagocytosis results in development of anemia and icterus without hemoglobinemia and hemoglobinuria (de la Fuente et al., 2001b). In the acute phase of infection 10% to 90% of the erythrocytes may be infected, depending upon the susceptibility of the host and the strain of *Anaplasma* (Aubry and Geale, 2011).

Clinical symptoms may include fever, weight loss, abortion, lethargy, anorexia, lower milk production, retardation of growth and death (Kessler and Schenk, 1998). The disease severity is associated with the degree of anemia, and includes pallor of the mucous membranes and increased heart and respiratory rates. The packed cell volume

declines in correspondence with increasing parasitemia (Kocan et al., 2010). *A. marginale* can infect cattle of all ages, but the severity of disease is age-dependant. For calves under six months illness is rare, but between the ages of six to 12 months they usually develop mild disease. Animals from one to two years of age suffer from acute but rarely fatal disease, but in adults over two years the illness is acute and often fatal (Aubry and Geale, 2011). Concerning cattle breeds, *Bos taurus* (Holstein, Brown Swiss, Hereford) are more likely to develop acute anaplasmosis than crossbred Zebu or Creole cattle (Aguirre et al., 1988). Cattle that recover from acute infection develop persistent infections characterized by cyclic low–level rickettsemia that cannot be detected microscopically (Kieser et al., 1990; Zaugg et al., 1986). In most instances carrier cattle (persistently infected cattle) have lifelong protection against clinical disease, (from homologous strains), but incidences of clinical cases can be associated with bimonthly increases of rickettsemia levels. These increases are due to cyclical multiplication of *A. marginale* caused by generation antigenic variants not recognized by the immune system of the host (Kieser et al., 1990) or because of other immunosuppressive factors (Ribeiro and Passos, 2002).

Once animals become infected, they serve as a reservoir for arthropod-borne transmission of *A. marginale* to susceptible cattle (Kieser et al., 1990; Zaugg et al., 1986).

1.5. Genetic diversity

A. marginale is currently the most completely characterized rickettsial pathogen in both its vertebrate and tick hosts (de la Fuente et al., 2001a). A recent study (Brayton et al., 2005) completed the sequence of the St. Maries strain of *A. marginale* genome and found it has a length of 1.197.687 bp. The sequencing of the complete genome showed that two superfamilies containing immunodominant proteins dominate the surface coat: the *msp*2 superfamily and the *msp*1 superfamily. Of the 949 annotated coding sequences in this *A. marginale* genome, 62 were predicted to be outer membrane proteins and only 49 belong to the major surface proteins *msp*1 and *msp*2 superfamilies. Major surface proteins are involved in parasite interactions with both vertebrate and invertebrate hosts and play a crucial role in their ability to cause infection (Aubry and Geale, 2011). Therefore, these genes are likely to evolve more rapidly than others because they are exposed to selective pressures exerted by two different host immune systems (Kocan et al., 2010). Another explanation for the genetic heterogeneity observed among *A. marginale* strains in endemic regions could

be cattle movement and maintenance of different genotypes by independent transmission events.

Six MSPs have been identified of *A. marginale* derived from bovine erythrocytes and were found to also be conserved on tick and cell culture-derived organisms. Three of these MSPs, namely MSP1a, MSP4 and MSP5, are from single gens, and do not vary antigenically within isolates, while MSP1b, MSP2 and MSP3 are from multigene families and may vary antigenically, most notably in persistently infected cattle (Kocan et al., 2003). These six proteins are responsible for the production of antibodies in animals that have been naturally infected or vaccinated with the initial bodies of *A. marginale* (Tebele et al., 1991a). The MSP1 complex has been shown to induce protective immunity in immunized cattle against homologous or heterologous challenge with geographically distinct *A. marginale* strains (Palmer et al., 1989). MSP1 is a heterodimer composed of two structurally unrelated proteins: MSP1a which is encoded by a single-copy gene, *msp1α* (Allred et al., 1990), and MSP1b which is encoded by members of the *msp1β* multigene family which varies within the strain (Barbet and Allred, 1991). MSP1a has been shown to be involved in adhesion to bovine erythrocytes and tick cells, whereas MSP1b which is encoded by at least two genes, *msp1β1* and *msp1β2*, has been suggested to be an adhesin only for bovine erythrocytes and not for tick cells (de la Fuente, 2001a; McGarey and Allred 1994a; McGarey et al., 1994b).

Structure of msp1α:

Msp1α has been found to be a stable genetic marker for the identification of *A. marginale* isolates within geographical regions, conserved in individual animals during the acute and chronic phases of infection, and before, during and after tick transmission (Palmer et al., 2001). Characterization of the function and evolution of MSP1a is essential for understanding host-parasite interactions of *A. marginale* and other rickettsial pathogens. The molecular weight of MSP1a varies among different *A. marginale* isolates due to a variable number of tandem 23-31 amino acids repeats located in the amino–terminal portion of the protein (de la Fuente et al., 2007). Variation in the sequence of the tandem repeats occurs within and among isolates, and may have resulted from evolutionary pressure exerted by ligand-receptor and host-parasite interactions. The slipped-strand mispairing (SSM) that occurs during replication in many organisms may have been the mechanism for the origin of tandem

repeats in *msp1α* (de la Fuente et al., 2001a). The *msp1α* genes are composed of an upstream promoter region and coding sequences for the MSP1a polypeptide (Allred et al., 1990).

Another characteristic of the *A. marginale* MSP1a is the microsatellites sequences which are located in the MSP1a 5`UTR between the Shine-Dalgarno sequence (GTAGG) (de la Fuente et al., 2001a) and the translation initial codon (ATG). A recent study (Estrada-Peña et al., 2009) demonstrated that microsatellites sequences were associated with world ecoregion clusters with specific and different environmental envelopes. This provides another piece of fundamental information toward understanding the evolution of the vector-borne pathogens.

2. *Babesia bovis, Babesia bigemina*

2.1. Taxonomy and morphology

The other agent of TPB is *Babesia*, an intraerythrocytic protozoan parasite of a wide range of domestic and wild animals and occasionally man (Bock et al., 2004). Babesiosis is also known as piroplasmosis, tick fever, red water, Texas fever, splenic fever, or tristeza, and is transmitted only by ixodid ticks (Ristic, 1988). In 1893 Smith and Kilborne identified the agent of Texas fever and named it *Pyrosoma bigemina* (later *B. bigemina*), thus demonstrating the first disease for which transmission by an arthropod to a mammal was implicated (Smith and Kilborne, 1893). The same year Starcovici (1893) gave these parasites the name of *B. bovis, B.ovis* and *B. bigemina* following the publication of his work babesiosis was identified all over the world.

Nowadays seven species of *Babesia* are recognized for cattle: *B. bovis, B. bigemina, B. divergens, B. major, B. ovata* , *B. occultans* and *B. jakimovi* (Uilenberg, 2006; Zweygarth et al., 2006). In Brazil only *B. bigemina* and *B. bovis* occur, both transmitted by the tick *R.* (*B.) microplus* (Ribeiro and Passos, 2002).

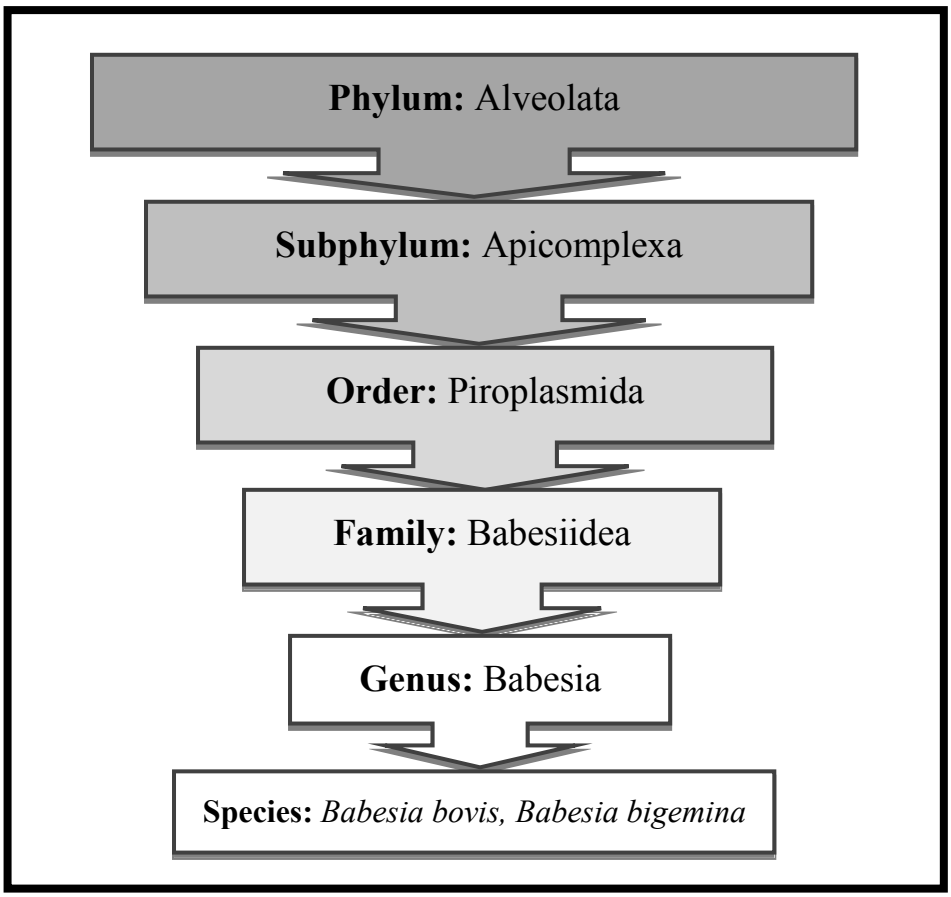

Figure 3: Taxonomy of the genus *Babesia* (adapted from Eckert et al., 2005)

Morphologically the different *Babesia* species can be divided into two distinct groups: the small (merozoites < 2.5 μm) and the large type (merozoites > 2.5 μm) (Eckert et al., 2005).

B. bigemina belongs to the large type, and the merozoites may be pyriform or pear shaped with the paired forms being at a more acute angle to each other or almost parallel. *B. bovis* is of the small type, which also may be pear shaped or round, usually centrally located in the erythrocytes and often found in pairs that are at an obtuse angle to each other (OIE, 2010; Ristic, 1988; Solorio-Riveira and Rodríquez-Vivaz, 1997).

2.2. Geographic distribution

B. bovis and *B. bigemina* are present in most areas of the world, with the greatest incidence between the latitudes of 32 °N and 30 °S, where their *Boophilus* tick vector commonly occurs (Ristic, 1988). They are found in most areas of Africa, Asia, Australia, Central and South America and Southern Europe. In Brazil, babesiosis is only transmitted biologically by the tick *R.* (*B.) microplus,* but in other areas of the world there are more vectors that infect domestic animals (Uilenberg, 2006).

Table 1: Vectors (ticks) for the transmission of the pathogens *Babesia bovis* and *Babesia bigemina*

Parasite	Vector (Tick)
Babesia bovis	*Rhipicephalus(Boophilus) microplus* *Boophilus annulatus* *Boophilus geigyi*
Babesia bigemina	*Rhipicephalus (Boophilus) microplus* *Boophilus decoloratus* *Boophilus annulatus* *Boophilus geigyi* *Rhipicephalus evertsi*

R. (B.) microplus is the most important and widespread vector worldwide, but in southern Africa *Boophilus decoloratus* interferes with its spread in colder and drier areas. Interbreeding between the two species produces sterile progeny; these create a zone which *R.* (*B.) microplus* has difficulty passing through (Sutherst, 1987).

Generally both parasites, *B. bovis* and *B. bigemina*, have the same distribution, but in Africa *B. bigemina* is more widespread than *B. bovis* because of the ability of *B. decoloratus* and *R. evertsi* to also act as vectors for this species (Friedhoff, 1988).

Babesiosis was the first disease to be eradicated from a continent, namely North America. The eradication program for *Boophilus annulatus*, the only *Boophilus* tick present in the U.S., was conducted from 1907 to 1940. With the exception of some areas in Florida, tick eradication was completed in 1940 from a 181300 km^2 area by dipping all cattle and other tick infested livestock in an arsenic solution every 2 weeks (Graham and Hourrigan, 1977).

2.3. Transmission and life cycle

As already mentioned, in Brazil babesiosis is transmitted biologically only by the tick *R.* (*B.) microplus;* mechanical transmission by blood transfusion can occur but is epidemiologically insignificant (Ristic, 1988). Only the female *R.* (*B.) microplus* can become infected with *B. bigemina* and *B. bovis* when ingesting blood cells containing parasites (Ribeiro and Passos, 2002). In the female tick the infection invades numerous organs including the ovaries, therefore allowing both *B. bigemina* and *B. bovis* to be transmitted transovarially to the next generation (Uilenberg, 2006). Only the larval stage can transmit *B. bovis* to the bovine host, and after transmission larvae lose the infection, whereas *B. bigemina* is transmitted during the nymphal and adult feeding period and the tick host remains infected for many generations (Dalgliesh and Stewart, 1983).

Babesia is pathogenic for the tick host, so a high level of parasites in cattle can result in a decrease in egg production or even death of the female ticks after infection. Another important factor for transovarial transmission is the temperature, with a lower percentage of eggs infected when the female ticks are incubated in lower temperatures. Higher temperatures result in higher levels of infection but also can induce death in the female ticks or reduce the duration of oviposition (Dalgliesh and Stewart, 1979).

The development of *Babesia* in the vertebrate host begins with the injection of tick saliva containing sporozoites, which then directly infect red blood cells (Guglielmone, 1995). As far as it is known, in the vertebrate host all *Babesia* species can only infect erythrocytes. Inside the erythrocytes the sporozoit develops into a round form, the trophozoit, which multiplies by merogony (binary fission) in two merozoites. The merozoites leave the host cell by disrupting the membrane, and each enters another red blood cell (Friedhoff, 1988). These cycles of assexual reproduction require around 8 hours, and continue either until death of the host or more usually, until recovery from infection (Ribeiro and Passos, 2002). Some of the merozoites within the red blood cells do not undergo merogony but instead acquire an unusual shape and form gametocytes. The transformation to gametocytes can occur either in bovine erythrocytes or in the gut lumen of the tick (Young and Morzaria, 1986).

The alimentary infection of the tick begins with the ingestion of blood cells containing the different stages, trophozoites, merozoites and gametocytes. In the lumen of the tick gut, trophozoites and merozoites are destroyed and only the gametocytes survive. Some of the intraerythrocytic gametocytes leave the erythrocytes and differentiate to

form “Strahlenkörper” stages, also known as ray bodies, which develop into male and female gametes in the tick gut (Uilenberg, 2006; Young and Morzaria, 1986). Within two to four days, the gametes fuse to form by gamogony a motile zygote, which then transforms into an ookinete. The ookinete invades gut epithelium to initiate asexual division (sporogony) and form sporokinetes (Ribeiro and Passos, 2002). Subsequently the sporokinetes are released from the gut epithelial cells and enter the hemolymph to invade other cells. The parasites can undergo further asexual division to produce more sporokinetes in various other cell types, such as haemocytes, cells of Malpighian tubules, muscle fibres, ovarian cells and epidermis (Young and Morzaria, 1986). In this way the infection passes through the ovaries to the egg and the next generation of the ticks (Uilenberg, 2006). The larvae eclose already infected with *Babesia* but the parasites appear to remain dormant; only when the larvea begin to feed do the sporokinetes start new cycles of sporogony (Young and Morzaria, 1986). The most significant development of *Babesia* occurs when ookinetes enter the salivary glands and are transformed into sporozoites (Mackenstedt et al., 1995).The sporozoites of the salivary gland are morphologically different from the sporokinetes, are incapable of division in any tick cells, and are only infective to the mammalian host (Young and Morzaria, 1986).

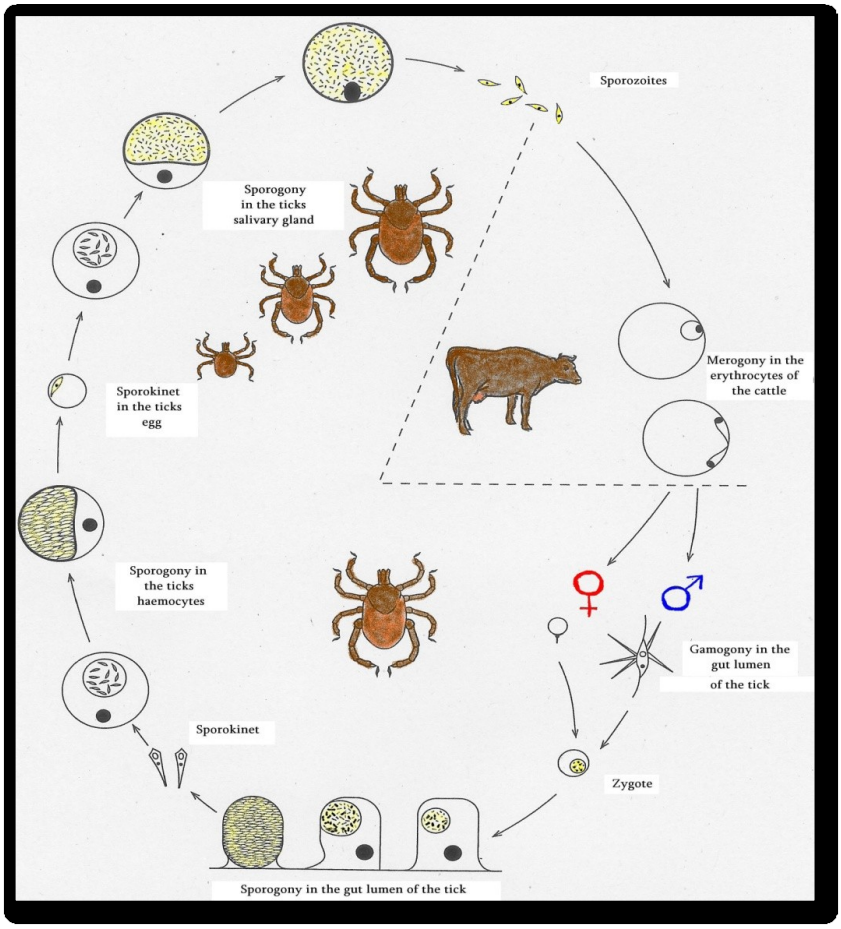

Figure 4: Life cycle of the genus *Babesia* (adapted from Schnieder, 2006)

2.4. Clinical symptoms

According to da Costa et al. (1997) the incubation period of *Babesia* spp varies from five to 14 days. However, heat stimulation (37 °C 3-5 days or 30 °C for 8 days) prior to attachment stimulates the development of *B. bovis* parasites to an infected stage in unfed larval ticks and can lead to shortened prepatent periods in summer (Dalgliesh and Stewart, 1979). In agreement with Mahoney et al. (1979) the clinical signs vary because of the different pathogenicity of each *Babesia* species and the strain, and also due to the susceptibility of the host, which can be influenced by age, breed and colostral antibodies.

In animals with acute *B. bigemina* infection more than 40% of erythrocytes may be infected, but only a relatively small proportion of cases are fatal (Callow, 1979). In contrast, mortality rates over 50% are common for animals infected with *B. bovis* (Wright, 1991).

Infections in cattle are characterized by fever, anorexia, listlessness, dehydration and progressive hemolysis, and may be followed by hemoglobinuria and hemoglobinemia resulting in jaundice (Zintl et al., 2005). Both *B. bigemina* and *B. bovis* have the above-named clinical signs in common, but show differences in pathogenesis and manifestation. Hence *B. bigemina* can be characterized as a peripheral babesiosis with severe anemia, where as *B. bovis* often induces a visceral babesiosis because of thrombus formation (Ribeiro and Passos, 2002). *B. bovis* infection usually runs a course of three to seven days, with fever (>40 °C) generally present for several days before other signs become obvious. The fever during infection can cause abortion in pregnant cows. In the acute phase, cattle show inappetence, depression, increased respiratory rate, weakness and a reluctance to move, and in more protracted cases anemia and jaundice (Bock et al., 2004). Muscle wasting, tremors and cerebral dysfunctions sometimes can be mistaken for other diseases like rabies or intoxication with plants, which also cause neurological signs (Soares et al., 2000).

