CHAPTER 1

Literature Review

1.1 Introduction

Equine piroplasmosis is an infectious tick-borne disease of Equidae (horses, mules, donkeys, zebras). Piroplasmosis in horses was first reported in South Africa around the turn of the 19th century when it was initially described as 'anthrax fever' (Henning, 1956; Theiler, 1901), 'biliary fever' (Hutcheon, 1890), 'a bilous form of African horsesickness' (Nunn, 1894) and in West Africa as 'equine malaria' (Henning, 1956). The first detailed study of equine piroplasmosis was the topic of Sir Arnold Theiler's doctoral dissertation (Theiler, 1901). At the beginning of the 20th century, Laveran (1901) recognized that the disease was caused by an intraerythrocytic protozoan parasite which he named *Piroplasma equi*. Two morphologically distinct species infecting horses in Zimbabwe were identified in 1904 (Koch, 1904). It was later shown that both parasites occurred in horses suffering from biliary fever and the name *Piroplasma caballi* was suggested for the larger parasite (Nuttall and Strickland, 1910; de Waal and van Heerden, 2004). The parasites were later renamed *Babesia caballi* (Nuttall and Strickland, 1910) and *Babesia equi* (Laveran, 1901). More recently, based on the clarification of the life cycle, it was recognized that *B. equi* more closely resembles *Theileria* species and it has thus been renamed *Theileria equi* (Mehlhorn and Schein, 1998).

1.2 Etiology

Babesia and *Theileria* belong to the phylum Apicomplexa and, in reference to intraerythrocytic forms that are pear-shaped in some species, they form a group called the piroplasms (Levine, 1985). The two piroplasm genera are usually distinguished by the lack of a pre-erythrocytic cycle in *Babesia* and the lack of transovarial transmission in *Theileria.* The piroplasms have complex life cycles that involve morphologically distinct developmental stages in the tick and mammalian host cells. A typical apicomplexan life cycle is characterized by three stages of reproduction: (a) gamogony, which is the formation and fusion of gametes inside the tick gut, (b) sporogony, asexual reproduction in the salivary glands and (c) merogony, asexual reproduction in the vertebrate host (Levine, 1985; Homer et al., 2000; Shaw, 2003).

1.2.1 *Life cycle of Theileria equi*

Theileria equi is present in the lymphocytes and erythrocytes of vertebrate hosts and is transmitted exclusively by the bite of vector ticks. Transmission occurs only through the bites of nymphs and adults as larvae are not pre-infected (i.e. transstadial transmission). Schizogony occurs in the vertebrate host, while gamogony and sporogony take place in ticks (Mehlhorn and Schein, 1998). The vertebrate host acquires the parasitic infection when sporozoites from infected nymphal or adult ticks are transferred during feeding. Sporozoites initially invade lymphocytes where they undergo schizogony, during which large macroschizonts and microschizonts occur, which give rise to merozoites. The merozoites enter erythrocytes and start reproducing by binary fission, frequently resulting in the formation of four pear-shaped stages in an arrangement referred to as a tetrad or maltese cross. *Theileria equi* merozoites are relatively small (2-3 µm long) and either round, amoeboid or piriform (Levine, 1985). Parasitaemias commonly range between 1-5 % but up to 80 % of erythrocytes may be infected in some cases (de Waal and van Heerden, 2004; Mehlhorn and Schein, 1998). When the erythrocytes rupture, the erythrocytic merozoites enter other erythrocytes where another phase of asexual reproduction is initiated. Ticks become infected by ingesting infected erythrocytes (gamonts) and the process of gametogenesis is initiated in the tick. Gametocytes undergo morphological changes within the tick's gut where they develop into ray bodies. Two ray bodies (gametes) fuse to form a zygote, which then develops into kinetes that initiate the process of sporogony when they invade the salivary glands. The development of *T. equi* has been studied in detail in the salivary glands of its tick vectors, *Rhipicephalus evertsi evertsi*, *Hyalomma detritum*, *Rhipicephalus turanicus* and *Rhipicephalus* (*Boophilus*) *microplus* (Guimaraes et al., 1998; Moltmann et al., 1983; Young et al., 1973). The sporonts persist unchanged in the salivary glands during the nymphal to adult moult. The maturation of sporozoites occurs once an infected adult tick attaches to a vertebrate host (de Waal and van Heerden, 2004; Mehlhorn and Schein, 1998).