The kinin-kallikrein-system has an important function related to the pathogenesis of *B. bovis.* At the beginning of infection there is an overproduction of kinin and other pharmacologically active agents, causing vasodilatation, hypotension, increased capillary permeability, oedema, vascular collapse, coagulation disorders, endothelial damage and circulatory stasis (Ahmed, 2002). Infected erythrocytes undergo sequestration by attachment to capillary endothelium and induce stasis in the microcirculation. Modifications of the membrane of erythrocytes, related to *Babesia* antigens, induce aggregation in capillary beds, thrombus and even hypotensive shock syndrome. Sequestration of infected erythrocytes leads to lesions and damage in organs and induce cerebral, renal and pulmonary dysfunction and failure (Wright et al., 1988).

Acute cases that are not fatal may take some weeks to recover from completely. Moreover, incidences of subacute infections can occur with less pronounced clinical signs, and are sometimes difficult to detect (Bock et al., 2004). Animals that survive *B. bovis* infections remain persistently infected and resistant to clinical disease (Brown et al., 2006).

The pathogenic effects of *B. bigemina* are more directly related to erythrocyte destruction and are characterized by hemolytic anemia and hemoglobinuria (Young and Morzaria, 1986). Acutely infected cattle are usually not as severely affected as those with *B. bovis* infections. There is no cerebral involvement, and coagulation

disorders, cytoadherence and a hypotensive state are also not features of *B. bigemina* infections (Wright et al., 1988). However, in some cases the disease can develop very rapidly with anemia and jaundice, and death may occur with little warning (Bock et al., 2004). The packed cell volume in most fatal infections with *B. bigemina* will be below 10%, whereas death can occur with *B. bovis* when the packed cell volume is 12% or higher (Vial and Gorenflot, 2006).

Once an animal recovers from primary infection, immunity to *B. bovis* is lifelong. Sterile immunity does not occur for *B. bovis* infection and cattle undergo continuous series of parasite recrudescence. Therefore the immune response may be stimulated continuously and a high antibody level can be detected up to four years after infection (Mahoney et al., 1979; Mahoney et al., 1973). However, sterile immunity does occur in *B. bigemina* infection, and a protective immune response is induced without a parasite recrudescence (Mahoney et al., 1973). In generally antibodies cannot be detected 18-21 months after infection, even though the animal is protected against subsequent *B. bigemina* infections (Wright et al., 1992).

3. Bovine tick-borne disease in Brazil (TPB)

3.1. Epidemiology

A. marginale and both species of *Babesia* have a high prevalence in the majority of Brazilian regions, resulting in natural endemic stability (Madruga et al., 2001; Soares et al., 2000; Souza et al., 2000a). Perry (1996) defined endemic stability as the state where the relationship between host, agent, vector and environment is such that clinical disease occurs rarely or not at all. However, some micro-regions of the south and northeast of Brazil are exceptions to this and are endemically unstable (Oliveira et al., 1992). In these areas ecological and climatic conditions impede the development of the vector *R.* (*B.) microplus,* with the consequence that transmission rates of *Babesia* and *Anaplasma* are insufficient to generate immunity for a majority of susceptible calves before the loss of calfhood resistance (Smith et al., 2000). The south of Rio Grande do Sul is free from TPB, and endemic instability is found in the states of Santa Catarina and Parana. The northeastern Brazilian states of Bahia, Pernambuco and Ceara are large areas with a semi-arid climate, unsuitable for tick development for several months of the year (Barros et al., 2005; Kessler and Schenk, 1998). Hence in Brazil we can find three different epidemiological situations relating to TPB (Mahoney, 1975; Mahoney and Ross, 1972).

Area of endemic stability: areas where the climatic conditions allow the presence of the ticks throughout the year. Calves become infected in the first months of life, at an age where they are relatively resistance to the clinical signs of disease, and develop immunity.

Area of endemic instability: areas where the climatic conditions and management interrupt the development of vectors, resulting in low population densities. Because of this some calves do not become infected until the window of innate resistance has passed, and have not developed immunity. If these animals are exposed later in life they may develop severe, life-threatening disease - therefore in these areas more clinical cases may be found.

Free area: areas where the climatic conditions are unfavorable for the maintenance of the tick population. These areas are localized below the 32 °S latitude, with a long cold period responsible for the absence of ticks. The animals are totally unprotected against TPB because they do have not developed immunity.

Mahoney (1974) estimated that if at least 75% of calves were exposed to *Babesia* spp. infection by six to nine months of age, the disease incidence would be very low and the state of natural endemic stability would exist. But an area of stability may transform to an area of instability and vice versa, depending on the prevalent cattle breed, management strategies, tick infection rates and acaricide treatment (D´Andrea et al., 2006). Moreover, it is important to determine parasite prevalence to characterize the epidemiological situation of a region and decide if preventive measures are required (Madruga et al., 2000).

3.2. Age and breed resistance

An innate resistance of calves to clinical disease until nine months of age is seen as an important factor for the maintenance of endemic stability (Zintl et al., 2005). Clinical signs like fever and anemia, as well as parasitemia levels, are milder and reduced compared with adult cattle (Goff et al., 2003). In neonates this age-related resistance may be acquired from maternal antibodies in the colostrum (Mahoney, 1967a). However, calves remain resistant for longer than colostral antibodies persist, and even calves from non-immune mothers exhibit resistance to *B. bovis* and *B. bigemina* (Carson and Phillips, 1981). The age-related resistance is somewhat counter-intuitive and there are several different possible explanations. Levy et al. (1982) demonstrated that blood from young animals contains a factor responsible for inhibition of parasite

multiplication of *B. bovis*. Another study by Montealegre et al. (1985) indicated that soluble products secreted by bovine blood mononuclear phagocytes after activation by soluble antigens could inhibit the *in vitro* growth of *B. bovis*. Goff et al. (2001) showed that the innate immune response of young calves to *B. bovis* involves the rapid induction of interleukin-12 and interferon-γ, and the presence of inducible nitric oxide synthase (iNOS) mRNA expression in the spleen. Some more possible resistance mechanisms have been suggested but not investigated in detail, such as the inhibitory effect of fetal hemoglobin or the different susceptibility of reticulocytes compared to mature erythrocytes (Langley and Gray, 1987; Levy et al., 1982).

The immune response of TPB involves humoral and cellular mechanisms, which is typical for protozoan parasites; furthermore immunity is T-cell dependent and has type-1 characteristics (Brown and Logan, 1992; Goff et al., 2002; Goff et al., 2003). The fact that splenectomised cattle develop a higher parasitemia to primary *Babesia* infections indicates that the spleen plays an important role in the immune response to *Babesia spp* (Goff, 2001; Wright et al., 1988; Wright and Kerr, 1977).

The phenomenon of breed resistance is thought to be a result of the evolutionary relationship between *Bos indicus* cattle, *R.* (*B.) microplus* and the parasites (Dalgliesh, 1993).

Comparative studies in *B. indicus* and *B. taurus* showed that there are some differences in the breed resistance to *B. bovis, B. bigemina* and *A. marginale*. The results of Bock et al. (1997) indicated that *B. bovis* infections were more pathogenic in *B. taurus* cattle and cross-breed cattle than in *B. indicus* cattle. In contrast *B. bigemina* infections were uniformly mild and *A. marginale* infections uniformly severe in all cattle breeds. However, other studies concluded that *B. indicus* were also much more resistant to *B. bigemina* and *A. marginale* (Bock et al, 1999; Parker et al., 1985). Thus there is some ambiguity in the literature concerning the relationship of these parasites to *B. indicus* and *B. taurus* breeds, and more studies would help to properly understand the different situations.

Figure 5: ***Bos indicus*** **(A. Pohl)**

3.3. Economic impact

The principal agents of TPB, *A. marginale, B. bovis* and *B. bigemina,* are responsible for important losses in the cattle industry of tropical and subtropical areas worldwide, including Brazil (Barros et al., 2005). The economic impact is not only a consequence of direct losses through mortality, abortions, and reduction in milk and meat yield, but also due to indirect losses such as the treatment costs, and the expense of prevention and control measures (Ribeiro and Passos, 2002). According to Lima (1991) the biggest disadvantage of TPB is the restriction it imposes on importing cattle from temperate to tropical areas to improve the production of milk and meat. Unless preventive measures are taken, *B. taurus* breeds imported into tropical developing countries to upgrade local livestock industries suffer significant losses due to tick-borne diseases including babesiosis and anaplasmosis (Callow, 1977). Grisi et al. (2002) estimated the annual losses in Brazil caused by babesiosis and anaplasmosis as 500 million US dollar.

Furthermore, global warming, if it does occur as predicted, may play an important role in worldwide patterns of disease occurrence. The distribution of vector-borne diseases may be expected to change in part as a result of climate change, which may influence tick movement and thus expand the range of tick-borne diseases over the world (Jonsson and Reid, 2000).

3.4. Diagnostic

The diagnosis of TPB may be based on geographic location, season, anamnesis, and clinical signs or necropsy findings observed in infected animals. This diagnosis should be confirmed by laboratory tests – these tests are fundamental to confirm the clinical diagnosis, identify the specific agent and determine specific measures of treatment and prevention (Jones and Brock, 1966; Kessler and Schenk, 1998).

3.4.1. Identification of the agent

Direct microscopic examination

During the acute phase of disease the recommended examination is blood and/or organ smears stained with Giemsa evaluated by light microscope (Ribeiro and Passos, 2002). To obtain the best results, blood films should be prepared from capillary blood, collected from the tip of the tail or margin of the ear, because blood of the general circulation may contain up to 20 times fewer *B. bovis* than capillary blood. In the case of *B. bigemina* and *A. marginale* infections, parasitized cells are distributed more evenly throughout the blood circulation (Böse et al., 1995). For post-mortem diagnosis in freshly dead animals, impression smears can be made from kidney, brain, heart muscle, spleen or liver, whereas in decomposing animals samples should be taken from the spleen or brain (Böse et al., 1995). *A. marginale* can be visualized as dense, rounded, intra-erythrocytic bodies situated mostly on or near the margin of the erythrocytes (OIE, 2008). *B. bovis* is a small parasite, usually centrally located in the erythrocytes and often found as pairs that are at an obtuse angle to each other, while *B. bigemina*, a much bigger parasite, is typically pear-shaped and often found as pairs at an acute angle to each other or almost parallel. Nevertheless, even though these two *Babesia* species show morphological variations, it is difficult to identify one from the other on morphological grounds alone (OIE, 2010).

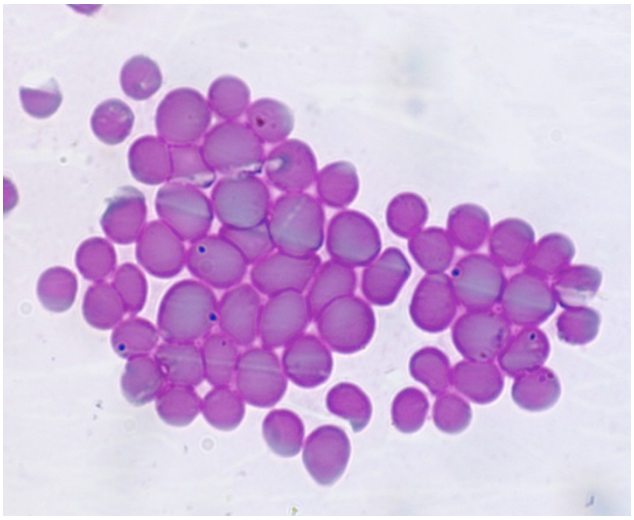

Figure 6: Blood smear with *Anaplasma marginale* (A. Pohl)

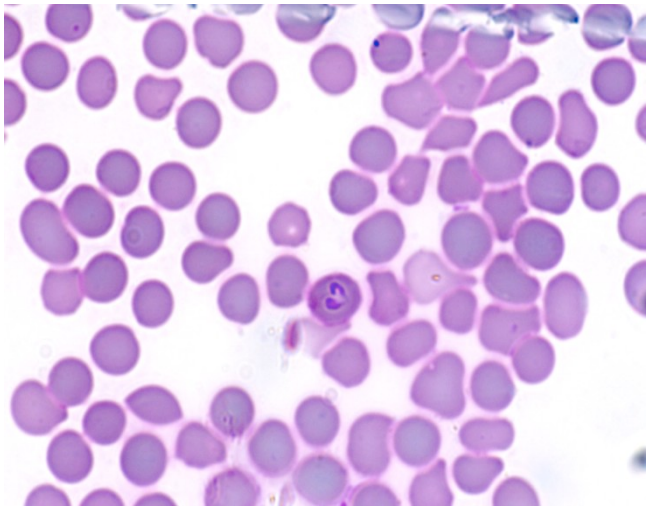

Figure 7: Blood smear with *Babesia bovis* (A. Pohl)

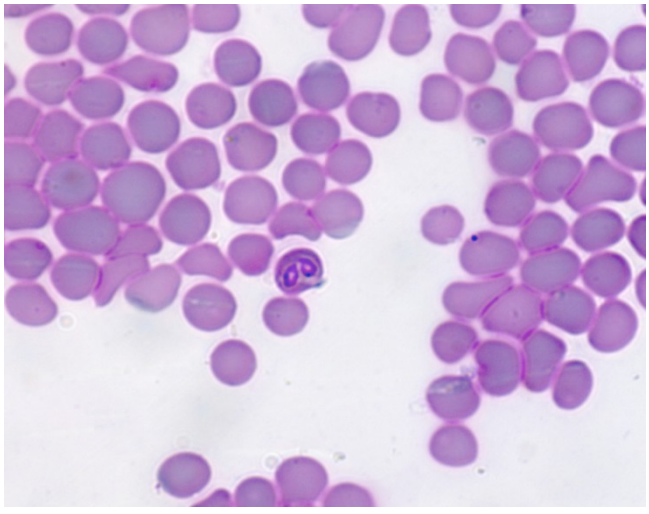

Figure 8: Blood smear with *Babesia bigemina* (A. Pohl)

Animal inoculation

In the past the gold standard to identify latent anaplasmosis or babesiosis infections was the sub-inoculation of blood from the animal with a suspected infection into a splenectomised calf. If the donor was infected, parasites would be observed in smears of the splenectomised animal after four to eight weeks. However this method is costly, requires experimental animals, and raises welfare issues because experimental calves become very ill and have to be euthanized (Aubry and Geale, 2011; Böse et al., 1995).

PCR

The polymerase chain reaction has a very high sensitivity and specificity, which makes it useful for validating results from other diagnostic techniques and for applications such as export certification of livestock. PCR tests have been developed which can detect *A. marginale, B. bovis* and *B. bigemina* in a single assay (Böse et al., 1995). However, at present PCR is unlikely to supplant serological tests as the method of choice, because it does not lend itself to large-scale testing and requires specialized laboratory equipment and trained personnel, increasing the costs (Aubry and Geale, 2011; OIE, 2010).

3.4.2. Serological tests

Blood smears are not reliable for detection of carrier animals; in these cases molecular detection methods, or serological diagnostic procedures to demonstrate specific antibodies, are required (Aubry and Geale, 2011). The different serological tests to detect specific antibodies for epidemiological studies are: complement fixation test (CF test), card agglutination test (CAT), indirect fluorescent antibody test (IFAT), as well as various enzyme-linked immunosorbent assays (ELISA) such as a competitive ELISA (cELISA), indirect ELISA (iELISA) and dot ELISA (Aubry and Geale, 2011). In practice only two of them, the IFAT and ELISA, are used routinely (Böse et al., 1995).

CF test

The complement fixation test is a serological test designed to measure serum levels of specific antibody to antigens. Through complement attachment to the antibody-antigen-complex, the fixed or free complement can be measured to demonstrate the presence of specific antibodies. The test is based primarily on the reaction of IgM antibodies, which are produced early in primary infections. As most of the animals

tested are chronically infected, the sensitivity of CF is low. Therefore it is no longer recommended as a reliable assay for detecting infected animals, except for equine *Babesia* infections (Böse et al.; OIE, 2008, 2010).

CAT

The CAT is sensitive for *A. marginale* and *B. bovis*, but cannot be applied for *B. bigemina*. The test may be undertaken either in the laboratory or in the field and gives a quick result; nevertheless non-specific reactions and subjectivity in interpreting assay reactions may be a problem. Furthermore the CAT antigen can be difficult to prepare, can vary from batch to batch and laboratory to laboratory, and requires the inoculation from splenectomized calves with *Anaplasma* or *Babesia* infected blood (OIE, 2008, 2010).

IFAT

The indirect fluorescent antibody test has been the test most widely used for diagnosis of all *Babesia* spp. and can also be applied for *A. marginale*. The test is well suited to low technology environments, the costs are low and most reagents are readily available or can be produced on site (Böse et al., 1995). However, it has disadvantages - low sample throughput, operator fatigue and the subjective judgment of the operator on the test results, which makes standardization difficult. In addition, non-specific fluorescence from antibodies adhering to infected erythrocytes may be a serious problem (OIE, 2008, 2010). Nonetheless, the test is still one of the most commonly used for diagnosis of TPB (Ribeiro and Passos, 2002).

ELISA

The ELISA can be a very sensitive and efficient test because of the objectivity it gives in interpretation of results, and the capacity to process high number of samples. It should be considered that high antigen quality is crucial for the development of sensitive and specific tests (Böse et al., 1995).

At present, the competitive ELISA is the most sensitive and specific serological test currently available for identifying *Anaplasma*-infected cattle. The test is based on a monoclonal antibody (mAb) that recognizes MSP5 antigen in *A. marginale, A. centrale* and *A. ovis.* The disadvantages are the cross-reactivity of the MSP5 mAb with multiple species of *Anaplasma* - therefore the test cannot differentiate between *A. marginale* and some other *Anaplasma* species (OIE, 2008).

For diagnosis of *B. bovis* infection, there is an ELISA which uses a whole merozoite antigen and has already undergone extensive evaluation which demonstrated a high sensitivity and specificity for the test (de Echaide et al., 1995; Molloy et al., 1998). Recently, indirect and competitive ELISAs have also been developed for *B. bovis*, but a reduction in specificity has been noted in some situations (OIE, 2010).

For *B. bigemina* there is still no well-validated ELISA available. The only ELISA reported to be in routine use is a competitive ELISA developed in Australia and the USA (OIE, 2010). Furthermore, due to the decline of antibody levels within months after infection, inconclusive results may occur which presents a diagnostic challenge (Goff et al., 2008).

3.5. Treatment

Antimicrobial therapy for bovine anaplasmosis indicates the use of tetracycline drugs at a dose of 8-11 mg/kg for three days, administered intramuscularly (Ribeiro and Passos, 2002). Oxytetracycline and chlortetracycline used at their recommended therapeutic doses are effective to limit the clinical signs of infection and reduce the level of parasitemia. However, therapy does not reliably eliminate persistent infections and there is no evidence that it prevents cattle from becoming infected with *A. marginale* (Aubry and Geale, 2011). Another agent is imidocarb, at a dose of 3 mg/kg subcutaneous or intramuscular, which has been used for over 30 years to treat the disease (McHardy and Simpson, 1974).

The drugs most commonly used for treatment of *B. bovis* and *B. bigemina* are diminazene diaceturate (at a dose of 3-5 mg/kg, Berenil®, Hoechst Ltd.) and imidocarb (at a dose of 1-3 mg/kg, Imizol®, Carbesia®, Schering-Plough) intramuscularly. The choice of treatment is also dependent on early diagnosis; animals infected with *B. bovis* which already present neurological signs do not have a favorable prognosis. *B. bigemina* infections treated with imidocarb may cure the infection and clear the host of parasites. Imidocarb also provides protection from clinical disease from three to six weeks but allows a sufficient level of infection to develop immunity, which is important in areas where babesiosis is endemic (Vial and Gorenflot, 2006).

3.6. Prevention

Control measures vary with geographical location and include maintenance of *Anaplasma*- and *Babesia*-free herds through import and movement control, administration of antibiotics and acaricide products, and vaccination.

3.6.1. Vector control

Tick and fly control is expensive and labor-intensive, and nowadays environmental pollution is becoming an important issue. In addition, repeated application of acaricides can result in the development of resistance in fly and tick populations (Kocan et al., 2000). Furthermore, to maintain the endemic stability of an area it is necessary to keep at least a minimal population of vectors (Smith et al., 2000). But acaricides are not the only way for vector control: alternatives could be pasture rotation, or implementation of forage with antibiosis and antixenosis mechanisms to affect the tick population, as well as biological control with insects, nematodes, bacterias and fungi (Kessler and Schenk, 1998).