1.2.2 *Life cycle of Babesia caballi*

Babesia caballi develops in the vertebrate host exclusively in erythrocytes. Merozoites are large, pear-shaped and vary in size by between 2 µm and 5 µm in length. Erythrocytes rarely contain more than two *B. caballi* parasites. The parasitaemia in *B. caballi* infections is very low, rarely exceeding 1% (de Waal and van Heerden, 2004).

As for *T. equi*, vector ticks become infected by ingesting infected erythrocytes. Development of *B. caballi* in *Dermacentor nitens* occurs in the epithelial cells of the gut in the female tick. Schizogony occurs resulting in the infection of a variety of cell types, including the ova. Nymphal ticks begin feeding shortly after *B. caballi* ookinetes (vermicules) enter the salivary glands where they develop into sporozoites. It has been indicated that *B. caballi* infections can be carried over from the nymphal to the adult stage as well as transovarially, to the following generation (de Waal and van Heerden, 2004; Homer et al., 2000).

1.3 Epidemiology

Equine piroplasmosis is thought to be indigenous to Asia (Friedhoff et al., 1990) but due to the global transport of horses for personal transport, as draft animals and for equestrian sports, both parasites have become distributed throughout the tropical and subtropical areas of the world where suitable tick vectors are present (Brüning, 1996). Endemic areas now include many parts of Europe, Arabia, South and Central America and Africa (Friedhoff et al., 1990; de Waal, 1992; Avarzed et al., 1997; Yin et al., 1997; Camacho et al., 2005; Asgarali et al., 2007; Heim et al., 2007; Salim et al., 2008; Karatepe et al., 2009). Many countries are free of the parasite including Canada, New Zealand, Australia, Japan, Germany, United Kingdom, Ireland, Netherlands, and the Scandinavian countries (Anonymous, 2008). Although Australia is considered free of equine piroplasmosis, *T. equi* has been introduced into Australia on several occasions. It was first introduced into the country by horses imported from Texas in the 1950s and 1960s and in Andalusian horses from Spain in the 1970s. The disease never established, however, due to the absence of suitable tick vectors (Martin, 1999). The establishment of equine piroplasmosis is thus greatly dependent on the distribution of the tick vectors. Both parasites frequently share the same vector, but *T. equi* has been reported to have a wider geographical distribution than *B. caballi* (Levine, 1985).

1.4 Transmission

1.4.1 Biological vectors

Fourteen species of ixodid ticks in the genera *Dermacentor, Rhipicephalus* and *Hyalomma* have been identified as vectors of either *T. equi* or *B. caballi* (de Waal, 1992). In South Africa, only two tick species, *Rhipicephalus evertsi evertsi* and *Hyalomma truncatum*, have been identified as vectors of *T. equi* and *B. caballi.* Both species are widely distributed throughout the country.

The more common vector, *R. evertsi evertsi* occurs throughout the Limpopo, North-west, Gauteng, Mpumalanga and KwaZulu-Natal provinces, the northern part of the Free State and along the coast of the Eastern and Western Cape. *Hyalomma truncatum* occurs throughout the western and northern parts of South Africa (de Waal, 1992; de Waal and van Heerden, 2004). *Rhipicephalus evertsi evertsi* transmits *B. caballi* as well as *T. equi* transstadially, whereas *H. truncatum* only transmits *B. caballi* transovarially (de Waal and Potgieter, 1987; de Waal, 1990).