3.6.2. Chemoprophylaxis

Chemoprophylaxis consists of measures to apply sub-therapeutic doses of a product to control an agent. The aim is to keep the agent at a sub-clinical level, so that high parasitemia is prevented and the carrier animal can deal with the infection (Ribeiro and Passos, 2002). Different studies using parenteral or oral tetracyclines have been done, but none have been approved for the elimination for persistent *A. marginale* infections in cattle (Aubry and Geale, 2011). For *Babesia* infections, the use of imidocarb at a dose of 1 to 2 mg/kg has been established with satisfactory results and subsequent protective immunity (Ribeiro and Passos, 2002).The disadvantage of chemoprophylaxis is that the continuous use of antibiotics may lead to resistance developing in other pathogens like *E. coli*.

3.6.3. Vaccination

Immunization has been an effective and economical way to control hemoparasites worldwide. Vaccination programs are recommended in areas where the number of vectors is not sufficient to develop immunity, or when susceptible animals are introduced into endemic areas (Ribeiro and Passos, 2002). Vaccines can be divided into two major types: live and killed vaccines. However, they do not prevent cattle from becoming persistently infected with TPB and acting as reservoirs of infections (Bock et al., 2004; Kocan et al., 2003).

Anaplasma Vaccines

In the 1960s the United States developed killed vaccines, but these were only marketed until 1999. Killed vaccines continue to be tested and have several advantages to live vaccines. Storage is inexpensive, postinoculation reactions are of minimal clinical relevance, and the risk of contamination with other infectious agents is low. Disadvantages are the higher cost of purification, the requirement for yearly boosters, and the lack of cross protection among isolates from widely separated geographic areas. Hence, despite the disadvantages of live vaccines, killed vaccines are not frequently used worldwide (Kocan et al., 2003).

Live vaccines, which were initiated by Sir Arnold Theiler in the early 1900s, involve the infection of cattle by inoculation with erythrocytes infected with *A. marginale*. For vaccine production, splenectomized calves under quarantine conditions are experimentally inoculated with defined strains, and serve as a source of infected blood for vaccines (Kocan et al., 2003). The number of parasites/ml of blood is adjusted to help to predict the period when the animals will present clinical signs and the likely intensity of the manifestation (Ribeiro and Passos, 2002). Treatment with low doses of tetracycline drugs is recommended during the initial phase of patent infection. Vaccinated animals develop persistent infections, which induce lifelong protective immunity and usually do not require vaccination to be repeated (Tebele and Palmer, 1991b). The classic way of immunization, already an antiquated and obsolete method, is still used in some areas of Brazil. The procedure consists of inoculation with blood from a donor animal infected with *Anaplasma* into a susceptible animal to develop immunity. The blood of the donor animal is inoculated subcutaneously or intramuscularly in the cattle to be immunized. After 21 to 38 days, the temperature of the immunized animal will rise due to the *Anaplasma* infection and the cattle will be treated with tetracycline drugs. This method shows inconsistent results due to the unknown number of parasites in the inoculate, different infectivity and virulence of the samples, and the risk of transmission of other infective diseases (Ribeiro and Passos, 2002).

The attenuation of *A. marginale* by passaging the organism in sheep or deer, or by irradiation, is another possibility for producing live vaccines (Kocan et al., 2003; Kuttler and Zaugg, 1988).

In Africa, Australia, Israel, and Latin American countries the most widely used strain of live vaccine is *A. centrale* (Aubry and Geale, 2011). *A. centrale* is less pathogenic for cattle than *A. marginale,* and Theiler observed in the early 1900s that cattle infected with *A. centrale* developed protective immunity against subsequent *A. marginale* infections (Theiler, 1911).

Another important point for the development of vaccines in the future is the *in vitro* cultivation of *A. marginale* isolates in continuous tick cell lines (IDE8) (Bastos et al., 2010; Zweygarth et al., 2006). Tick cell *in vitro* systems have a high potential for development of improved vaccines and diagnostic assays, particularly in areas with endemic *A. marginale* infections where maintaining animal donors free from hemoparasites, is difficult and expensive. Moreover, the large scale application of tick cell lines could lead to a reduction in the use of experimental animals (Passos, 2012).

Babesia Vaccines

Researchers noticed early that cattle develop a durable, long-lasting immunity after a single infection with *Babesia,* and therefore used blood from recovered animals for immunization (Connoway and Francines, 1899). The disadvantages of this method - variable infectivity, high virulence, and contamination with other pathogenic organisms - led to changes in the production of vaccines. Australia initiated new techniques for production of live vaccines for *Babesia* (Callow, 1977), which were subsequently introduced in other countries. Today most of the available live vaccines are produced in government-supported production facilities (Bock et al., 2004). The procedure for *B. bovis* attenuation involves rapid passage of the strain through susceptible splenectomised calves; attenuation is usually obtained after 20 passages. In contrast, *B. bigemina* attenuation is obtained by slow passage through intact calves, which are then splenectomised in the chronic phase of disease (Ribeiro and Passos, 2002). Most of the live vaccines are provided in a chilled form, and are popular due to the ease of production and use, and their low cost. However, the disadvantage is a very short shelf-life, which does not allowed post-production testing of potency and safety before dispatch. This lead to a change to frozen vaccines (Bock et al., 2004). Frozen vaccines can be stored in liquid nitrogen for up to 18 months, and the long shelf-life makes it possible to test safety and affectivity before vaccine release (Waal and Combrink, 2006).

Killed vaccines could be an alternative which would overcome the difficulties in production, transport and use of live vaccines (Bock et al., 2004). One of the first attempts to use non-living vaccines was made by Mahoney (1967b), and established the use of killed parasites mixed with Freunds adjuvant. Further studies used exoantigens from cell culture of *B. bovis* and *B. bigemina* to develop killed vaccines which gave a high degree of protection (Kuttler et al., 1982; Montenegro-James et al., 1992; Patarroyo et al., 1995). However, other studies have shown that the protection conferred by these antigens was considerable less than those of live vaccines (Timms et al., 1983). Recombinant antigens are potentially an alternative to live vaccines, but *Babesia* has developed a variety of mechanisms such as antigen variation to evade the immune response, and future studies of recombinant vaccines will need to contain several antigens to provide protection (Bock et al., 2004).

In Brazil two attenuated vaccines exist for TPB, one in chilled form and the other frozen in liquid nitrogen. Both are trivalent vaccines containing packed red cells infected with *B. bovis, B. bigemina* and *A. centrale.* The registered and commercialized Brazilian vaccine (frozen) EMBRAVAC® was developed by the Empresa Brasileira de Pesquisa Agropecuaria (Embrapa) (Brazilian Enterprise for Agricultural Research) (Kessler et al., 2002).

However, repeated use of live vaccines may lead to possible side effects like neonatal hemolytic anemia: vaccinated cattle may develop erythrocytic isoantibodies, which when ingested by calves in colostrum, may cause hemolytic anemia. Therefore repeated vaccinations should not be performed (Dennis et al., 1970; Dimmock and Bell, 1970). Furthermore, pregnant cows should be vaccinated only after calving because of the accompanying fever, which may cause abortion if they develop severe vaccine reactions (Waal and Combrink, 2006).

4. The ticks in Brazil

4.1. Taxonomy

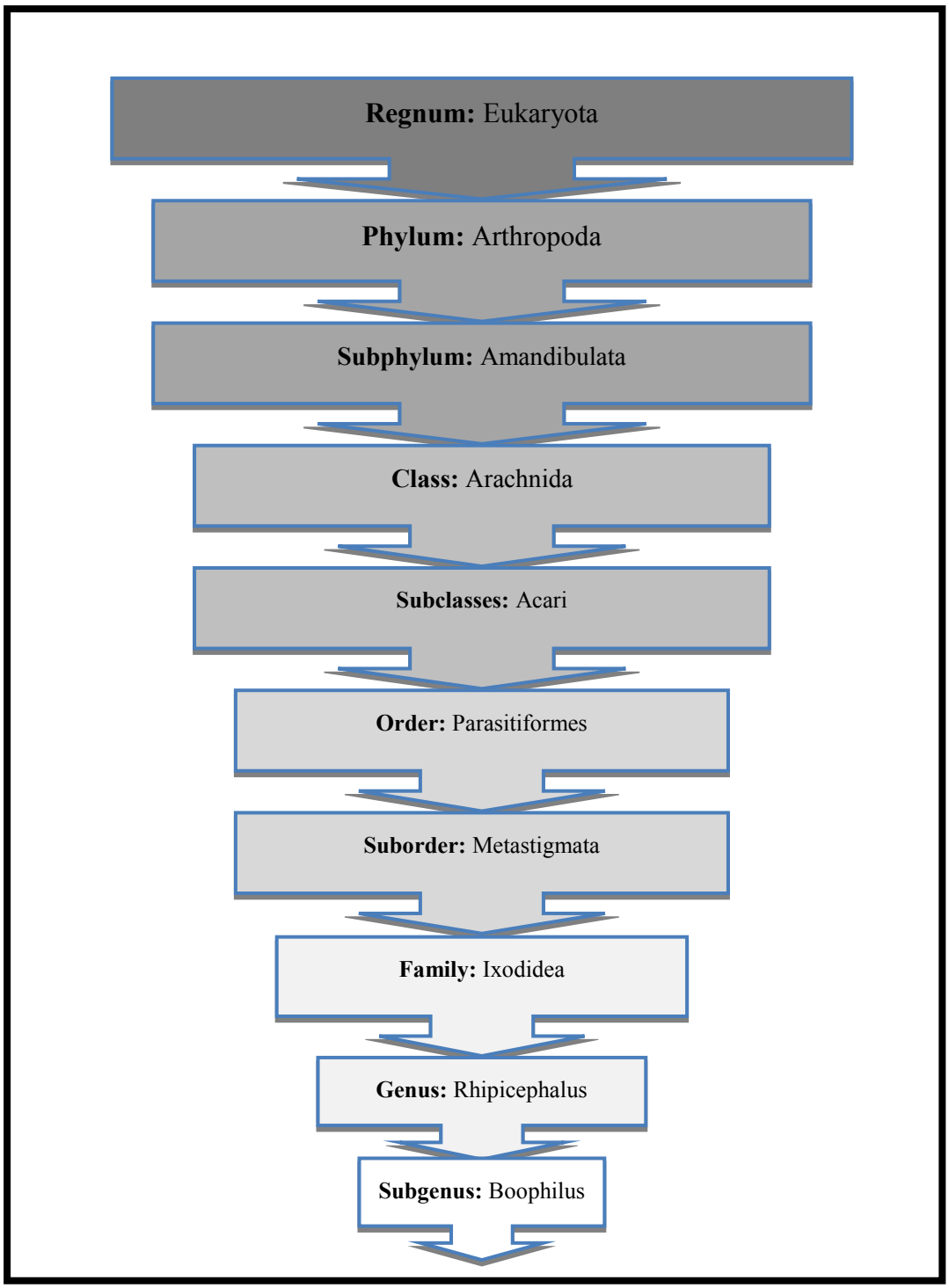

Figure 9: Taxonomy of the tick *Boophilus* (adapted from Eckert et al., 2005).

4.2. Prevalence

The subgenus *Boophilus* contains five species, which affect cattle and in rare cases other animals like horses, sheep and wild ruminants. The most important species are *R. (B.) microplus* (engl. *cattle tick*), *B. decoloratus* (engl. *blue tick*) and *B. annulatus* (engl. *Texas fever tick*) (Eckert et al., 2005).

The tick *R. (B.) microplus* originated from Asia, mainly from India and the Java Islands, and its expansion is related to historical transfers of animals and wares (Kessler and Schenk, 1998). Nowadays *R. (B.) microplus* is considered the tick with the most important economic significance in South America (Nari, 1995). In Brazil the tick can be found all over the country, differing in its density according to climatic variations, pasture, husbandry practices, tick control strategies, and the cattle breed farmed in each area (Kessler and Schenk, 1998; Nari, 1995).

4.3. Life cycle

R. (B.) microplus is a one-host-tick which remains on the host from the larval and nymphal stages until they become adults (Eckert et al., 2005). The development to the adult tick takes about three weeks and each stage (larval, nymphal and adult) feeds only once. After feeding adult males become sexually mature and mate with adult females. The females drop off the host after mating, deposit a batch off eggs in the environment, and die after ovipositing. The life cycle restarts with the eclosion of the larvae and their attachment to a new host (Health, 2007). Under favorable conditions (28 °C, > 80% relative air humidity) five to six generations of ticks can be found in one year (Eckert et al., 2005).

4.4. Economic impact

The initial damage, perhaps of low importance, caused by *R.* (*B.) microplus* is its blood sucking alimentation which can interfere with weight increase of the host animal (Kessler and Schenk, 1998). Furthermore as already mentioned, the tick can transmit at least two infectious agents, the rickettsia *Anaplasma* spp. and the protozoa *Babesia* spp. responsible for TPB. The third direct damage from the tick can be seen on leather made from the host the animal. The feeding behavior of the tick involves cutting a hole in the host's epidermis to insert their hypostome, injuring the leather and abetting the infestation with cutaneous myiasis. These lesion leads to losses in the leather industry. The export of crude and tanned leather, and leather products, is an important aspect for the Brazilian industry - but the ticks constitute serious detriment

to processing and commercialization of these products (Kessler and Schenk, 1998). In addition to the disadvantages caused directly by the tick there are also indirect ones: expenses for employers of equipments and acaricide treatments for control and prevention strategies in combating the parasites (Nari, 1995). The complex consequences of tick infestation make it very difficult to define the economic losses to the cattle industry worldwide.

III. MATERIALS AND METHODS

1. Location

The study was carried out from December 2010 to January 2011 on a farm in the South of Minas Gerais, Brazil (see figure 10). The farm “Fazenda do Porto“ is located near the city Cordislandia (latitude 21° 79′ longitude 45° 67′, 816 m) and counts with its 1500 hectare to a midsized farm in Brazil. In this area the average temperature is about 21.05 °C and receives on average of 1080.7 mm of precipitation annually (www.agritempo.gov.br/agroclima/sumario). The region where the farm is located, is characterized by two well defined seasonal periods. From March to September there is a decrease in temperature and rainfall what leads to a reduction of the population of vectors. The period from October to February coincides with an increase in temperature and rain, resulting in an increased number of vectors (Melo et al., 2001).

Among the 520 dairy cattle, that are the principal income, the farm also conducts with horse breeding, winery and production of coffee and “cachaça”.

The dairy cattle of the different pure breeds Canadian Holstein, Jersey and Gir and the crossbred Gir-Holanda are kept in a system of semi-confined free stall and pasture with 290 hectare. They are fed with corn silage, damp grain, soya and citrus pulp. The daily production of milk is about 4,400 liters and the milk is sold to the cooperation of “Boa Esperança” (CAPEBE). The animals are vaccinated annually against brucellosis, aftosis, leptospirosis, rabies and mastitis and additionally they received antihelmintics.

The above information was obtained through a questionnaire answered by the manager of the farm.

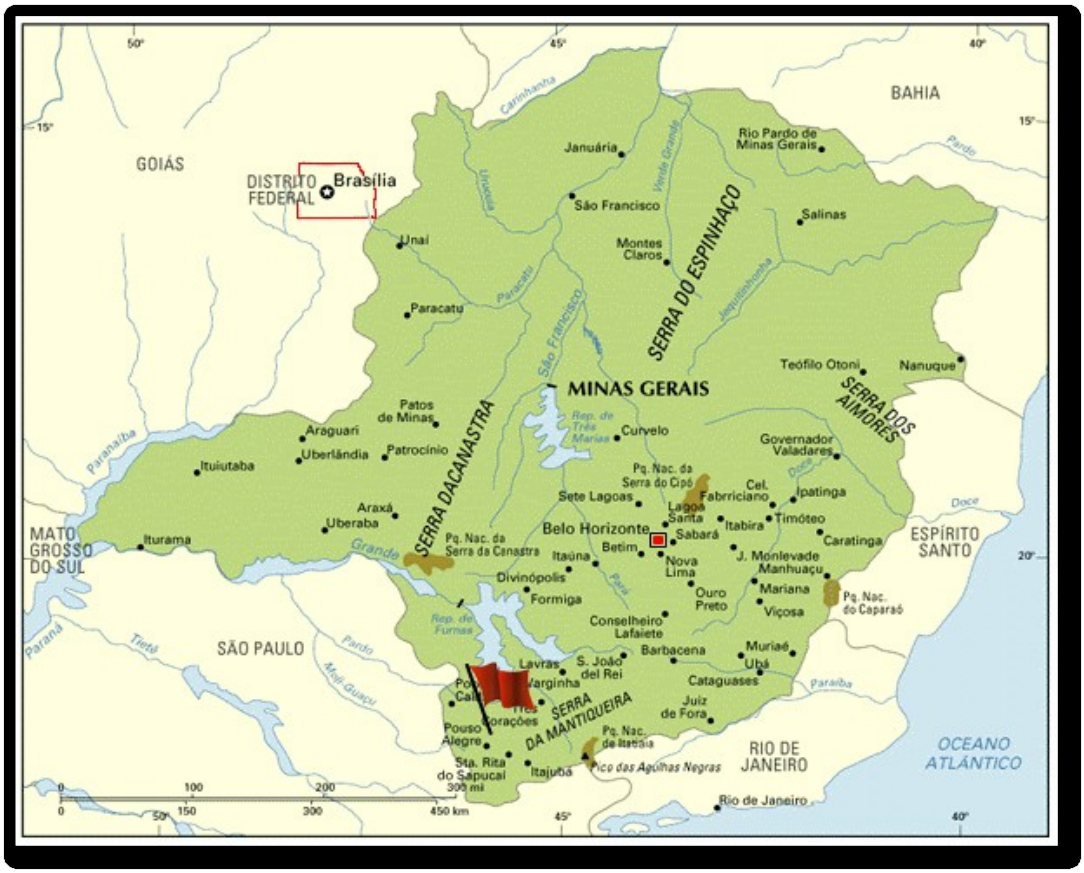

Figure 10: Map of Minas Gerais, Brazil and location of the farm (adapted from www.v-brazil.com/tourism/minas-gerais/map-minas-gerais.html)

1.1. Animals

The 100 examined calves were of the different breeds Canadian Holstein, Jersey and Gir and the crossbreed Gir-Holanda. They were hold in seven different stables divided according to their age and breed (see figure 11 and 12).

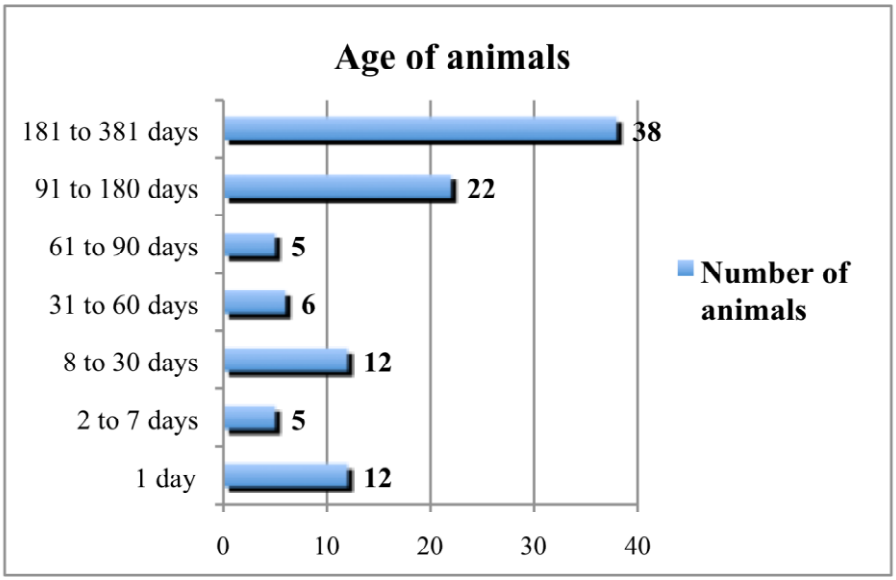

Figure 11: Number of animals with the different ages

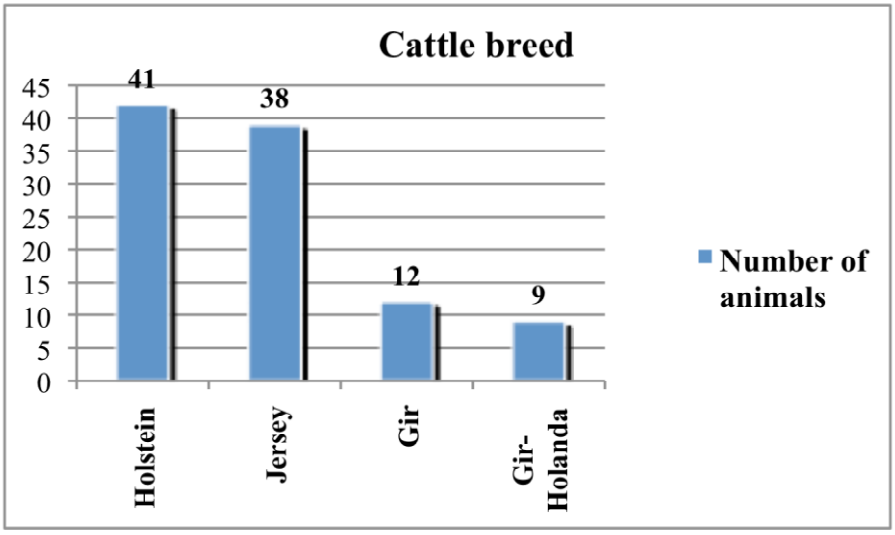

Figure 12: Number of animals with the different breeds

Maternity ("maternidade")

The calves stayed in the maternity from the day they were born until they completed seven or eight days. The area was composed of a cemented and covered place with an attached three meters long and seven meters wide pasture. The cemented place also was used as hospital ward and claw trimming of the adult cattle. For general cleaning the ground was hosed with water and chalk in powder form was spread over it. The calves received two liters milk from their mothers twice a day and water *ad libitum*. In the course of the research 14 calves of the age of one day to seven days were

examined and sampled in the maternity.

Figure 13: Maternity (A. Pohl)

Calf stable 1 ("bezerreio 1")

With the age of one week the calves were transferred from the maternity to a calf stable and stayed there until they were weaned with the age of 120 days or reached a weight of more or less 70 kilos. The animals which were sick, too small or light for their age stayed a bit longer in the calf stable 1. The covered stable was composed of a 15 meters long and five meters wide cemented, lightly lowered floor surrounded with a 1.5 meters high wall. Water and concentrate were disposal *ad libitum*. During the day the calves had access to a pasture of 100 square meters as well equipped with water and concentrate *ad libitum*. In the morning and the evening they were fed with three liters of milk from the pail. Animals with diarrhea received saline solution to prevent dehydration. In the course of the research 22 calves of all breeds (Canadian Holstein, Jersey, Gir and Gir-Holanda) and with the age of 14 days to 122 days were examined and sampled in the calf stable 1.

Figure 14: Calf stable 1 exterior view (A. Pohl)

Figure 15 Calf stable 1 interior view (A. Pohl)

Calf stable 2 ("bezerreio 2")

The calf stable 2 was constituted of a covered free stall with a 200 square meters pasture. The feeding ground was a cemented and covered tray with a concrete floor. The calves were fed with corn silage, damp grain and soya and water *ad libitum*. The nine animals were of the age 123 to 245 days and of all breeds.

Figure 16: Calf stable 2 and 3 (A. Pohl)

Calf stable 3 ("bezerreio 3")

The composition of the stable was the same as of calf stable 2 as well the feeding with corn silage, dump grain and soya. The eleven animals of the breeds Canadian Holstein and Jersey were of the age of 162 to 245 days.

Calf stable 4 ("bezerreio 4")

The calf stable 4 corresponded in structure and feeding with the calf stable 3, however, was twice as big and had a pasture with 400 square meters. The 21 calves with the age of 217 to 377 days were of the breeds Canadian Holstein and Jersey.

Figure 17: Calf stable 4 (A. Pohl)

Heifer 1 ("novilheiro")

The area for the heifer was composed of a 400 square meters pasture and a cemented and covered feeding place. They were fed with corn silage, dump grain and soya and water *ad libitum*. The animals remained in the heifer until they were old enough for insemination. In the course of the research seven heifers of the breeds Canadian Holstein and Jersey with the age of 258 to 381 days were examined and sampled.

Figure 18: Heifer 1 (A. Pohl)

Stable of the Gir ("curral do Gir")

As its name implies in the stable were only reared calves of the breed Gir (*B. indicus*). The covered free stall with concrete floor was attached to a 200 square meters pasture to which the animals had access during the day. The age group of the 16 calves was between one to 177 days. Up to the age of 120 days the animals suckled twice a day two liters at their mothers udder and received corn silage, dump grain and soya for feeding and water *ad libitum*.

Figure 19: Stable of the Gir (A. Pohl)

2. Collection of samples

2.1. Blood samples

A total of 100 blood samples were collected from 100 calves of the different breeds (Canadian Holstein, Jersey, Gir, Gir-Holanda) and different ages (from one to 381 days old). The blood samples were taken from the jugular vein using disposable needles for each animal and stored in 10 ml tubes containing EDTA (ethylenediaminetetraacetic acid). Of each sample the packed cell volume (PCV) was determinate and the EDTA-tubes were immediately refrigerated. The processing of the blood samples and the hematological techniques were carried out at the Laboratório de Doenças Parasitárias do Departamento de Medicina Veterinária Preventiva (DMVP) da Escola de Veterinária and no Departamento de Parasitologia (ICB) da Universidade Federal de Minas Gerais, Belo Horizonte. For the subsequent DNA-extraction 0.9 ml blood of each sample were separated with a sterile pipette into a 1.5 ml Eppendorf tube and stored at -20 °C. In addition the samples were centrifuged at 4,500 x g for ten minutes to obtain the plasma samples which were stored in 1.5 ml Eppendorf tubes at -20 °C.

Figure 20: Calves of the breed Canadian Holstein, Jersey, Gir and Gir-Holanda (A. Pohl)

2.2. Blood smears

The blood smears were prepared with blood from the tail of each animal with the aim to detect the presence of hemoparasites. After air-drying they were fixed in methanol and stained with Giemsa (Schalm et al., 1975). The smears were examined under light microscope (Olympus BX40) with a magnification of 100 times under oil immersion. The parasitemia was determined by counting infected erythrocytes in 20 fields containing approximately 300 to 400 erythrocytes.

2.3. Packed cell volume (PCV)

One of the typical symptoms of anaplasmosis and babesiosis is anemia. Therefore hematocrit values can serve as a reliable and significant measurement parameter for the health status of the animal.

The packed cell volume was determined using the microhematocrit technique (Schalm et al., 1975). Capillary tubes were filled with blood of each sample, the unfilled ends were sealed and the tubes were centrifuged at 10,000 rpm for five minutes. Afterwards the hematocrit values were determined with the aid of a microhematocrit scale.

2.4. Clinical examination

With the objective of a clearer judgment of the health status of each animal additional clinical examination were made. The rectal temperature was taken from each calf. Temperatures between 38.0-39.5 °C were considered as normal. Animals with temperatures > 39.5 °C were considered to have fever and those with temperatures < 38.0 °C to have subnormal temperature. Furthermore the mucous membrane of the eye was evaluated with a scale from one to three (1-normal, 2-anaemic, 3-icteric).

3. Laboratory analysis

3.1. DNA-Extraction

DNA-Extraction from each blood sample was carried out using the commercial Wizard® Genomic DNA Purification kit according to manufacturer's instructions (Promega, Madison, USA) with modifications.

Each frozen blood sample (1.5 ml Eppendorf tube) was defrosted, gently rocked and a volume of 300 µl blood was added to a sterile 1.5 ml Eppendorf tube containing 900 µl of "Cell Lysis Solution" (Promega, Madison, USA). Subsequently the tubes were inverted five to six times and incubated for ten minutes at room temperature to lyse the red blood cells. Afterwards the mixture was centrifuged at 13,000-16,000 x g for 20 seconds at room temperature and as much supernatant as possible was removed without disturbing the visible white pellet. If the pellet appeared to contain red blood cells and was not white, the steps above were repeated until all erythrocytes were lysed. Then the tube was vortexed, 300 µl of "Nuclei Lysis Solution" was added and the solution was pipetted five to six times to lyse the white blood cells. If clumps of cells were visible after mixing, the solution was incubated at 37 °C until the clumps were disrupted. After incubation 100 µl of "Protein Precipitation Solution" was added and mixed vigorously for 10-20 seconds in the vortex. Subsequently the sample was centrifuged at 13,000-16,000 x g for three minutes at room temperature and the supernatant was transferred to a clean 1.5 ml Eppendorf tube containing 300 µl isopropanol (room temperature). The solution was mixed gently by inversion and centrifuged as before for one minute at room temperature. The supernatant was decanted carefully, 300 µl of 70% ethanol was added to the pellet, mixed and centrifuged at the same conditions for one minute. The excessive ethanol was aspirated carefully and the tube containing the DNA pellet was inverted on a clean absorbent paper and air-dried for one day. Finally the DNA was rehydrated with 30 µl

"DNA Rehydration Solution" by incubation at 65 °C for one hour. The extracted DNA was stored at -20 °C until further use.

3.2. Indirect Fluorescent Antibody Test (IFAT)

The main principle of the IFAT consists in the attachment of antibodies on a tissue (microscope slide) fixed antigen. A secondary antibody (sheep-anti-bovine-IgG-FITC) conjugated with fluorescein-isothiocyanat makes it possible to examine and evaluate the complex in the fluorescence microscope. The microscope slides, which served as antigen, had been previously prepared with blood from experimentally infected cattle with *A. marginale, B.bovis* and *B. bigemina.* The test was carried out using the following protocol (IICA, 1987).

The microscopic slides, which served as antigen, and serum samples (1.5 ml Eppendorf tubes) of each cattle were taken out of the freezer and defrosted. The different microscopic slides were marked with letters corresponding to the infected blood primed on it (A – *Anaplasma marginale*, Bi – *Babesia bigemina*, Bo – *Babesia bovis*) and tagged with 18 circles using a good visible nail polish and air- dried. For evaluation of sera, serum and positive and negative control were applied on the circles (figure 21).

The plasma samples were diluted in a microtiterboard with PBS in a concentration of 1/40. Furthermore undiluted PBS was used for a negative control and blood sera of positive calves were used for the positive control group. Of each diluted test sera and the control groups one drop was applied in the nail polish circles of the microscopic slides. The microscopic slides were stored in a special humid and lightproof box and incubated in a cabinet drier at 37 °C for 30 minutes. Afterwards the slides were washed two times with PBS (cover with PBS, let impact for three minutes, drain off) and one time with distillated water and dried with a ventilator. 100 µl of Evansblue solution were mixed with 1 µl of Anti-bovine-IgG conjugate and one drop of the mixture was applied on each circle with the sample. The microscopic slides were incubated again for 30 minutes at 37 °C in the same box and washed the same way two times with PBS and one time with distillated water and dried. Some drops of glycerin were put on the slides, a cover slip was added and the microscopic slides were examined in the fluorescence microscope.

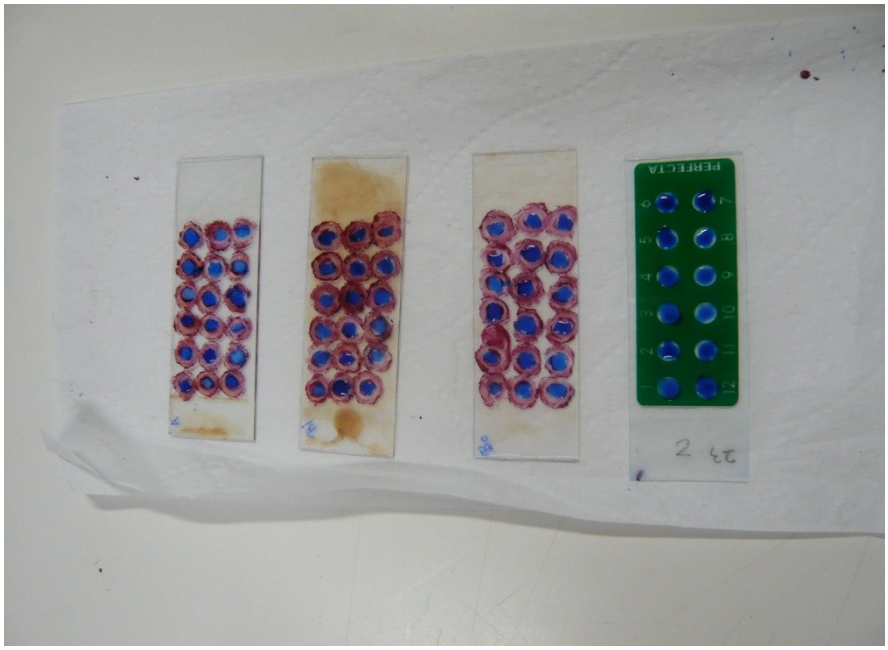

Figure 21: Microscopic slides for IFAT evaluation (A. Pohl)

3.3. DNA concentration

For verification of the quality and quantity of the DNA extraction the samples were measured in a spectrophotometer (NanoDrop®ND-1000, Erlangen, Germany) at the laboratory of the Institute for Comparative Tropical Medicine and Parasitology, Ludwig-Maximilians-Universität (LMU) München, Germany.

3.4. Polymerase Chain Reaction

3.4.1. Principle of the Polymerase Chain Reaction

The polymerase chain reaction is based on the enzymatic amplification of a defined DNA sequence using two flanking oligonucleotides primer and a thermo stable DNA polymerase. In general the process includes 30 to 50 amplification cycles and is carried out in a thermo cycler. Each amplification cycle requires a repetitive series of three fundamental steps which defines one PCR cycle.

Denaturation: The double-stranded DNA is denatured into two complementary single strands by heating to 94-95 °C.

Annealing: After cooling down the primer hybridize with the complementary part of the single-stranded DNA.

Extension: The DNA polymerase incorporates free nucleotides into the DNA to form a new double-stranded DNA.

The three-step process is repeated in several cycles what leads, under ideal conditions, to an exponential increase of the target sequence flanked by the two primers. In a final step the PCR products are made visible with agarose gel electrophoresis.

3.4.2. Real-time PCR

The real-time PCR combines amplification and detection in a single step that enables to collect data throughout the PCR process as it occurs. The PCR products are made visible already during the amplification process by adding unspecific DNA-binding fluorescent dyes or special fluorescent hybridization probes. The target amplification is first detected when fluorescence intensity is greater than background fluorescence. The intensity of the fluorescence correlates proportionally with the concentration of the PCR products and does not require post-amplification with agarose gel electrophoresis what reduces the risk of contamination (Wong and Medrano, 2005). Sequence-specific fluorescence probes are a quantification method used to measure the present DNA concentration and are more specific as DNA binding dye (SYBR Green I). Fluorescence probes are oligonucleotids marked with a fluorophore complementary to the target sequence and after hybridization create a fluorescent signal that is measured. The probes are labeled with an acceptor dye (quench molecule) on the 3′-end and a donor dye (reporter molecule) on the 5′-end leading to an increase in fluorescence resonance energy transfer (FRET= Förster resonance energy transfer) when bound to the target sequence (Wong and Medrano, 2005).

The most common types of probes are those:

Hybridization probes: They bind to the sequence between the two primers. The reporter dye absorbs light of a specific wavelength and transmits the energy to the acceptor. The signal of the acceptor becomes stronger when the distance between acceptor and donor is diminished (Wong and Medrano, 2005).

TaqMan® probes: Half-moon-shaped hydrolysis probes. The quencher dye at the 3′-end reduces the intensity of the reporter dye on the 5′-end in the intact probe. When annealed to the target sequence and degraded by the DNA polymerase during the extension step the reporter and the quencher are separated resulting in a increased fluorescence emission by the diminished quencher (Wong and Medrano, 2005).

Molecular beacons: They consist of a sequence-specific region "loop" flanked by two inverted repeats. When the beacon is free in solution, reporter and quencher are attached to each other by the hairpin formation causing a reduction in fluorescence emission via contact quenching (FRET). By binding to the target sequence, quencher and reporter are separated allowing emission of fluorescence (Wong and Medrano, 2005).

Furthermore examples for probes are Scorpions, Sunrise and Light upon extension (LUX) which are a combination of primer and probe.

The probe used for the real-time PCR for detection of *A. marginale* in the study was a TaqMan® primer with a 6-FAM as reporter molecule and a BHQ1 as quencher molecule.

3.4.3. Nested PCR

Nested PCR is a variation of the polymerase chain reaction with two amplification cycles succeeding each other. The first pair of primers amplifies a fragment similar to a standard PCR. During the second cycle the amplified product serves as a template DNA and the second primer binds inside the fragment allowing amplification of a shorter product. By this way unspecific byproducts are minimized and the sensitivity is increased.

3.5. Polymerase Chain Reaction for identification of *Anaplasma marginale* DNA

3.5.1. Real-time PCR for detection of the *msp1β* gene of *Anaplasma marginale*

For the initial screening a real-time PCR protocol was chosen from Carelli et al. (2007) and slightly modified. The target sequence lies on the major surface protein 1b (MSP1b) and forms with the MSP1a the MSP1 complex (de la Fuente et al., 2003). The *msp1β* gene is a member of a polymorphic multigene family and has been shown to be involved in adhesion to bovine erythrocytes (de Andrade et al., 2004). The primer AM-For and AM-Rev and the TaqMan® probe AM-Pb hybridize with the conserved region of the gene and generated a 95-bp fragment (table 1). The amplification was carried out in a 7500-Fast-Real-Time PCR System (Applied Biosystems, Darmstadt, Germany).

Table 2 and 3 show the reaction and cycling conditions used. Sterile water was included as negative control.

Table 2: Primers for real-time PCR for detection of the *msp1β* gene of *Anaplasma marginale*

Primer	Oligonucleotide sequence	Reference
AM-For	5′-TTGGCAAGGCAGCAGCTT-3′	Carelli et al., (2007)
AM-Rev	5′-TTCCGCGAGCATGTGCAT-3′	
AM-Pb	5′-6FAM-TCGGTCTAACATCTCCAGGCTTTCAT-BHQ1-3′ labeled 5′-6-carboxy-fluoroscein (6FAM) 3′-blackhole (BHQ1)	

Table 3: Reaction conditions for real-time PCR for detection of the *msp1β* gene of *Anaplasma marginale*

Reagent	Volume	
Gene Expression TaqMan MMX	15.0	µl
Primer f AM-f (10 µM)	2.25	µl
Primer r AM-r (10 µM)	2.25	µl
Sonde AM-p (10 µM)	0.5	µl
Template DNA	5.0	µl
Total Volume	**25.0**	**µl**

Table 4: Cycling condition for real-time PCR for detection of the *msp1β* gene of *Anaplasma marginale*

Reaction	Temperature	Duration	Cycle
Incubation	50 °C	2 min	1x
Activation	95 °C	10 min	1x
Denaturation	95 °C	15 sec	40x
Annealing Extension	60 °C	1 min	40x

3.5.2. Hemi-nested PCR for the detection of the *msp1α* gene of *Anaplasma marginale*

As it was intended to gain an overview of the genetic diversity present on the farm, a second hemi-nested PCR targeting the *msp1α* gene was run. The protocol, chosen from Lew et al. (2002), was applied to 30 of the samples showing a positive *msp1β* result and products were subsequently sequenced.

The same forward primer 1733F–5′-TGTGCTTATGGCAGACATTTCC-3′ was used in both amplification cycles with two different reverse primers (3134R–5′-TCACGGTCAAAACCTTTGCTTACC-3′ and 2957R–5′-AAACCTTGTAGC CCCAACTTATCC-3′) using primer 3134R in the first and 2957R in the second PCR. Reactions were performed in an automated DNA thermal cycler (Eppendorf Mastercycler® , gradient) with the reaction mix and cycling conditions as shown in tables 5 and 6.