Rhipicephalus evertsi evertsi, the red-legged tick, has a two-host life cycle in which the larva to lymph moult occurs on the host. The parasitic protozoa are unable to pass from an infected adult through the eggs to the larvae of *R. evertsi evertsi*. The ticks thus pick up the parasites in their larval or nymphal stages and transmit them to a new host in the adult stage (Norval, 2004). Horses are thus the reservoir of parasites. *Hyalomma truncatum* is also a two-host tick, but because transovarial transmission of *B. caballi* occurs, infected immatures are able to transmit the disease and moult into infected adults that will infect susceptible horses. In this instance, ticks serve as a reservoir of the parasite (de Waal and van Heerden, 2004).

1.4.2 Mechanical transmission

An additional feature of piroplasmosis is that the disease can be transmitted mechanically in infected blood. Infection can therefore, also be induced by inoculating infected blood into a susceptible animal via the intravenous (i/v), intramuscular (i/m) or subcutaneous routes. Accidental transmission by the use of contaminated hypodermic needles is also a possibility (de Waal and van Heerden, 2004; Gerstenberg et al., 1998; Phipps and Otter, 2004).

1.4.3 Intrauterine infections

Intrauterine infections of unborn foals are a common cause of equine abortions as carrier mares potentially infect their foals throughout their breeding life (de Waal and van Heerden, 2004). Infection of foals *in utero* may result in abortion with the foetus showing lesions characteristic of equine piroplasmosis, or in the birth of foals showing signs of the disease (de Waal and van Heerden, 2004; Phipps and Otter, 2004; Allsopp et al., 2007). Phipps and Otter (2004) reported on the transplacental transmission of *T. equi* to two foals born in the UK to an infected mare which had been imported from Portugal, where *T. equi* is endemic. Placental damage or the occurrence of reverse erythroblastosis foetalis has been implicated as the cause of transplacental

transmission (Du Plessis and Basson, 1966; Erbsloh, 1975). However, a DNA probing assay carried out on six naturally born foals suggested that parasite transmission also occurs in pregnancies where placentation is normal (Allsopp et al., 2007). Due to the persistent nature of the infection, the carrier status for *T. equi* infections is assumed to be life-long, while animals may remain carriers of *B. caballi* for up to four years (de Waal and van Heerden, 2004).

1.5 Pathogenesis

Although not much research has been done on elucidating the pathogenesis of *T. equi* and *B. caballi* infections in horses, it is believed that these parasites have a similar pathogenesis as described for other *Babesia* and *Theileria* species (de Waal and van Heerden, 2004). The development of anaemia in horses is a typical clinical sign of *T. equi* infections where hypophosphataemia has been implicated in playing a role. Red blood cells are an energy source for piroplasm parasites. The increased uptake of phosphorus by red blood cells may thus be responsible for the development of a hypophosphataemic state, which may in turn contribute to the weakening of erythrocytic cell membranes causing haemolysis (de Waal and van Heerden, 2004). The haemolysis may further result in haemoglobinaemic nephrosis and uraemia in severely infected horses. Various degrees of thrombocytopenia, hypoferronaemia and bilirubinaemia have also been reported (de Waal and Potgieter, 1987). A study conducted on the erythrocyte-associated haemato-biochemical changes in *T. equi* confirmed that during acute babesiosis, the biochemical composition of the erythrocyte membrane is altered thus leading to haemolysis and anaemia (Ambawat et al., 1999).

The pathogenesis of *B. caballi* infections was investigated by analyzing cytokine production in experimentally infected horses. In *B. caballi* infected horses, nitric oxide (NO), tumor necrosis factor alpha (TNF-α) and other cytokines may enhance protective immune responses against the parasite if produced in optimal quantities, but overproduction of these molecules may contribute to pathogenesis (Hanafusa et al., 1998). The accumulation of parasitized red blood cells in small blood vessels and capillaries is also implicated in the pathogenesis of *B. caballi* infections and the development of an acute hypotensive state may be the cause of peracute deaths. Pulmonary oedema has also been associated with hyperacute infections and death. The absorption of endotoxins may possibly be the cause of the development of laminitis and the occurrence of gastrointestinal stasis (de Waal et al., 1987; de Waal and van Heerden, 2004).