Table 5: Reaction conditions for hemi-nested PCR for detection of the *msp1α* gene of *Anaplasma marginale*

Reagent	**Volume**	
Buffer (10x15 mM)	5.0	µl
$MgCl_2$ (10 µM)	1.0	µl
dNTPs (10 mM each)	1.0	µl
Primer f (10 µM)	0.5	µl
Primer r (10 µM)	0.5	µl
HotStar Taq Plus (5 U/µl)	0.25	µl
Pure H_2O	36.75	µl
Template DNA	5.0	µl
Total Volume	**50.0**	**µl**

Table 6: Cycling conditions for hemi-nested PCR for detection of the *msp1α* gene of *Anaplasma marginale*

Reaction	**Temperature**	**Duration**	**Cycle**
Primary amplification			
Initial denaturation	95 °C	5 min	1x
Denaturation	94 °C	30 sec	40x
Annealing	55 °C	1 min	40x
Extension	72 °C	2 min	40x
Final extension	72 °C	7 min	1x
Second amplification			
Initial denaturation	95 °C	5 min	1x
Denaturation	94 °C	30 sec	40x
Annealing	60 °C	1 min	40x
Extension	72 °C	2 min	40x
Final extension	72 °C	7 min	1x

3.6. Agarose gel electrophoresis

Conventional PCR products were electrophoresed on 2% agarose gel stained with Gel Red® (Biotium, USA, Gel 50 ml, GelRed 5 µl) using 50 bp and 100 bp ladders as size markers (Ladder Mix 1/12 Loading-dye, Fermentas Life Science, Leonrot, Germany). They were visualized under UV light.

3.7. DNA Purification

The purification of PCR products was carried out with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

3.8. Sequencing and Sequence Analysis

After purification all PCR products were send off for sequencing (Eurofins, MWG, Operon, Ebersberg). The results were evaluated with Chroma©Lites (www.technelysium.com.au/chromas_lite.html), and sequence similarities were searched by using BLASTn analysis (www.ncbi.nlm.nih.gov.library.vu.edu.au/BLAST/).

IV. RESULTS

No cases of clinical anaplasmosis or babesiosis were observed in the calves or any other animals of the farm during the study period.

1. Direct examination of the blood smears

The direct examination of the blood smears from the 100 calves showed an overall rate of positivity of 48% for *A. marginale.* From the 48 positive calves, 40 had levels of bacteremia below 1% and only seven calves had levels > 1% with the range from 1.1% to 6.5%. A single calf showed a value of 16% of infected erythrocytes.

The results in the light microscope for babesiosis revealed that only 14 (14%) animals were infected with *B. bovis*, while 18 (18%) were infected with *B. bigemina*.

Regarding the age of the animals infected with *B. bovis*, the youngest calf was 122 days and the oldest one 375 days old, the average age was 204 days. For *B. bigemina* infection the average age was 179 days and the youngest calf was 38 days and the oldest one 375 days old.

Only five (5%) animals had concurrent infections of *A. marginale*, *B. bovis* and *B. bigemina*.

2. Packed cell volume and infection rate

The analysis of the packed cell volume revealed that 88 (88%) calves had a normal PCV with values from 24% to 46%.

11 (11%) animals were anaemic and had a PCV < 24% with values between 18% and 23%. Of those 11 animals, six were positive in the blood smears to *A. marginale*, and one of them had a concurrent infection with *A. marginale* and *B. bovis*.

Only one calf had a higher PCV than normal (52%) with hemorrhagic symptoms. That animal was positive for *A. marginale* in the blood smear.

3. Clinical examinations

The majority of the calves (79%) had a normal temperature with values ranging from 38.0 to 39.5 °C. But even so, 18 (18%) animals had a elevated temperature (fever) with values between 39.6-40.9 °C and three (3%) calves had subnormal temperature with values from 37.8 to 37.9 °C. The animals with fever were of the age from 29 to 324 days. The three animals with under temperature were two newborn calves and one 14 days old calf.

The evaluation of the mucous membrane of the eye revealed that eight (8%) calves were evaluated on a scale from one to three with two, what means they were anaemic.

4. IFAT

Of 100 serum samples tested, 99 (99%) showed a positive result to specific antibodies of *A. marginale*. Only a newborn Jersey-calf had a negative antibody level.

Concerning the antibody levels of *B. bovis*, in 90 (90%) serum samples were detected positive antibody levels. The animals with negative antibody levels for *B. bovis* were of ages from one to 177 days.

The results for antibody levels to *B. bigemina* revealed 92 (92%) positive animals. Here also the calves with a negative level were of ages between one to 285 days of life.

5. Real-time PCR for *Anaplasma marginale*

94 blood samples were amplified in the real-time PCR for detection of the *msp1β* gene of *A. marginale*. Samples were considered negative when amplificated later than cycle 40. However, some samples remained doubtful due to late amplification between cycles 38 and 40 and had to been run a second time for confirmation. Figure 22 demonstrates the results of real-time PCR protocol with 40 cycles.

From the 94 samples, 66 were positive in real-time PCR outlining a rate of 70.2% for *A. marginale*.

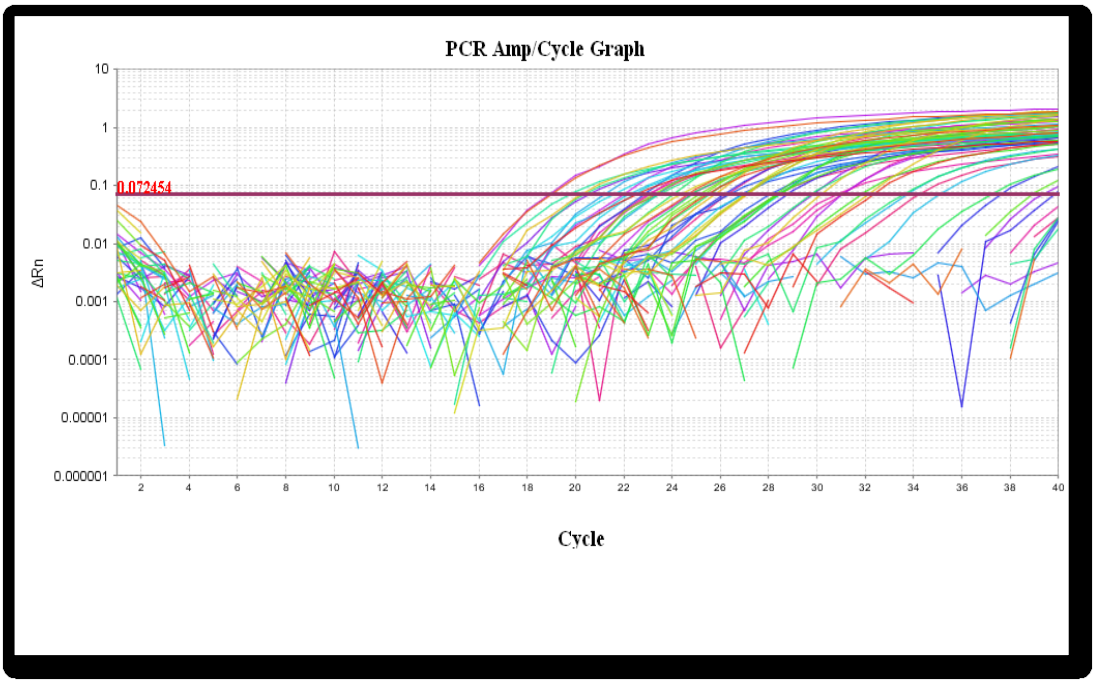

Figure 22: *Msp1β* real-time PCR results with 40 cycles

5.1. Real-time PCR and age

The correlation of PCR and the age in the different age groups can be featured out as shown in the following table 7.

An increasing PCR positivity can be identified with the increasing age.

In four calves with the age of one day could be detected an intrauterine infection of *A. marginale*.

Table 7: Detection of *Anaplasma marginale* in the real-time PCR in relation with the age

Age of animals (days)	**Animals**		
	Tested	**Positive (%)**	**Negative (%)**
0 to 7	16	4 (25)	12 (75)
8 to 29	12	2 (16.7)	10 (83.3)
38 to 107	13	8 (61.5)	5 (38.5)
108 to 381	53	52 (98.1)	1 (1.9)
Total number of animals	**94**	**66 (70.2)**	**28 (29.8)**

6. Hemi nested-PCR for *Anaplasma marginale*

30 samples, positive in the real-time PCR for *A. marginale* were selected to run a hemi-nested PCR targeting the *msp1α* gene of *A. marginale*.

Out of the 30 samples, 28 yielded bands of the correct nucleotide size in the gel electrophoresis.

Figure 23 shows the results in the gel electrophoresis.

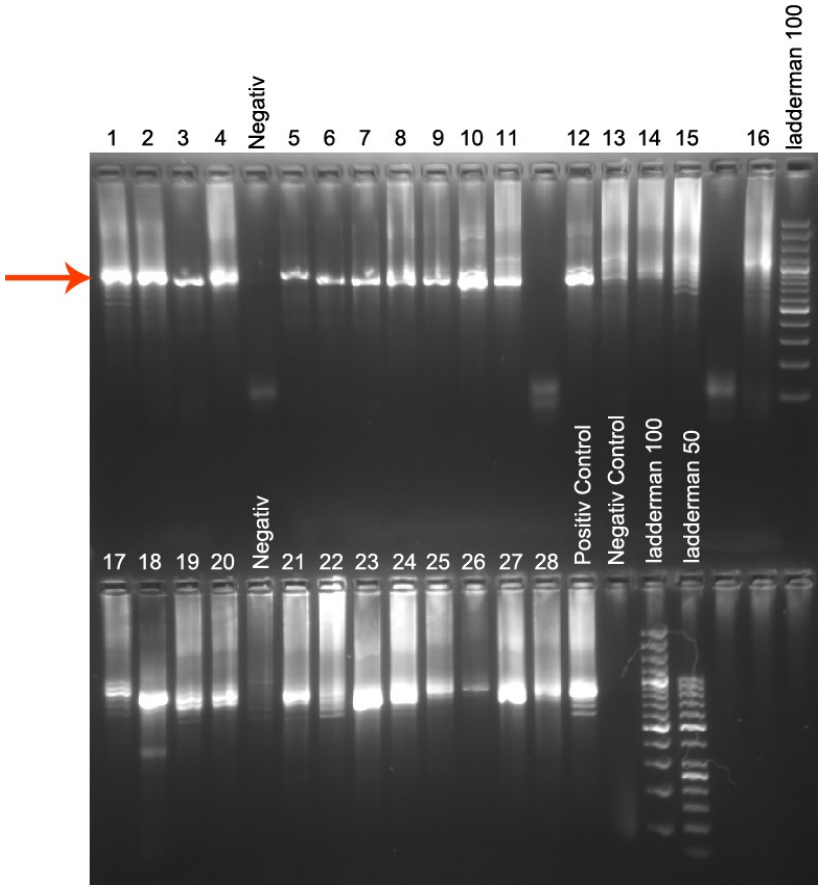

Figure 23 Results of the hemi-nested PCR targeting the *msp1α* gene (→) of *Anaplasma marginale* in the gel electrophoresis

(Samples 1 – 28 were positive in the gel electrophoresis)

7. Publication

The results of the sequencing analyses of *A. marginale* have been accepted for publication (Pohl et al., 2013) by the journal Revista Brasileira de Parasitologia Veterinária as follows:

Detection of genetic diversity of *Anaplasma marginale* isolates in Minas Gerais, Brazil

Detecção de diversidade genética de isolados *Anaplasma marginale* em Minas Gerais, Brasil

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Detection of genetic diversity of *Anaplasma marginale* isolates in Minas Gerais, Brazil.

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Abstract

Bovine anaplasmosis, caused by the tick-borne rickettsia *Anaplasma marginale*, is endemic in tropical and subtropical regions of the world and results in economic losses in the cattle industry. Major surface proteins (MSPs) have been used as markers for the genetic characterization of *A. marginale* strains and demonstrate that many isolates may occur in a given geographic area. However, in Brazil, little is known about the genetic diversity of *A. marginale* isolates within individual herds. This study was designed to examine the genetic variation among *A. marginale* infecting calves in a farm in the south of Minas Gerais State, Brazil. Blood samples collected from 100 calves were used to prepare Giemsa-stained smears that were microscopically examined for the presence of *A. marginale*. From each blood sample, DNA was extracted and analyzed by a polymerase chain reaction (PCR), followed by sequencing to determine diversity among the isolates. Examination of blood smears showed that 48% of the calves were infected with *A. marginale*, while the real-time PCR detected

70.2% positivity. Congenital infections were found in four calves. The microsatellite and tandem repeat analyses showed high genetic diversity among the isolates.

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Keywords: *Anaplasma marginale*, MSP1a, DNA sequencing, microsatellites, tandem repeats, Brazil.

Resumo

A anaplasmose bovina, causada pela rickettsia *Anaplasma marginale* e transmitida por carrapatos, é endêmica em regiões tropicais e subtropicais no mundo e causa grandes perdas econômicas na industria de bovinos. Proteínas principais de superfície (MSPs) foram usados como marcadores para a caracterização genética de amostras de *A. marginale*, demonstrando que diferentes isolados podem ocorrer numa certa região geográfica. Porém, no Brasil pouco se sabe sobre a variedade genética de isolados de *A. marginale* em rebanhos individuais. Este estudo teve como objetivo investigar a ocorrência de variação genética entre bezerros infectados com *A. marginale* numa fazenda do sul de Minas Gerais, Brasil. Amostras de sangue coletadas de 100 bezerros foram utilizadas para o preparo de esfregaços sanguíneos, corados pelo Giemsa, para detecção da infecção por *A. marginale*. Amostras de DNA extraídas de cada amostra foram analisadas através de PCR seguido de sequenciamento. O exame dos esfregaços demonstrou que 48% dos bezerros estavam infectados com *A. marginale*, enquanto que o PCR detectou 70,2% de positividade. Infecção congênita foi detectada em quatro bezerros. As análises de microsatélites e 'tandem repeats' comprovaram uma grande diversidade genética entre os isolados.

Palavras-chave: *Anaplasma marginale*, MSP1a, sequenciamento de DNA, microsatélites, tandem repeats, Brasil.

Introduction

Anaplasmosis is a tick-borne disease of ruminants, caused by the obligate intra-erythrocytic bacterium *Anaplasma marginale* with a widespread distribution in tropical-to-temperate climates (AUBRY; GEALE, 2011). Ticks are the biological vectors of *A. marginale* and the one-host tick *Rhipicephalus (Boophilus) microplus* is considered to be the main vector in Brazil (KESSLER; SCHENK, 1998). The pathogen can also be transmitted mechanically by biting flies, blood-contaminated fomites, or congenitally by transplacental transmission (ZAUGG; KUTTLER, 1984; PASSOS; LIMA, 1984). Transplacental transmission of *A. marginale* may therefore contribute to the epidemiology of this disease in some regions (KOCAN et al., 2003). Cattle of all ages can become infected and remain persistently infected carriers for life, with clinical signs varying from asymptomatic to acute cases with fever, anaemia, abortion, weight loss, lowered milk production or death (KESSLER; SCHENK, 1998; RIBEIRO; PASSOS, 2002).

Direct diagnosis can be made by microscopic examination of Giemsa-stained blood smears, but this method can only detect levels of $>10^7$ infected erythrocytes per ml of blood (PALMER et al., 2000).

For epidemiological surveys, indirect and direct methods, such as the Indirect Fluorescent Antibody Test (IFAT) and the real-time PCR are more appropriate to reveal the status of a herd.

Brazil is considered to be an endemic area where calves, being less susceptible to clinical disease than adults, acquire the infection shortly after birth which seems to be an important factor for endemic stability (AUBRY; GEALE, 2011). However, although anaplasmosis is endemic in Minas Gerais State, the occurrence of outbreaks causes huge economic losses to the cattle industry (RIBEIRO; REIS, 1981).

Many different geographic strains of *A. marginale* have been identified, which differ as regards to biology, genetic characteristics and transmissibility by ticks (DE LA FUENTE et al., 2005, 2007). The *A. marginale* major surface protein 1a (MSP1a) was shown to be involved in vector-pathogen and host-pathogen interactions and to have evolved under positive selection pressure (DE LA FUENTE et al., 2003a). Among different strains, the MSP1a differs in molecular weight due to a variable number of tandem 23- to 31-amino-acid repeats, and it has been proven to be a stable marker of strain identity (ESTRADA-PEÑA et al., 2009).

The understanding of *A. marginale* epidemiology, including the characterization of the genetic diversity of strains in a region, provides knowledge for the development and implementation of control measures.

Therefore the aim of the present study was to determine the occurrence of genetic diversity within a herd in a dairy farm in Brazil.

Materials and Methods

Samples and microscopic examination

The study was carried out from December 2010 to January 2011 on a dairy farm located near Cordislândia (latitude 21° 79′ longitude 45° 67′, 816 m), in Minas Gerais State, Brazil. Blood samples were collected from the jugular vein into EDTA from all the calves (100) on the farm, which were of the breeds Holstein, Jersey and crossbreed. The calves were classified into 4 groups according to their ages: Group 1 (17 calves from 1 to 7 days old), Group 2 (12 calves from 8 to 30 days old), Group 3: (15 calves from 31 to 107 days old) and Group 4: (56 calves from 108 to 381 days old). Giemsa-stained blood smears were prepared and bacteremia was calculated as the percentage of *A. marginale* infected erythrocytes detected in 20 microscopic fields.

Genomic DNA isolation and PCR

DNA was extracted from 94 blood samples using the commercial Wizard® Genomic DNA Purification kit according to the manufacturer's instructions (Promega, Madison, USA). DNA concentrations were determined using the spectrophotometer NanoDrop®ND-1000 (PepLab, Erlangen, Germany). For the initial screening a real-time PCR was used, as reported by CARELLI et al. (2007) with modifications for targeting the *msp1β* gene. All amplifications were performed in a 7500-fast-Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) and were carried out in a 25 µl reaction mixture containing 5 µl of DNA template, 15 µl TaqMan® Gen Expression Master Mix (Applied Biosystems, USA), 2.25 µl (10 µM) of each primer (AM-forward: 5′ TTGGCAAGGCAGCAGCTT 3′ and AM-reverse: 5′ TTCCGCGAGCATGTGCAT 3′) and 0.50 µl (10 µM) of the probe (AM-probe: 6FAM-5`-TCGGTCTAACATCTCCAGGCTTTCAT-3`-BHQ1). Cycling was performed under the following conditions: 2 min/50°C; 10 min/95°C and 40 cycles of

15 sec/95°C and 1 min/60°C.

For sequencing, 13 positive samples from calves of different ages were selected and further analyzed by a hemi-nested PCR targeting the *msp1α* gene following the protocol of Lew et al. (2002). Reactions were performed in an automated DNA thermal cycler (Eppendorf Mastercycler® , gradient) using the primers 1733F (5′ TGTGCTTATGGCAGACATTTCC 3′), 3134R (5′ TCACGGTCAAAACCTTT GCTTACC 3′) and 2957R (5′ AAACCTTGTAGCCCCAACTTATCC 3′). The primary amplification cycle, following an initial denaturation at 94°C for 5 min, consisted of 40 cycles at 94°C of 30 sec, 1 min at 55°C and 2 min at 72°C, followed by a final extension step for 7 min at 72°C. The same conditions were used in the second amplification cycle except that the annealing temperature was changed to 60°C. For visualization, PCR products were electrophoresed on a 2% agarose gel stained with Gel Red® (Biotium, USA). In addition, amplified fragments were purified using a commercial kit (QIAquick PCR purification Kit, Qiagen, Hilden, Germany) and sent for sequencing of both strands (Eurofins, MWG, Operon, Ebersberg).

DNA sequence analysis

All MSP1a sequences of *A. marginale* isolates from Minas Gerais available in GenBank were included for the genetic diversity analysis. A microsatellite was located in the MSP1a 5'UTR between the putative Shine-Dalgarno sequence (GTAGG) and the translation initiation codon (ATG). The structure of the microsatellite (**bold**) was GTAGG **(G/A TTT)m (GT)n** T ATG (ESTRADA-PEÑA et al., 2009). The UFMG-1 and UFMG-2 isolates were not included in the microsatellite analysis because the 5'UTR was missing. The tandem repeat analysis was performed following the nomenclature proposed by DE LA FUENTE et al. (2007).

The database nucleotide collection (nr/nt) using the Megablast (optimized for highly similar sequences) from the BLAST server (http://blast.ncbi.nlm.nih.gov/) was applied to find homologies with our sequences. Nucleotide sequences were aligned using BLAST (ZHANG et al., 2000) and protein sequences were aligned using the multiple-alignment program CLUSTALW (THOMPSON et al., 1994). Nucleotide sequences were translated to amino acid (aa) sequence by the ExPASy translation tool of the Swiss Institute of Bioinformatics (ExPaSy Translation Tool, 2011).