1.6 Clinical signs

The incubation period for *T. equi* infections is 12 to 19 days (de Waal and van Heerden, 2004), and for *B. caballi*, it is 10 to 30 days (de Waal and Potgieter, 1987; de Waal, 1990), with *T. equi* infections being more pathogenic. Equine piroplasmosis is generally characterized by fever, malaise, anorexia, icterus, haemoglobinaemia/ haemoglobinuria, pale mucous membranes, tachycardia, and tachypnoea.

The more frequent, acute cases are often characterized by fever, usually greater than 40ºC, varying degrees of anorexia and malaise and elevated respiratory and pulse rates. Subacute cases show varying degrees of anorexia, malaise, weight loss, elevated or normal temperature and increased pulse and respiratory rates. Petechia or ecchymosis may be present on mucous membranes. The infected animal may become constipated, but this is soon followed by severe diarrhoea. The urine is also often discoloured as a result of the haemoglobin and bile pigments. The spleen is also often enlarged. Chronic cases usually show non-specific clinical signs that include weight loss, poor performance and mild inappetance (de Waal, 1992; Irby, 2002).

Secondary complications that may arise from equine piroplasmosis include acute renal failure, pneumonia, colic, enteritis, laminitis, infertility, and abortion. In rare, peracute forms, horses die within 24-48 hours of the onset of clinical signs (de Waal, 1992).

1.7 Economical impact of equine piroplasmosis - Globally and in South Africa

Globally, equine piroplasmosis poses a problem in the international movement of horses through trading and equestrian sport, from endemic to disease-free areas such as the United States, Canada, Australia, Japan, England and Ireland (Sluyter, 2001; Irby, 2002). The introduction of parasitic organisms in non-infested areas poses a serious risk in the economic loss of valuable race and tournament horses susceptible to the disease (de Waal, 1992).

The economic importance of equine piroplasmosis in South Africa has not been fully assessed due to the lack of an adequate reporting system. However, the number of cases of piroplasmosis exceeds that of any other infectious disease of horses in South Africa (de Waal and van Heerden, 2004). Due to the high seroprevalence of asymptomatic carrier animals in the region, many countries restrict the importation of horses from South Africa. The South African thoroughbred

racing industry is thus particularly affected by this restriction and also by acute piroplasmosis infections which result in missed training sessions and races and therefore a loss of income (Allsopp et al., 2007).

1.8 Control and treatment

A large variety of drugs is available for the treatment of equine piroplasmosis, each with varying degrees of success. Diminazene administered at a dose rate of 11 mg/kg body weight by deep intramuscular injection is usually effective in eliminating *B. caballi* but not *T. equi* infections (de Waal, 1992). Imidocarb, which is considered to be the safest of all drugs available, is effective in treating clinical cases of both organisms (Belloli et al., 2002). However, a recent study showed that even high dose treatment with imidocarb may not be capable of eliminating *B. caballi* and *T. equi* infections from healthy carriers (Butler et al., 2008). The acridine dyes (euflavine and others) at a recommended dose of 4 to 8 ml/100 kg body weight of a 5% solution with a maximum dose of 20 ml, administered i/v are therapeutically helpful against both *B. caballi* and *T. equi* (de Waal and van Heerden, 2004; Brüning, 1996).

Preliminary research indicates that parvaquone, an antitheilerial drug, can be effective in the treatment of acute *T. equi* infections, but that the drug is unable to sterilize the infection (Kuttler et al., 1987). Chemosterilization is rarely recommended, but may be indicated when horses are to be moved from an endemic area to a disease-free area (de Waal, 1992).