The phylogenetic analysis was performed as follows: nucleotide sequences were

aligned with MUSCLE (v3.7) configured for highest accuracy (EDGAR, 2004). After alignment, ambiguous regions (i.e., containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b) (CASTRESANA, 2000). The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT) (GUINDON; GASCUEL, 2003, ANISIMOVA; GASCUEL, 2006). Reliability for internal branch was assessed using the bootstrapping method (100 bootstrap replicates). Graphical representation and editing of the phylogenetic tree were performed with TreeDyn (v198.3) (CHEVENET et al., 2006).

Results

Detection of A. marginale infections

A. marginale could be detected by microscopic examination of blood smears in 48 out of 100 animals (48%) with bacteremia ranging from 0.1 to 16.1%.

The results obtained by real-time PCR showed that 66 out of 94 animals (70.2%) were positive for *A. marginale* and were thus this method was more sensitive than direct examination of blood smears. *A. marginale* infection was detected in all the age groups.

Unexpectedly, congenital infection was detected in four 1-day old calves. All four calves were positive in the hemi-nested PCR and in the real-time PCR. Only one of them was negative by blood smear examination.

Table 1 shows the percentage of animals in each age group that were positive by blood smear and in the real-time PCR. From the MSP1a PCR an amplicon of approximately 0.8 Kb was isolated corresponding to the expected size of the targeted *msp1 α* gene fragment (Table 2).

MSP1a microsatellite analysis

The analysis of MSP1a microsatellite sequences resulted in five different genotypes amongst *A. marginale* strains from Minas Gerais (Table 3). The different microsatellite sequences produced SD-ATG distances between 19 and 23 nucleotides (Table 3). The predominant genotype was **E** and it was distributed among animals of different ages and breeds (Table 4). In sequences previously reported from Minas Gerais, the genotypes **G**, **D**, **C** and **E** were found (DE LA FUENTE et al., 2002).

Genotype **C** was not found in the present study and genotype **B** is reported for the first time in Minas Gerais isolates.

MSP1a tandem repeats and phylogenetic analysis

Differences were found in the tandem repeat sequences and in the structure of the gene *msp1α* among the different isolates from Minas Gerais. One MSP1a tandem repeat resulted in a new sequence with amino acid changes as shown in Figure 1. Twenty four different tandem repeats were found in all the analyzed sequences (Figure 2). Nine of them were not previously reported in Minas Gerais, but had been detected in Argentina, Mexico, South Africa and Israel (DE LA FUENTE et al., 2007), and one was a new sequence when compared with the tandem repeats reported worldwide for *A. marginale* MSP1a.

Interestingly, the tandem repeats σ, C, F, N, μ and 42, previously already reported in Minas Gerais (DE LA FUENTE et al., 2007), were not found in the MSP1a sequences analyzed in this study (Figure 2).

Using the 0.8 Kb fragment of MSP1a a maximum likelihood tree was built. The tree shows clusters among the different isolates from Minas Gerais and the phylogenetic relationship between them. Four clusters of MSP1a genes were found as shown in the phylogenetic tree (Figure 3).

Discussion

The analysis of MSP1a repeats sequences, which has provided evolutionary information about geographically distinct *A. marginale* strains (DE LA FUENTE et al., 2001, 2005, 2007), was used in the present study to characterize pathogen genetic diversity within a Brazilian dairy farm.

The results reported here confirm the presence of *A. marginale* infections among all age groups tested, and demonstrate that calves acquire the infection shortly after birth, suggesting that there is endemic stability for anaplasmosis in this dairy farm. On the other hand, the results reveal the presence of several *A. marginale msp1a* genotypes within the herd (Table 2). These results are consistent with the findings of high *A. marginale* genetic diversity in endemic areas worldwide (DE LA FUENTE et al., 2007).

MSP1a contains a variable number of tandemly repeated peptides in the amino-terminal region, while the remainder of the protein is highly conserved between isolates. The number of repeats varies among geographic isolates of *A. marginale* but is constant within an isolate and has been used as a stable genetic marker of isolate identity (DE LA FUENTE et al., 2003b).

Based on analysis of MSP1a tandem repeats, we found fourteen different strains in isolates from Minas Gerais (Figure 2), eight of them reported for the first time. Comparisons with the tandem repeats previously reported for MSP1a showed one new tandem repeat sequence in this study, which has been named 72 (Figure 1), following the nomenclature proposed by DE LA FUENTE et al. (2007).

One common structure (72-62-61) for MSP1a tandem repeats was found in six of the Minas Gerais isolates (Minas 6-10 and Minas 12), representing the most frequent strain in our study. The maximum likelihood phylogenetic tree shows that the MSP1a from Minas Gerais falls into four separated clusters. The tandem repeats 72-62-61 and C-F-N, contained in the isolate Brazil-5, reveal minor changes in amino acids (data not shown) and the phylogenetic tree shows that they fall in the same Cluster (Cluster 2), suggesting a common ancestor for both isolates. All the isolates containing at least one tandem repeat type β fall in the MSP1a Cluster 1 based on the phylogenetic tree, supporting the idea that strains containing the tandem repeat type β have common origins in Minas Gerais. Two of the strains containing tandem repeat type β found in our study, Minas-1 and Minas-3, belonged to animals with congenital *A. marginale*.

The phylogeographic clustering of some genotypes of the MSP1a microsatellite has been reported. The genotype **B** was exclusively associated with areas of southern Brazil but genotypes **C**, **D**, **E** and **G** have been also found in other geographic regions (ESTRADA-PEÑA et al., 2009).

The five genotypes found in Minas Gerais isolates were previously reported for this geographic region. The predominant genotype **E** was present in all of the phylogenetic clusters of MSP1a except for Cluster 3. The length of the MSP1a microsatellite could have affected the expression of MSP1a, thus influencing the infection and transmission of *A. marginale*. Higher expression levels were reported for SD-ATG distances of 23 and 29 nucleotides and lower expression for 19 nucleotides (ESTRADA-PEÑA et al., 2009). Almost all the tandem repeats found in the present study were 21 or 23 nucleotides long. It is noteworthy that all the SD-ATG distances in the congenitally transmitted *A. marginale* were 23 nucleotides. This suggests a high

capacity for infection and transmission of the *A. marginale* strains found in this area and particularly of the isolates transmitted congenitally.

Based on the analysis of MSP1a microsatellite and tandem repeat structure we found a high genetic diversity in the Minas Gerais *A. marginale* isolates. The genetic diversity of *A. marginale* MSP1a could be explained not only by evolutionary pressures exerted by ligand-receptor and host-parasite interactions (DE LA FUENTE et al., 2001) but also by constant movements of cattle or independent transmission (DE LA FUENTE et al., 2005). In the case of this farm, evolutionary pressure and independent transmission should be the main explanations for the genetic diversity, since no new animals had been introduced into the herd over the previous 20 years. Nevertheless, one animal with congenital *A. marginale* (isolate Minas-3) had a tandem repeat structure previously reported in Mexico (DE LA FUENTE et al., 2001).

Regarding congenital transmission, each of the four infected newborn calves carried a different strain of *A. marginale* (Minas-1 to -4) (Figure 2), but only two genotypes, **E** and **G**, based on the *msp1α* microsatellites (Table 3). The genotype **E** predominated in this and the other age groups, showing it to be the predominant genotype at the study site. This genotype was found in one sequence previously reported in Minas Gerais (Brazil-12) (Table 3).

Conclusions

From the results presented here we can conclude that (1) genetic diversity of *A. marginale* is high in Minas Gerais and (2) congenital transmission appears to be an important phenomenon that contributes to the persistence of several *A. marginale* strains within a herd. Further investigations should address the influence of such elevated genetic variation of *A. marginale* strains on epidemiological aspects of bovine anaplasmosis. This might contribute to a better understanding of the epidemiology of the disease and consequently to the development and implementation of appropriate control and prevention measures.

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Figures

Figure 1. New sequence of MSP1a tandem repeat (72) found in *A. marginale* isolates from Minas Gerais. The one letter amino acid code was used to depict the differences found in MSP1a repeats. Asterisks indicate identical amino acids and gaps indicate deletion/insertions. The repeat form B was taken as a model to compare the new repeat.

Figure 2. The structure of the MSP1a repeat regions, according to the nomenclature proposed by de la Fuente et al. (2007).

Figure 3. Unrooted phylogenetic tree based on the MSP1a protein sequences of *A. marginale* isolates from Minas Gerais. The tree shows the clusters of MSP1a. Bootstrap values are show as % in the internal branch (only values equal or higher than 50%).

Tables

Table 1. Positivity (%) for *A. marginale* detected by direct examination (blood smears) and by molecular analysis (RT-PCR).

Table 2. Isolates of *A. marginale* from Minas Gerais, Brazil included in the study.

Table 3. The *msp1α* microsatellite sequences were analyzed in 13 *A. marginale* isolates. The microsatellite (sequence in bold) was located between the Shine-Dalgarno (SD; sequence in brackets) and the translation initiation codon (ATG) with the structure: GTAGG (G/ATTT)m (GT)n T ATG. The SD-ATG distance was calculated in nucleotides as (4xm) + (2xn) +1.

Table 4. *A. marginale* genotype frequency per breed and age of the calves.

Table 1. Positivity (%) for *Anaplasma marginale* detected by direct examination (blood smears) and by molecular analysis (RT-PCR).

Group (age range in days)	+ Blood smears (%)	+ RT-PCR (%)
1 (1-7)	17.6	25
2 (8-30)	8.3	16.7
3 (31-107)	46.7	61.5
4 (108-381)	67.9	98.1

Table 2. Isolates of *Anaplasma marginale* from Minas Gerais, Brazil included in the study.

Isolate	Age (days)	Breed	Age group	GenBank accession number
Minas-1	0	Holstein	1	JX844205
Minas-2	1	Jersey	1	JX844206
Minas-3	1	Holstein	1	JX844207
Minas-4	1	Jersey	1	JX844208
Minas-5	14	Jersey	2	JX844209
Minas-6	29	Jersey	2	JX844210
Minas-7	40	Jersey	2	JX844216
Minas-8	78	Jersey	3	JX844211
Minas-9	78	Holstein	3	JX844217
Minas-10	103	Holstein	3	JX844212
Minas-11	253	Jersey	4	JX844213
Minas-12	280	Jersey	4	JX844214
Minas-13	355	Jersey	4	JX844215
Brazil	N/D	N/D	N/D	AF428092
Brazil-5	N/D	N/D	N/D	AY283198
Brazil-9	N/D	N/D	N/D	AY283199
Brazil-12	N/D	N/D	N/D	AY283200
UFMG-1	N/D	N/D	N/D	EU676175
UFMG-2	N/D	N/D	N/D	EU676176

N/D: Not Defined

Table 3. Structure of the *Anaplasma marginale msp1α* microsatellites

Isolates	Genotype	m*	n**	SD-ATG distance (nucleotide)
Minas-1	E	2	7	23
Minas-2	E	2	7	23
Minas-3	E	2	7	23
Minas-4	G	3	5	23
Minas-5	B	1	9	23
Minas-6	E	2	7	23
Minas-7	D	2	6	21
Minas-8	E	2	7	23
Minas-9	D	2	6	21
Minas-10	E	2	7	23
Minas-11	E	2	7	23
Minas-12	E	2	7	23
Minas-13	E	2	7	23
Brazil	G	3	5	23
Brazil-5	D	2	6	21
Brazil-9	C	2	5	19
Brazil-12	E	2	7	23

* m is the number of repetitions of the nucleotide sequence G/A TTT

** n is the number of repetitions of the nucleotide sequence GT

Table 4. *Anaplasma marginale* genotype frequency per breed and age

Genotype	Isolates	Breed		Group (age range in days)			
		Holstein	Jersey	1 (1-7)	2 (8-30)	3 (31-107)	4 (108-381)
B	1	0.00	1.00	0.00	1.00	0.00	0.00
D	2	0.50	0.50	0.00	0.50	0.50	0.00
E	9	0.33	0.66	0.33	0.11	0.22	0.33
G	1	0.00	1.00	1.00	0.00	0.00	0.00

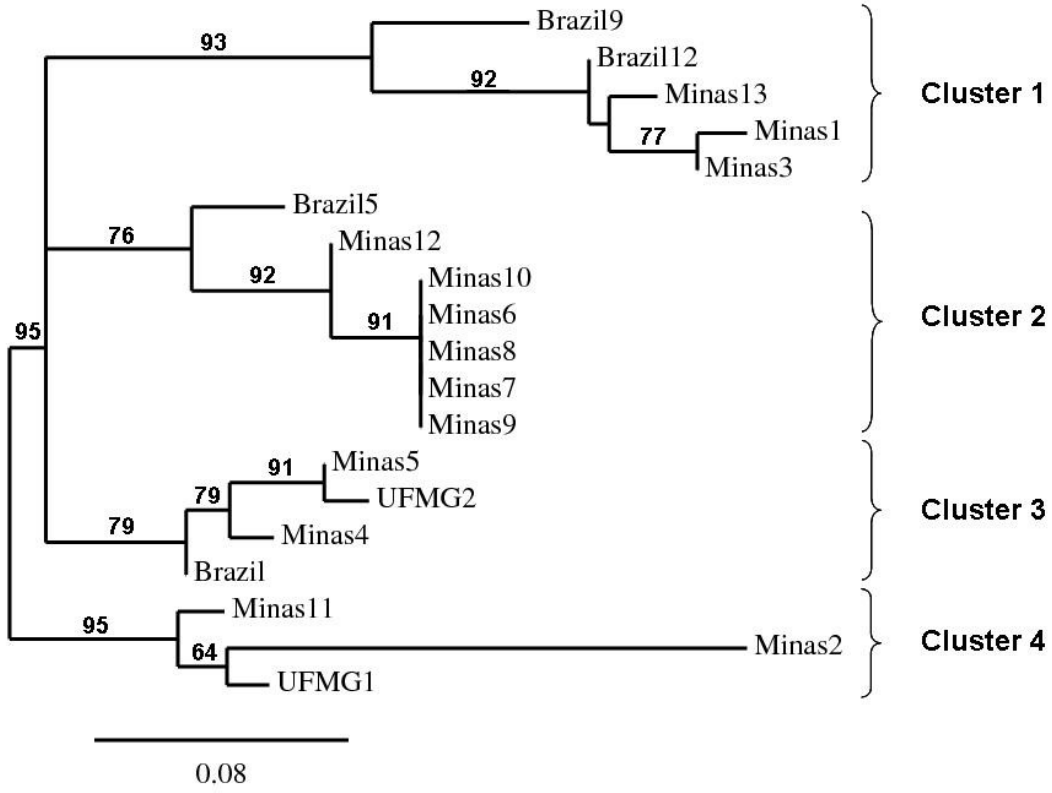
Figure 1. New sequence of MSP1a tandem repeat found in *Anaplasma marginale* isolates from Minas Gerais.

Repeat form	Encoded sequence
B	A D S S S A G G Q Q Q E S S V S S Q S D Q A S T S S Q L G
72	* * * * * * * D * * * * * * * * * * * * G - * * * * * * * *

Figure 2. Structure of the MSP1a repeat regions.

Isolates	Structure of MSP1a tandem repeats					No. of repeats
Minas-1	τ	57	β	β	γ	5
Minas-2	Is9	24	24	25	31	5
Minas-3	α	β	β	γ		4
Minas-4	B	Q	B	M		4
Minas-5	13	27	27	27		4
Minas-6	72	62	61			3
Minas-7	72	62	61			3
Minas-8	72	62	61			3
Minas-9	72	62	61			3
Minas-10	72	62	61			3
Minas-11	τ	57	13	18		4
Minas-12	72	62	61			3
Minas-13	α	β	β	13		4
Brazil	B	B	Q	σ	μ	5
Brazil-5	C	F	N			3
Brazil-9	α	β	τ	M		4
Brazil-12	α	β	β	N		4
UFMG-1	13	42	13	18		4
UFMG-2	13	27	27			3

Figure 3. Phylogenetic tree based on MSP1a protein.



V. DISCUSSION

The occurrence and distribution of tick-borne disease caused by hemoprotozoans *B. bovis* and *B. bigemina,* concomitant with the rickettsia *A. marginale,* is a widespread problem in the cattle industry and responsible for important economic losses in Brazil (Grisi et al., 2002). The economic impact is not only a consequence of direct losses such as mortality and reduction in meat and milk production; it also consists of indirect ones such as the cost of prevention and control measures. Furthermore, the importation of *B. taurus* breeds and general cattle movement from anaplasmosis- and babesiosis-free areas into endemic areas can lead to outbreaks with severe, life-threatening disease.

Several studies have been carried out on the epidemiological situation of anaplasmosis and babesiosis in specific regions of Brazil, but still little is known about the occurrence and diversity within a specific herd. Regarding the epidemiological survey and distribution of these pathogens in a defined herd, the study was performed to investigate the occurrence of tick-borne disease within a herd on a farm in Minas Gerais, Brazil, with a focus on the age of the calves and the genetic diversity of *A. marginale*.

For this purpose, 100 calves of different ages were tested microscopically and serologically for *A. marginale, B. bovis* and *B. bigemina.* Additionally molecularly testing was done for *A. marginale*, and the gene sequences compared with previously published isolates from Minas Gerais to identify differences between them and any newly identified sequences.

The results of the study revealed a high occurrence of *A. marginale*, *B. bovis* and *B. bigemina* within the herd, comparable to studies in other regions in Brazil (Barros et al., 2005; Madruga et al., 2001; Soares et al., 2000; Souza et al., 2000a; Souza et al., 2000b). Despite the high infection rate detected among the animals by blood smears and the PCR, no acute clinical infection could be determined. However, this does not exclude the possibility of subclinical infections of the calves, which could affect the clinical health status, fertility and milk production in later life.

The findings from this study have shown that the most positive diagnoses from blood smears were for *A. marginale,* with an overall infection rate of 48%. The majority of the *A. marginale* positive blood smears, 83.33%, had bacteremia levels below 1%.

Only seven calves had higher levels, ranging from 1.15% to 6.5%; a single calf had a high value of 16% which could indicate an acute infection. Depending upon the susceptibility of the host, 10% to 90% of the erythrocytes may be parasitized in the acute stage of *A. marginale* infection (Aubry and Geale, 2011). The low number of infected erythrocytes recorded here could be an explanation why no clinical signs of anaplasmosis were seen at the farm. Also, Giemsa-stained blood smears can only detect infection at levels above 10^7 infected erythrocytes per ml blood (Gale et al., 1996; Palmer et al., 2000) and so are not reliable for detecting pre-symptomatic or carrier animals, which are characterized by levels of bacteremia $10^{2,5}$ to 10^7 infected erythrocytes per ml blood (Kieser et al., 1990). Even so, the results showed that almost half of the calves were positive by blood smear. This is a very high number, leading to the conclusion that some of the calves could be negatively affected in their further development because of subclinical infections. Consequently, calves that are negatively influenced (feeble and puny) in their early development and life can lead to economic losses in the future due to fertility problems, reduced milk production and susceptibility for further infections. Thus it is very important to support the early development of the calves by a good calf rearing management, to avoid infections and to ensure healthy and robust animals in the future.

There were lower rates of direct detection of *B. bovis* and *B. bigemina* in blood smears, with only 14% and 18% positivity respectively. In five (5%) animals a concurrent infection with the three pathogens *A. marginale, B. bovis* and *B. bigemina* was detected. As with *A. marginale*, stained blood smears are usually adequate for detection of acute infections, but not for detection of carriers where the parasitemias are mostly very low (OIE, 2010). Even so, the sensitivity of blood smears is such that it can detect parasitemias as low as one parasite in 10^6 erythrocytes (Böse et al., 1995). Also, the number of erythrocytes infected with *B. bovis* or *B. bigemina* was lower compared to *A. margianle*-infected samples, which impedes the diagnosis by Giemsa-stained blood smears. However, the low results do not exclude subclinical infections which as already mentioned could influence the further development of the animals.

The study was carried out from December to January, the period in Minas Gerais which coincides with the rainy season and causes the most favorable climatic conditions for the development of populations of vectors. Therefore the calves were exposed to the ticks at an early age and could acquire the primary infection during their first days of life.

Through constant exposure of calves with ticks and tick-transmitted pathogens, the animals become chronically infected and develop immunity for *A. marginale, B. bovis* and *B. bigemina*. During the period from March to September (Autumn-Winter) a decrease in temperature and rainfall leads to less favorable climatic conditions for the vectors. Consequently not all of the animals born in this period acquire the infection in their first days of life. As susceptibility to anaplasmosis and babesiosis increases with age and decline of colostral antibodies (Aubry and Geale, 2011; Roby et al., 1961), calves born between April and August could therefore be more vulnerable developing clinical disease at the beginning of the rainy season (October)., when the vector population rises. Because of this, controlling the disease could be helped by targeting preventive measures to protect calves born in the winter from having clinical infections in the beginning of the rainy season.