None of the drugs available is 100% effective in sterilizing the parasitic infections and the dosage required usually approaches toxic levels that are dangerous to use on valuable horses (de Waal, 1992). Depending on the severity of the disease, supportive therapy that includes blood or glucose infusions and the administration of essential phospholipids may be required (Hailat et al., 1997). The regular application of acaricides may also contribute to the prevention of piroplasmosis by eliminating contact between the tick vector and the equine host (de Waal, 1992; Brüning, 1996).

1.9 Diagnosis

Several techniques have been developed for the diagnosis of equine piroplasmosis, including the detection and differentiation of protozoa based on clinical signs, the direct demonstration of parasites in blood smears, serological assays, cell-culture techniques, xenodiagnosis, DNA probes and nested PCR assays. The clinical signs of equine piroplasmosis are often nonspecific, making it easy to confuse the disease with a variety of others that include equine influenza, encephalosis virus infection and equine infectious anaemia. It is also not possible to distinguish between *T. equi* and *B. caballi* infections based on clinical signs alone and mixed infections do occur (de Waal, 1992).

1.9.1 Blood smear examination

Traditionally, thick and thin blood smears are methods of choice for the detection and identification of blood parasites infecting horses (de Waal and van Heerden, 2004). In cases where the parasitaemias are low, thick blood smears are useful (de Waal and van Heerden, 2004; Böse et al., 1995).

1.9.2 In vitro culture techniques

Advances in the *in vitro* culture methods for equine piroplasmosis have enabled researchers to identify both *T. equi* and *B. caballi* carrier animals that were microscopically and serologically negative (Holman et al., 1993; de Waal and van Heerden, 2004; Holman et al., 1994; Zweygarth et al., 1997). Although the sensitivity of the culture technique has not been compared to the sensitivities of the PCR techniques currently available, parasitaemias as low as 10^{-8} have been reported (de Waal and van Heerden, 2004; Böse et al., 1995). Despite the high sensitivities and specificities, *in vitro* culture techniques require expensive culture facilities and skilled personnel, and can only be performed on freshly collected blood samples. Furthermore, culture techniques are time-consuming, resulting in lower sample throughput, thus further emphasizing their limitation as a diagnostic test.

1.9.3 Serological diagnosis

1.9.3.1 IFAT, CFT and ELISA

Due to the difficulties experienced in detecting low numbers of parasites by microscopy in subclinically infected or carrier animals, serological methods such as the complement fixation test (CFT), indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) have been developed to aid in the diagnostic process (de Waal and van Heerden, 2004; Phipps and Otter, 2004). The CFT does have some drawbacks, however, in that false-positive and negative results are possible and that not all infected animals, especially those that have been treated, can be identified (de Waal and van Heerden, 2004; Brüning, 1996). The IFAT is considered to be more sensitive than the CFT and it can be used to distinguish between *T. equi* and *B. caballi* infections. Although recognizing a strong positive IFAT result is simple, differentiation between weak positive and negative reactions can be difficult. An indirect ELISA has also been used to detect antibody to both species of parasites in experimentally infected horses, but cross-reactions that occur between *B. caballi* and *T. equi* prevent the test from being recognized as a differential diagnostic assay (Weiland, 1986). Despite these limitations, IFAT and ELISA have been used to determine the epidemiology of equine piroplasmosis in the north-west region of Spain, in Turkey, Sudan, Israel and Mongolia (Shkap et al., 1998; Camacho et al., 2005; Boldbaatar et al., 2005; Acici et al., 2008; Salim et al., 2008; Karatepe et al., 2009).

1.9.3.2 cELISA

Recombinant *T. equi* and *B. caballi* merozoite proteins and monoclonal antibodies to these proteins have subsequently been used in the development of competitive inhibition ELISAs (cELISA), with promising results.

Merozoite surface proteins play pivotal roles in the recognition, attachment and penetration of host erythrocytes by parasites (Knowles et al., 1991). The surface-exposed, immunodominant protein expressed during the *T. equi* merozoite stage, equi merozoite antigen-1 (EMA-1), is an important candidate for the development of effective diagnostic assays (Knowles et al., 1991). Xuan et al. (2001) reported a high degree of homology between amino acid sequences of EMA-1 from 19 *T. equi* strains from various countries and homologous gene sequences have been described for a number of other *Theileria* species (Katende et al., 1990; Kawazu et al., 1992; Matsuba et al., 1995; Shiels et al., 1995; d'Oliveira et al., 1996). The cELISA employing a monoclonal antibody (MAb 36/133.97) to recombinant EMA-1 has been shown to reliably detect

antibody to *T. equi* in the sera of infected horses from 19 countries (Knowles et al., 1992; Shkap et al., 1998; Sevinc et al., 2008).

Similarly, apicomplexan parasites secrete proteins from their apical organelles, which include the rhoptry-associated protein-1 (RAP-1) (Sam-Yellowe, 1996). RAP-1 proteins were initially described in *Babesia bovis* and *Babesia bigemina*, but have subsequently been described in other *Babesia* parasites (Dalrymple et al., 1993; Skuce et al., 1996; Suarez et al., 1998; Kappmeyer et al., 1999; Ikadai et al., 1999). The RAP-1 protein family contains several immunogenic epitopes, and antibodies directed against these protein epitopes have been shown to inhibit merozoite invasion (Ikadai et al., 1999; Machado et al., 1999; Yokoyama et al., 2006). This observation suggests that RAP-1 proteins are important targets of the protective immune response (Suarez et al., 2003). A monoclonal antibody to recombinant RAP-1 was used in the development of the cELISA for the detection of *B. caballi* antibody in infected horses (Kappmeyer et al., 1999). This assay has been reported to be successful in the detection of *B. caballi* antibody from sera of infected horses in North and South America and various European countries (Kappmeyer et al., 1999; Sevinc et al., 2008).

The cELISA proved to be superior to CFT in the detection of long-term *T. equi* and B. *caballi* infected animals. Additionally, cELISA has been shown to have a higher specificity for both *T. equi* and *B. caballi*, when compared to the indirect ELISA (Shkap et al., 1998; Kappmeyer et al., 1999).

1.9.4 Molecular diagnosis

1.9.4.1 DNA Probes

DNA probes have been developed for the detection of either *B. caballi* or *T. equi* parasites in blood, tissues and tick organs (Posnett and Ambrosio, 1989; Posnett and Ambrosio, 1991; Böse et al., 1995; de Waal and van Heerden, 2004). Although these probes were able to detect parasites in carrier animals (Posnett et al., 1991), it was recommended that their sensitivity must be increased before they could be used to certify animals free from both *B. caballi* and *T. equi.* An improvement on the radioisotope labeled DNA probe (Posnett and Ambrosio, 1991) for the detection of *B. caballi* was observed with the subsequent development of the biotin-labeled *Bc1* DNA probe (Sahagun-Ruiz et al., 1997), which, used in conjunction with the polymerase chain reaction (PCR) and Southern blot analysis, showed an increase in sensitivity of the test.

Oligonucleotide probes based on sequence differences in the small subunit ribosomal RNA (18S rRNA) genes have been developed and were used in a preliminary study to demonstrate transplacental transmission of *T. equi* (Allsopp et al., 2007).