The results for the hematocrit measurements in this study did not show any correlation with *A. marginal, B. bovis* or *B. bigemina* positive blood smears. The majority of the calves (88%) had a normal packed cell volume (PCV) with values between 24% and 46%. Even so, 11 (11%) animals had PCV values < 24% and of those, six were positive in the blood smears to *A. marginale*, and one of them had a concurrent infection with *A. marginale* and *B. bovis*. A possible explanation for finding no correlation between hematocrit and infection rate could be that the packed cell volume declines coincidental with increasing parasitemia and is therefore a better indicator for acute infections than chronically infected animals. During a latent infection the hematocrit ranges are within normal physiological value, such as those seen here. This supports the assumption of sub-clinical infections of some calves within the herd studied.

The clinical examinations found a normal, stable health status in the majority of the calves. 79 (79%) animals had a normal temperature with values between 38.0 and 39.5 °C and even 92 (92%) calves had mucous membrane of the eye in normal range, without being anaemic or icteric. An explanation for the good health status despite 48% of the animals being positive by blood smear for *A. marginale*, could be in the relationship between host, agent, vector and environment. As the climatic conditions during the period of the study were favourable for the vector population, the calves could acquire the infections before the loss of calfhood resistance and so without developing clinical signs. According to the person in charge of the cattle at the farm, previous cases of tick-borne disease (“Tristeza Parasitária Bovina”) were most notable

in calves with ages between 120-140 days. The principal clinical signs are fever, reduced appetite (anorexia) and isolation from the other animals in the group. In most cases they did not die and recovered from the infection after antibiotic treatment, but their further development was often hindered. At the farm the principal cause of fatal infections in calves is pneumonia followed by "Tristeza Parasitária Bovina". While the majority of the calves had a good health status, nevertheless 18 (18%) calves had an elevated temperature (fever) with values ranging from 39.6 to 40.9 °C suggesting there were also subclinical infection with *A. marginale*, *B. bovis* or *B. bigemina* or with other pathogens.

The results obtained by IFAT revealed that the majority of animals had high levels of antibodies against *A. marginale* (99%), *B. bovis* (90%) and *B. bigemina* (92%). No age-related pattern of specific antibody could be detected for *B. bovis* and *B. bigemina*, as we found antibody-negative samples in all age groups. Only one animal was antibody-negative for *A. marginale*, namely a newborn one-day-old Jersey calf. In this case no passive transfer of maternal antibodies to the calf has occurred. Considering the criteria defined by Mahoney and Ross (1972), the studied farm can be characterizing as an enzootically stable area. In such conditions cattle maintain high levels of antibodies of *A. marginale*, *B. bovis* and *B. bigemina* throughout the year and outbreaks of clinical disease rarely occur. The high antibody levels against the three pathogens can be explained by the favorable temperature and humidity for *R. (B.) microplus* to complete its biological cycle throughout the year, with optimal conditions from October to February. These results coincide with studies in other states of Brazil where the tick *R (B.) microplus* has the same conditions (Barros et al., 2005; Folly et al., 2009; Madruga et al., 1985; Melo et al., 2001). However, there are some particular situations in Brazil, such as areas with low tick load, lack of rain and high aridity, where the antibody levels are lower characterizing areas of enzootic instability (Artiles et al., 1995; Oliveira et al., 1992). An area of enzootic stability, or in our case a farm, may transform to an area of instability depending on the prevalent cattle breed, management strategies, tick infection rates, acaricide treatment and other factors and this possibility should always kept in mind. Also even in an enzootically stable area, the import of animals from anaplasmosis- and babesiosis-free areas can always be a problem as it may lead to clinical outbreaks in the animals without immunity.

The initial screening with the real-time PCR targeting the *A. marginale msp1β* gene revealed that 66 out of 94 animals (70.2%) were positive for *A. marginale*. These real-time PCR results were higher than those from direct examination of blood smears (48%). The real-time PCR technology has been proven to be highly specific, giving a precise estimation of bacteremia levels (Carelli et al., 2007), which explains the higher positivity when compared with the rates from blood smears. *A. marginale* infections were detected in all age groups with the highest infection rate in the group of the oldest animals (98.1%). The increasing real-time PCR positivity in relation to increasing age can be explained by the susceptibility to anaplasmosis infection rising with the age of the calves and the decline of the colostral antibodies (Roby et al., 1961). These results coincide with previous studies in endemic areas of Brazil (Madruga et al., 1985; Melo et al., 2001; Pacheco et al., 2004) with elevated bacteremias with the increasing age of the calves. The PCR is a more sensitive technique for identifying carrier animals when compared to the detection of *A. marginale* in blood smears, but is currently still expensive and restricted to well equipped laboratories (OIE, 2008).

Interestingly, congenital transmission of *A. marginale* was detected in four one-day-old calves (breeds Jersey and Holstein) by the real-time PCR. Three of these animals were also positive by blood smears, but the levels of bacteremia were very low, ranging from 0.1% to 0.8%. None of the calves showed clinical signs of infection. During the study 12 newborn calves were tested for *A. marginale*, so the results show 33.3% *in utero* transmission. Even if we do not know anything about the infection status of the mother cows because they were not tested for *A. marginale* infections, the incubation period (7-50 days) of *A. marginale* proves that transplacental transmission has occurred. The results confirm that congenital transmission appears to be an important phenomenon that contributes to the transmission of *A. marginale* in calves and the spread of the infection, concurring with other studies (Bird, 1973; Passos and Lima, 1984; Ribeiro et al., 1995). The description of anaplasmosis outbreaks in areas where the tick *R. (B.) microplus* was eradicated (Guglielmone, 1995; Kocan et al., 2003) supports the view that congenital transmission can play an important role in the epidemiology of *A. marginale*, in addition to mechanically transmissions through e.g. biting flies.

A further aim of the study was to characterize Brazilian isolates of *A. marginale* to determine genetic diversity among infected calves within the farm. 13 samples from

calves with different ages were amplified by a hemi-nested PCR targeting the *msp1α* gene and send for sequencing. The results for the MSP1a repeat sequences revealed a high level of genetic diversity within the herd.

The major surface proteins provide evolutionary information about geographically distinct *A. marginale* strains and have been used in several studies to characterize pathogen genetic diversity (Estrada-Peña et al., 2009; Kocan et al., 2004; Palmer et al., 2000) and demonstrate that many isolates may occur in a given area (de la Fuente et al., 2001a; de la Fuente et al., 2005; de la Fuente et al., 2007). Furthermore the MSP1a sequences can provide relevant information about the biology of *A. marginale,* as MSP1a has been shown to be an adhesin for bovine erythrocytes and ticks cells (McGarey and Allred, 1994a), and thus plays a role in pathogen infection and tick transmission. It is a target of vaccine research to design vaccines with cross-protective capacity for endemic areas (Kocan et al., 2003). MSP1a contains a variable number of tandemly repeated sequences in the amino terminal region, while the remainder of the protein is highly conserved among isolates. The number of repeats varies among geographic isolates of *A. marginale* but is constant within an isolate and has been used as a stable genetic marker of isolate identity (de la Fuente et al., 2003). Based on MSP1a tandem repeat analysis we found eight different strains among the animals on the farm. Comparisons with the tandem repeats reported for MSP1a worldwide showed one new tandem repeat sequence in this study, which has been named 72 (see Annex) following the nomenclature proposed by de la Fuente et al. (2007). In six of the Minas Gerais isolates (Minas 6-10 and Minas 12) one common structure (72-62-61) for MSP1a tandem repeats was found, representing the most frequent strain in our study. The maximum likelihood phylogenetic tree showed that the MSP1a from Minas Gerais fall in four separated clusters (Cluster 1-4). All the isolates containing at least one tandem repeat type ß fall in the Cluster 1, supporting the idea that the strains containing tandem repeats type ß have common origins in Minas Gerais. Two of the strains containing tandem repeat type ß, Minas-1 and Minas-2, belong to animals with congenital *A. marginale* infection, which could further support the idea of common origins in Minas Gerais.

The analysis of our MSP1a microsatellite sequences from Minas Gerais found four different genotypes (**B**, **D**, **E**, **G**) among *A. marginale* strains, with SD-ATG distances between 21 and 23 nucleotides. The genotypes **D**, **E** and **G** have been previously reported in sequences from Minas Gerais (de la Fuente et al., 2002, 2004). The

genotype **B** was reported here for the first time in Minas Gerais isolates. The predominant genotype was **E** and it was spread among animals of different ages and breeds. The genotype **E** has a SD-ATG length of 23 nucleotides. The SD-ATG distance can affect the expression of MSP1a, thus influencing the infection and transmission of *A. marginale*. A higher expression level was reported for SD-ATG distances of 23 to 29 nucleotides and lower expression for a distance of 19 nucleotides (Estrada-Peña et al., 2009). It is noteworthy that the SD-ATG distances in the congenital transmission of *A. marginale* were also 23 nucleotides, suggesting a high infection and transmission capacity of the strains found in this area and particularly in the isolate transmitted congenitally.

Based on the analysis of MSP1a tandem repeats and microsatellites we found a high genetic diversity in the Minas Gerais isolates. The genetic diversity of *A. marginale* MSP1a could be explained not only by evolutionary pressures exerted by ligand-receptor and host-parasites interactions (de la Fuente et al., 2001a) but also by constant movements of cattle (de la Fuente et al., 2005). In the case of our studied farm, the evolutionary pressure and the independent transmission should be the main reason to explain the genetic diversity, since no new animals had been introduced into the herd since the last 20 years. Nevertheless, one animal with a congenital *A. marginale* infection (isolate Minas-3) had a tandem repeat structure previously reported by de la Fuente et al. (2001a) in Mexico. Regarding congenital transmission, each of the four newborn calves infected with *A. marginale* carried a different strain but only two genotypes (**E** and **G**) based on the microsatellites. Furthermore, the genotype **E** was the most predominant genotype in this and the other age groups, so overall the predominant genotype in the site of study.

VI. CONCLUSION

The results confirmed a high frequency of the three pathogens *A. marginale*, *B. bovis* and *B. bigemina* within the studied herd in Minas Gerais and contributed to the genetic characterization of *A. marginale* strains present in the farm.

The high antibody levels in the IFAT for *A. marginale*, *B. bovis* and *B. bigemina* as well the results in the PCR for *A. marginale* characterized the farm "Fazenda do Porto" as an enzootically stable area with a herd of immunity for *A. marginale*, *B. bovis* and *B. bigemina*.

Regarding the genetic diversity within the herd it would be interesting to conduct investigations in other herds to find out if the genetic diversity is as high as in the one studied here.

Also further investigations should address the influence of such elevated genetic variation of *A. marginale* strains on epidemiological aspects of bovine anaplasmosis. This might contribute to a better understanding of the epidemiology of the disease and consequently for the development and implementation of appropriate control and prevention measures.

Furthermore it should be kept in mind that every enzootically stable area may transform to an area of instability depending on different factors. Because of the risk of clinical outbreaks, the import of animals from anaplasmosis- and babesiosis-free areas should be well-considered first. Moreover the congenital transmission appears to be an important phenomenon that contributes to the transmission and persistence of several *A. marginale* strains within a herd.

VII. SUMMARY

The present study investigated the occurrence of the complex of tick-borne disease known in Brazil as “Tristeza Parasitária Bovina” caused by the pathogens *Anaplasma marginale*, *Babesia bovis* and *Babesia bigemina* within a farm (Fazenda do Porto) in Minas Gerais, Brazil. Blood samples were collected from 100 calves of ages ranging from one day to 381 days and the health status of each animal was evaluated. For each blood sample the packed cell volume (PCV) was determined and Giemsa-stained blood smears were analyzed microscopically for the presence of hemoparasites. The plasma was tested by the Indirect Fluorescent Antibody Test (IFAT) for specific antibodies against *A. marginale*, *B. bovis* and *B. bigemina*. In addition, DNA was extracted from the blood and a real-time PCR targeting the *msp1ß* gene of *A. marginale* was performed to screen for infections. 30 samples positive by RT-PCR were chosen for a second hemi-nested PCR detecting the *msp1α* gene. PCR *msp1α* products were sequenced and compared with each other and with previously publishes *msp1α* sequences in Genbank to determine the diversity among them. The microscopic examination of the blood smears showed *A. marginale* had the highest infection rate, with 48% of samples positive, while only 18% were positive for *B. bigemina* and 14% for *B. bovis*. However, no correlations were seen between positive blood smears and PCV values, and the evaluation of the health status of each animal did not reveal any acute clinical infection.The serological results showed high levels of antibodies with 99% of samples positive for *A. marginale*, 90% for *B. bovis,* and 92% for *B. bigemina,* characterizing the farm as a herd with stable immunity for the three pathogens. The results obtained by RT-PCR revealed that 66 (70.2%) out of 94 animals were positive for *A. marginale,* indicating that this method was more sensitive than the direct examination of blood smears (48% positivity). Congenital infections of *A. marginale* were detected in four one-day-old calves; all were positive in the RT-PCR and the hemi-nested PCR, only one was negative by blood smear examination. This suggests that congenital transmission might to be an important phenomenon that contributes to the epidemiology of *A. marginale*. Sequence analysis of the hemi-nested PCR products revealed high genetic diversity in the microsatellite and tandem repeats of the *A. marginale msp1α* gene within the herd.

VIII. ZUSAMMENFASSUNG

In der vorliegenden Studie wurde das Vorkommen der von Zecken übertragenen Erkrankung, in Brasilien bekannt als “Tristeza Parasitária Bovina”, verursacht durch die Erreger *Anaplasma marginale*, *Babesia bovis* und *Babesia bigemina*, auf einer Farm (Fazenda do Porto) in Minas Gerais, Brasilien, untersucht. Blutproben wurden von 100 Kälbern im Alter von einem Tag bis zu 381 Tagen genommen und der Gesundheitsstatus jedes Tieres wurde beurteilt. Für jede Blutprobe wurde der Hämatokrit bestimmt und mit Giemsa gefärbte Blutausstriche wurden mikroskopisch auf das Vorkommen von Blutparasiten durchsucht. Mittels indirektem Immunofluoreszenzantikörpertest (IFAT) wurde das Blutplasma auf spezifische Antikörper gegen *A. marginale*, *B. bovis* und *B. bigemina* getestet. Die DNA wurde aus dem Blut extrahiert und anhand eines real-time PCR mit dem Zielgen *msp1ß* von *A. marginale* ein Screening durchgeführt. 30 Proben, positiv im RT-PCR, wurden ausgewählt und ein zweiter hemi-nested PCR zur Detektion des *msp1α* Gens erfolgte. Die sequenzierten PCR Produkte wurden miteinander und mit bekannten Sequenzen aus der Gendatenbank verglichen um Unterschiede festzustellen. Die mikroskopische Untersuchung der Blutausstriche ergab die höchsten positiven Werte für *A. marginale* mit 48%, wohingegen nur 18% für *B. bigemina* und 14% für *B. bovis* positiv waren. Es konnte kein Zusammenhang zwischen den Blutausstrichen und dem Hämatokrit festgestellt werden und die Beurteilung des Gesundheitsstatus der Tiere wies keine akuten klinischen Infektionen auf. Die serologischen Ergebnisse zeigten hohe Antikörperspiegel, 99% der Proben waren positiv für *A. marginale*, 90% für *B. bovis* und 92% für *B. bigemina,* was die Farm als eine Herde mit stabiler Immunität gegen die drei Erreger charakterisiert. Die Ergebnisse des RT-PCR ergaben, dass 66 (70,2%) von 94 Tieren positiv für *A. marginale* waren, folglich war diese Methode damit sensitiver als die direkte Beurteilung der Blutausstriche (48% positiv). Kongenitale Infektionen von *A. marginale* wurden bei vier ein Tage alter Kälber festgestellt, alle waren positiv im RT-PCR und hemi-nested PCR, nur ein Kalb war negativ im Blutausstrich. Demzufolge scheint die kongenitale Übertragung ein wichtiges Phänomen in der Epidemiologie von *A. marginale* zu sein. Der Vergleich der Sequenzen der hemi-nested PCR Produkte untereinander und mit anderen Sequenzen ergab hohe genetische Unterschiede der Mikrosatelliten und Tandem Repeats von den *A. marginale msp1α* Gen in der untersuchten Herde.

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X. ABBREVIATIONS

A. central	*Anaplasma centrale*
Aqua dest.	distilled water
A. marginale	*Anaplasma marginale*
B. bigemina	*Babesia bigemina*
B. bovis	*Babesia bovis*
B. inducus	*Bos indicus*
B. ovis	*Babesia ovis*
B. taurus	*Bos taurus*
BHQ1	blackhole quencher 1
bp	Base pare
°C	degree Celsius
CAT	card agglutination test
CF test	complement fixation test
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	diaminoethanetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FRET	fluorescent resonance energy transfer
g	gramm
H_2O	water
IBGE	Instituto Brasileiro de Geografia e Estatística
IFAT	immun fluorescence assay test
mg	milligram
$MgCl_2$	magnesium chloride
min	minutes
ml	milliliter
mm	millimeter
mM	milimolar
mRNA	messenger ribosomal ribonucleic acid
msp	membranous surface protein
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
NaCl	sodium chloride
OIE	World Organization for Animal Health
K_2HPO_4	dipotassium hydrogen phosphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	packed cell volume
RT-PCR	real time polymerase chain reaction

sec	seconds
Taq	*Thermus aquaticus*
TPB	Tristeza Parasitária Bovina
Tris	Trishydroxymethylaminomethane
6-FAM	6-carboxyfluoroscein

XI. FIGURES

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XIII. ANNEX

1. Materials

1.1. Kits, oligonucleotids and chemicals

Wizard® Genomic DNA Purification Kit	(Promega, Madison, USA)
TaqMan® Gene Expression Master Mix	(Applied Biosystems, USA)
dNTP mix	(Qiagen, Hilden, Germany)
QIAquick PCR Purification Kit	(Qiagen, Hilden, Germany)
Ladder Mix 1/12 Loading-dye	(Fermentas Life Science, Leonrot, Germany)
Magnesium chloride	(Qiagen, Hilden, Germany)
Ethanol > 99.8 %	(Roth, Karlsruhe, Germany)
Evans-Blau	(Fluka Chemie AG, Neu Ulm, Germany)
Tween® 20	(Roth, Kalrsruhe, Germany)

1.2. Enzymes

HotStarTaq DNA polymerase	(Qiagen, Hilden, Germany)

1.3. Buffer and solution

1.3.1. Buffer and solution for the agarose gel electrophoresis

50x TAE buffer: 50 mM Tris
20 mM sodium acetate
1 mM EDTA
With concentrated acetic acid adjusted to pH=8,3

5 µl Gel Red (Biotium, Germany) for staining (1/100 dilution)

1.3.2. Buffer and solution for the indirect fluorescent antibody test

1x PBS buffer	10,7 mM $MgCl_2$	(Merck, Darmstadt, Germany)
	135 mM NaCl	(Merck, Darmstadt, Germany)
	15 mM K_2HPO_4 x 3 x H_2O	(Merck, Darmstadt, Germany)
	2,8 mM K_2HPO_4	(Merck, Darmstadt, Germany)
	ad 1000 ml *Aqua dest*. to dissolve	

PBS/Tween 0.05 %	PBS buffer
	Tween 0.05 % v/v

2. Sequencing data

2.1. *msp1α* sequences of *Anaplasma marginale*

All *msp1α* sequences of *Anaplasma marginale* obtained in this study and submitted to the GenBank

```
031J    ---GTTGTGCTCCCCAATTGTTAAAATTTAGTATATTAATCTTGCGATTACACGTTCCGT 57
375H    ---GTTGTGCTCCCCAATTGTTAAAATTTAGTATATTAATCTTGCGATTACACGTTCCGT 57
378H    -CGGTTGTGCTCCCCAATTGTTAAAATTTAGTATATTAATCTTGCGATTACACGTTCCGT 59
034J    ----------------------------AGTATATTAATCTTGCGATTACACGTTCCGT 31
033J    ----------------------------AGTATATTAATCTTGCGATTACACGTTCCGT 31
009J    ------GTGCTCCCCAATTGTTAAAATTTAGTATATTAATCTTGCGATTACACGTTCCGT 54
999J    ACGGTTGTGCTCCCCAATTGTTAAAATTTAGTATATTAATCTTGCGATTACACGTTCCGT 60
385H    ------GTGCTCCCCAATTGTTAAAATTTAGTATATTAATCTTGCGATTACACGTTCCGT 54
386H    ----------------------------AGTATATTAATCTTGCGATTACACGTTCCGT 31
039J    ----------------------------AGTATATTAATCTTGCGATTACACGTTCCGT 31
012J    ------------------------------------------------------------GT 2
037J    ----------------------------AGTATATTAATCTTGCGATTACACGTTCCGT 31
PampJ   -------------------------------------------------CACGTTCCGT 10

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375H    ATGTTACAATCGGGCTGCCGGTGTGGTAGCGTGCTGGTTGTGTGGTTGTCCTCTTTCCCG 117
378H    ATGTTACAATCGGGCTGCCGGTGTGGTAGCGTGCTGGTTGTGTGGTTGTCCTCTTTCCCG 119
034J    ATGTTACAATCGGGCTGCCGGTGTGGTAGCGTGCTGGTTGTGTGGTTGTCCTCTTTCCCG 91
033J    ATGTTACAATCGGGCTGCCGGTGTGGTAGCGTGCTGGTTGTGTGGTTGTCCTCTTTCCCG 91
009J    ATGTTACAATCGGGCTGCCGGTGTGGTAGCGTGCTGGTTGTGTGGTTGTCCTCTTTCCCG 114
999J    ATGTTACAATCGGGCTGCCGGTGTGGTAGCGTGCTGGTTGTGTGGTTGTCCTCTTTCCCG 120
385H    ATGTTACAATCGGGCTGCCGGTGTGGTAGCGTGCTGGTTGTGTGGTTGTCCTCTTTCCCG 114
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039J    ATGTTACAATCGGGCTGCCGGTGTGGTAGCGTGCTGGTTGTGTGGTTGTCCTCTTTCCCG 91
012J    ATGTTACAATCGGGCTGCCGGTGTGGTAGCGTGCTGGTTGTGTGGTTGTCCTCTTTCCCG 62
037J    ATGTTACAATCGGGCTGCCGGTGTGGTAGCGTGCTGGTTGTGTGGTTGTCCTCTTTCCCG 91
PampJ   ATGTTACAATCAGGCCGCCGGTGTGGTAGCGTGCTGGTTGTGTGGTTGTCCTCTTTCCCG 70

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375H    ATGTTGGGTCGTTCGTTTTACGTCTCACAAGTTTGTACGCTGTGCCCCTGGCAGTGTAGG 177
378H    ATGTTGGGTCGTTCGTTTTACGTCTCACAAGTTTGTACGCTGTGCCCCTGGCAGTGTAGG 179
034J    ATGTTGGGTCGTTCGTTTTACGTCTCACAAGTTTGTACGCTGTGCCCCTGGCAGTGTAGG 151
033J    ATGTTGGGTCGTTCGTTTTACGTCTCACAAGTTTGTACGCTGTGCCCCTGGCAGTGTAGG 151
009J    ATGTTGGGTCGTTCGTTTTACGTCTCACAAGTTTGTACGCTGTGCCCCTGGCAGTGTAGG 174
999J    ATGTTGGGTCGTTCGTTTTACGTCTCACAAGTTTGTACGCTGTGCCCCTGGCAGTGTAGG 180
385H    ATGTTGGGTCGTTCGTTTTACGTCTCACAAGTTTGTACGCTGTGCCCCTGGCAGTGTAGG 174
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039J    ATGTTGGGTCGTTCGTTTTACGTCTCACAAGTTTGTACGCTGTGCCCCTGGCAGTGTAGG 151
012J    ATGTTGGGTCGTTCGTTTTACGTCTCACAAGTTTGTACGCTGTGCCCCTGGCAGTGTAGG 122
037J    ATGTTGGGTCGTTCGTTTTACGTCTCACAAGTTTGCACGCTGTGCCCCTGGCAGTGTAGG 151
PampJ   ATGTTGGGTCGTTCGTTTTACGTCGCACAAGTTTGTACGCTGTGCCCCTGGCAGTGTAGG 130
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031J     GTTTGTTTGTGTGTGTGTGTGTTATGTCAG---AGT------------------------ 210
375H     GTTTGTTTGTGTGTGTGTGTGTTATGTCAG---AGT------------------------ 210
378H     GTTTGTTTGTGTGTGTGTG--TTATGTCAG---AGT------------------------ 210
034J     GTTTGTTTGTGTGTGTGTG--TTATGTCAG---AGT------------------------ 182
033J     GTTTGTTTGTGTGTGTGTGTGTTATGTCAG---AGT------------------------ 184
009J     GTTTGTTTGTGTGTGTGTGTGTTATGTCAG---AGT------------------------ 207
999J     GTTTGTTTGTGTGTGTGTGTGTTATGTCAG---AGT------------------------ 213
385H     GTTTGTTTGTGTGTGTGTGTGTTATGTCAG---AGT------------------------ 207
386H     GTTTGTTTGTGTGTGTGTGTGTTATGTCAG---AGTT---GTCCCTTCAGCCAACTGATA 205
039J     GTTTGTTTGTGTGTGTGTGTGTTATGTCAG---AGT---CGTCCCTTCAGCCAACTGATA 205
012J     GTTTGTTTGTGTGTGTGTGTGTTATGTCAG---AGT---TGTCCCTTCAGCCAACTGATA 176
037J     GTTTGTCTGTGTGTGTGTGTGTTATGTCAG---AGT---TGTCCCTTCAGTCAACTGATA 205
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375H     ---------------------------------ATGTGT-----CCA----GCCAGCC-- 226
378H     ---------------------------------ATGTGT-----CCA----GCCAGCC-- 226
034J     ---------------------------------ATGTGT-----CCA----GCCAGCC-- 198
033J     ---------------------------------ATGTGT-----CCA----GCCAGCC-- 200
009J     ---------------------------------ATGTGT-----CCA----GCCAGCC-- 223
999J     ---------------------------------ATGTGT-----CCA----GCCAGCC-- 229
385H     ---------------------------------ATGTGT-----CCA----GCCAGCC-- 223
386H     GCTCGTCAGCGAGTGGTCAGCAGCAAGAGAGTAGTGTGTTATCTCCAAGTGGTCAGGCCA 265
039J     GCTCGTCAGCGAGTGGTCAGCAGCAAGAGAGTAGTGTGTTATCTCAAAGTAGTCAGGCCA 265
012J     GCTCGTCAGCGAGTGGTCAGCAGCAAGAGAGTAGTGTGTTATCTCCAAGTGGTCAGGCCA 236
037J     GCTCGTCAGCGAGTGGTCAGCAGCAAGAGAGTAGTGTGTTATCTCAAAGTGATCAGGCCA 265
PampJ    GCTCGTCAGCGGGTGGTCAGCAGCAAGAGAGTAGTGTGTCATCTCAAAGTGATCAGGCCA 250

031J     -------------------AGCTGATAGCTCGTCAGCG---------------------- 245
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378H     -------------------AGCTGATAGCTCGTCAGCG---------------------- 245
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033J     -------------------AGCTGATAGCTCGTCAGCG---------------------- 219
009J     -------------------AGCTGATAGCTCGTCAGCG---------------------- 242
999J     -------------------AGCTGATAGCTCGTCAGCGAGTGGT----------------- 254
385H     -------------------AGCTGATAGCTCGTCAGCGAGTGGT----------------- 248
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039J     GTACATCGTCTCAATTAGGAGCTGATAGCTCGTCAGCG---------------------- 303
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037J     GTACATCGTCTCAATTAGGAGCTGATAGCTCGTCAGCG---------------------- 303
PampJ    GTACATCGTCTCAATTAGGAGCTGATAGCTCGTCAGCG---------------------- 288

031J     ------------------------------------------------------------
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378H     ------------------------------------------------------------
034J     ------------------------------------------------------------
033J     ------------------------------------------------------------
009J     ------------------------------------------------------------
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385H     --GTGTTATCTCAAAGTGGTCAGGCCAGTACATCGTCTCAATTAGGAACTGATAGCTCGT 306
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039J     ------------------------------------------------------------
012J     ------------------------------------------------------------
037J     ------------------------------------------------------------
PampJ    ------------------------------------------------------------
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031J     -----GGTGATCAGCAGCAAGAGAGTAGTGTGTCATCTCAAAGTGGT---GCCAGTACAT 297
375H     -----GGTGATCAGCAGCAAGAGAGTAGTGTGTCATCTCAAAGTGGT---GCCAGTACAT 297
378H     -----GGTGATCAGCAGCAAGAGAGTAGTGTGTCATCTCAAAGTGGT---GCCAGTACAT 297
034J     -----GGTGATCAGCAGCAAGAGAGTAGTGTGTCATCTCAAAGTGGT---GCCAGTACAT 269
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009J     -----GGTGATCAGCAGCAAGAGAGTAGTGTGTCATCTCAAAGTGGT---GCCAGTACAT 294
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385H     CAGCGGGTGATCAGCAGCAAGGGAGTGGTGTGTCATCTCAAAGTGGTCAGGCCAGTACAT 366
386H     CAGCGGGTGATCAGCAGCAAGGGAGTGGTGTGTCATCTCAAAGTGGTCAGGCCAGTACAT 445
039J     -----GGTAATCAGCAGCAAGAGAGTAGTGTGTTACCTCAAAGTGGTCAGGCCAGTACAT 358
012J     -----AGTGGTCAGCAGCAAGAGAGTAGTGTGTTATCTCCAAGTGGTCAGGCCAGTACAT 329
037J     -----AGTGGTCAGCAGCAAGAGAGTAGTGTGTTATCTCAAAGTGATCAGGCCAGTACAT 358
PampJ    -----AGTGGTCAGCAGCAAGAGAGTAGTGTGTCATCTCAAAGTGATCAGGCCAGTACAT 343

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375H     CGTCTCAATTAGGGACTGATAGCTCGTCAGCGGGTGATCAGCAGCAAGAGAGTAGTGTGT 357
378H     CGTCTCAATTAGGGACTGATAGCTCGTCAGCGGGTGATCAGCAGCAAGAGAGTAGTGTGT 357
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009J     CGTCTCAATTAGGGACTGATAGCTCGTCAGCGGGTGATCAGCAGCAAGAGAGTAGTGTGT 354
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039J     CGTCTCAATCAGGAGCTGATAGCTCGTCAGCGGGTAATCAGCAGCAAGAGAGTAGTGTGT 418
012J     CGTCTCAATCAGGAACTGATAGCTCGTCAGCGAGTGGTCAGCAGCAAGAGAGTAGTGTGT 389
037J     CGTCTCAATTAGGAGCTGATAGCTCGTCAGCGAGTGGTCAGCAGCAAGAGAGTAGTGTGT 418
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378H     CATCTCAAAGTGAT---GCCAGTACATCGTCTCAATTAGGGACTGATAGCTCGTCAGCGG 414
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033J     CATCTCAAAGTGAT---GCCAGTACATCGTCTCAATTAGGGACTGATAGCTCGTCAGCGG 388
009J     CATCTCAAAGTGAT---GCCAGTACATCGTCTCAATTAGGGACTGATAGCTCGTCAGCGG 411
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385H     CATCTCAAAGTGGTCAGGCCAGTACATCGTCTCAATTAGGGACTGATAGCTCGTCAGCGA 486
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378H     GTGATCAGCAGCAAGAGAGTAGTGTGTCATCTCAAAGTGGT---GCCAGTACATCGTCTC 471
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385H     GTGGTCAGCAGCAAGAGAGTAGTGTGTCATCTCAAAGTGAT---GCCAGTACATCGTCTC 543
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039J     GTGGTCAGCAGCAAGAGAGTAGTGTGTTATCTCAAAGTAGTCAGGCCAGTACATCGTCTC 538
012J     GTGGTCAGCAGCAAGAGAGTAGTGTGTTATCTCAAAGTGATCAGGCCAGTACATCGTCTC 509
037J     GTGGTCAGCAGCAAGAGAGTAGTGTGTTATCTCAAAGTGATCAGGCCAGTACATCGTCTC 538
PampJ    GTGGTCAGCAGCAAGAGAGTAGTGTGTCATCTCAAAGTGGTCAGGCCAGTACATCGTCTC 523
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375H     AATTAGGAGCTGAT---------------------------------------------- 485
378H     AATTAGGAGCTGAT---------------------------------------------- 485
034J     AATTAGGAGCTGAT---------------------------------------------- 457
033J     AATTAGGAGCTGAT---------------------------------------------- 459
009J     AATTAGGAGCTGAT---------------------------------------------- 482
999J     AATTAGGGACTGAT---------------------------------------------- 566
385H     AATTAGGAGCTGAT---------------------------------------------- 557
386H     AATTAGGAGCTGAT---------------------------------------------- 636
039J     AATTAGGAGCTGATAGCTCGTCAGCGGGTAATCAGCAGCAAGAGAGTAGTGTGTCATCTC 598
012J     AATCAGGAACTGAT---------------------------------------------- 523
037J     AATTAGGGACTGAT---------------------------------------------- 552
PampJ    AATTAGGGACTGAT---------------------------------------------- 537

031J     ------------------------------------TGGCGGCAAGAGGTGCACTCCA 507
375H     ------------------------------------TGGCGGCAAGAGGTGCACTCCA 507
378H     ------------------------------------TGGCGGCAAGAGGTGCACTCCA 507
034J     ------------------------------------TGGCGGCAAGAGGTGCACTCCA 479
033J     ------------------------------------TGGCGGCAAGAGGTGCACTCCA 481
009J     ------------------------------------TGGCGGCAAGAGATGCGCTCCA 504
999J     ------------------------------------TGGCGGCAAGAGATGTGCTCCA 588
385H     ------------------------------------TGGCGGCAAGAGGTGCACTCCA 579
386H     ------------------------------------TGGCGGCAAGAGGTGCACTCCA 658
039J     AAAGTGATGCCAGTACATCGTCTCAATTAGGGACTAATTGGCGGCAAGAAATGCGCTCCA 658
012J     ------------------------------------TGGCGGCAAGAGATGCGCTCCA 545
037J     ------------------------------------TGGCGGCAAGAGATGCGCTCCA 574
PampJ    ------------------------------------TGGCGGCAAGAGATGCGCTCCA 559

031J     AGGTTGCGAGTGTTGAGTACATTTTGGCTGCTCGTGCCCTTATCTCTGTAGGGGTCTATG 567
375H     AGGTTGCGAGTGTTGAGTACATTTTGGCTGCTCGTGCCCTTATCTCTGTAGGGGTCTATG 567
378H     AGGTTGCGAGTGTTGAGTACATTTTGGCTGCTCGTGCCCTTATCTCTGTAGGGGTCTATG 567
034J     AGGTTGCGAGTGTTGAGTACATTTTGGCTGCTCGTGCCCTTATCTCTGTAGGGGTCTATG 539
033J     AGGTTGCGAGTGTTGAGTACATTTTGGCTGCTCGTGCCCTTATCTCTGTAGGGGTCTATG 541
009J     AGGTTGCGAGTGTTGAGTACATTTTGGCTGCTCGTGCCCTTATCTCTGTAGGGGTCTATG 564
999J     AGGTTGCGAGTGTTGAGTACATTTTGGCTGCTCGTGCCCTTATCTCTGTAGGGGTCTATG 648
385H     AGGTTGCGAGTGTTGAGTACATTTTGGCTGCTCGTGCCCTTATCTCTGTAGGGGTCTATG 639
386H     AGGTTGCGAGTGTTGAGTACATTTTGGCTGCTCGTGCCCTTATCTCTGTAGGGGTCTATG 718
039J     AGGTTGCGAGTGTTGAGTACATTTTGGCTGCTCGTGCCCTTATCTCTGTAGGGGTCTATG 718
012J     AGGTTGCGAGTGTTGAGTACATGTTGGCTGCTCGTGCCCTTATTCTCTGTA---------- 595
037J     AGGTTGCGAGTGTTGAGTACATTTTGGCTGCTCGTGCCCTTATCTCTGTAGGGGTCTATG 634
PampJ    AGGTTGCGAGTGTTGAGTACATGTTGGCTGCTCGTGCCCTTATTCTCTGTAGGGGTCTATG 619

031J     CTGCTCAGAGAATGATCGCGCAATCGCAAGGGTGTGCTTCCCTGAGTGTTGCAGAAGTCG 627
375H     CTGCTCAGAGAATGATCGCGCAATCGCAAGGGTGTGCTTCCCTGAGTGTTGCAGAAGTCG 627
378H     CTGCTCAGAGAATGATCGCGCAATCGCAAGGGTGTGCTTCCCTGAGTGTTGCAGAAGTCG 627
034J     CTGCTCAGAGAATGATCGCGCAATCGCAAGGGTGTGCTTCCCTGAGTGTTGCAGAAGTCG 599
033J     CTGCTCAGAGAATGATCGCGCAATCGCAAGGGTGTGCTTCCCTGAGTGTTGCAGAAGTCG 601
009J     CTGCTCAGGAAGAGATCGCGAAATCGCTAGGGTATGCTCCCCTGCGTGTTGCAGAAGTCG 624
999J     CTGCTCAGGAAGAGATCGCGAGATCGCTAGGGCATACTCCCCTGCGTGTTGCAGAAGTCG 708
385H     CTGCTCAGAGAATGATCGCGCAATCGCGAGGGTGTGCTTCCCTGAGTGTTGCAGAAGTCG 699
386H     CTGCTCAGAGAATGATCGCGCAATCGCGAGGGTGTGCTTCCCTGAGTGTTGCAGAAGTCG 778
039J     CTGCTCAGGAAGAGATCGCGAGATCGCTAGGGTATGC----------------------- 755
012J     ------------------------------------------------------------
037J     CTGCTCAGGAAGAGATCGCGAGATCGCTGGGGCATACTCCCCTGCGTGTTGCAGAAGTCG 694
PampJ    CTGCTCAGGGAGAGATCGCGAGATCGCGAGGGTGTGCTCCCCTGCGTGTTGCAGAAGTCG 679
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031J     AAAAAATCGTGAGGGATAGCCTTGTACGCAGCCACTTTCATGATAGTGGCCTTTCACTAG 687
375H     AAGAAATCGTGAGGGATAGCCTTGTACGCAGCCACTTTCATGATAGTGGCCTTTCACTAG 687
378H     AAGAAATCGTGAGGGATAGCCTTGTACGCAGCCACTTTCATGATAGTGGCCTTTCACTAG 687
034J     AAGAAATCGTGAGGGATAGCCTTGTACGCAGCCACTTTCATGATAGTGGCCTTTCACTAG 659
033J     AAAAAATCGTGAGGGATAGCCTTGTACGCAGCCACTTTCATGATAGTGGCCTTTCACTAG 661
009J     AAGAAATCGTGAGGGATAGCCTTGTACGCAGCCACTTTCATGATAGTGGCCTTTCACTAG 684
999J     AAGCAATCGTGAGGGATAGCCTTGTACGCAGCCACTTTCATGATAGTGGCCTTTCACTAG 768
385H     AAGAAATCGTGAGGGATAGCCTTGTACGCAGCCACTTTCATGATAGTGGCCTTTCACTAG 759
386H     AAGAAATCGTGAGGGATAGCCTTGTACGCAGCCACTTTCATGATAGTGGCCTTTCACTAG 838
039J     ------------------------------------------------------------
012J     ------------------------------------------------------------
037J     AAGCAATCGTGAGG---------------------------------------------- 708
PampJ    AAGAAATCGTGAGGGATGGCCTTGTACGCA------------------------------ 709

031J     GCTCCATACGACT--- 700
375H     GCTCCATACGACTC-- 701
378H     GCTCCATACGACTC-- 701
034J     GCTCCATACGACTC-- 673
033J     GCTCCATACGACTCGT 677
009J     GCTCCATACGACTC-- 698
999J     GCTCCATACGACTC-- 782
385H     GCTCCATA-------- 767
386H     GCTCCATACGACTC-- 852
039J     ----------------
012J     ----------------
037J     ----------------
PampJ    ----------------
```

2.2. New sequence of MSP1a tandem repeat found in *Anaplasma marginale* isolate

Repeat form	Encoded sequence
B	A D S S S A G G Q Q Q E S S V S S Q S D Q A S T S S Q L G
72	* * * * * * * D * * * * * * * * * * * G _ * * * * * * * *

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