1.9.4.2 PCR techniques

1.9.4.2.1 Conventional and nested PCR techniques

The polymerase chain reaction (PCR) has been applied for the detection of many species of *Babesia* and *Theileria* and PCR assays have been reported to have higher sensitivity and specificity compared with serological assays (Geysen et al., 2003; Buling et al., 2007; Jefferies et al., 2007). The stability of ribosomal RNA genes makes them an attractive target for species discrimination. A PCR assay based on the design of species-specific oligonucleotide primers within the 18S rRNA gene was thus developed for the detection of *T. equi* and *B. caballi* (Bashiruddin et al., 1999). The authors reported an estimated parasitaemia of 0.0083% for *T. equi,* and parasitaemias of approximately 0.0017% for *B. caballi*, from cases previously undetected by microscopy. Nested PCR assays have subsequently been developed for the specific detection of the equi merozoite antigen gene (*ema-1*) of *T. equi* from the blood of infected horses and in *Rhipicephalus* (*Boophilus*) *microplus* ticks infected with *T. equi* and *B. caballi* (Nicolaiewsky et al., 2001; Battsetseg et al., 2002). The ability of nested PCR to diagnose sub-clinical infections is significant and could contribute toward controlling the exportation of infected animals as well as in determining the efficiency of medical treatments (Rampersad et al., 2003). Because of the sensitivity of the PCR assays, however, false-positives due to contaminants are always possible. Another shortcoming of PCR is that unless it is a specific multiplex PCR (e.g. Alhassan et al., 2005), several separate assays are required to detect mixed infections and the presence of novel species and/or genotypes can be overlooked (Gubbels et al., 1999). To overcome these problems, a reverse line blot assay (RLB) based on PCR amplification of the 18S rRNA gene, followed by simultaneous probing with species-specific oligonucleotide probes, has been developed (Gubbels et al., 1999; Nagore et al., 2004a).

1.9.4.2.2 Reverse Line Blot hybridization

A highly sensitive and specific reverse line blot (RLB) hybridization assay has been developed for the improved diagnosis of piroplasmosis in horses (Nagore et al., 2004a). RLB has proven to be a very powerful tool in detecting subclinical infections, which are usually undetectable by light microscopy. Furthermore, this assay enables the identification of mixed infections and has

also proven to be a valuable tool in the identification of novel piroplasm species or genotypes (Georges et al., 2001; Criado-Fornelio et al., 2004; Nagore et al., 2004a; Nagore et al., 2004b; Nijhof et al., 2005).

1.9.4.2.3 Quantitative real-time PCR assays

The recent development of quantitative real-time PCR (qPCR) has greatly improved the molecular detection and diagnosis of many organisms of veterinary and medical importance (Jeong et al., 2003; Lindh et al., 2007; O'Grady et al., 2008; Wengi et al., 2008). Quantitative PCR assays provide several advantages over the use of conventional PCR and probe-based assays, which are relatively sensitive but involve complex procedures that are time consuming and labour intensive (Allsopp et al., 1993; Bashiruddin et al., 1999; Nicolaiewsky et al., 2001; Rampersad et al., 2003; Alhassan et al., 2005). Detection and quantification of a qPCR product takes place in a single tube during the cycling process, thus eliminating the need for post-PCR manipulation and reducing the risk of contamination. Quantitative PCR tests have recently been developed for a number of haemoparasitic disease agents including *Theileria sergenti* (Jeong et al., 2003), *Babesia bovis* and *Babesia bigemina* (Buling et al., 2007), *Anaplasma marginale* (Carelli et al., 2007) and *Theileria parva* (Sibeko et al., 2008). These qPCR assays have significantly improved the sensitivity and specificity of parasite detection. A qPCR assay, based on the 18S rRNA gene, was recently developed for the detection of *T. equi* infections in horses (Kim et al., 2008). This assay proved to be highly sensitive and specific for *T. equi*, and it allowed for the simultaneous detection and quantification of experimental samples.

Several parasite outer membrane protein gene sequences have also been targeted in the development of molecular diagnostic assays for equine piroplasmosis (Nicolaiewsky et al., 2001; Ueti et al., 2003; Alhassan et al., 2005; Alhassan et al., 2007; Heim et al., 2007). A qPCR assay targeting the gene (*ema-1*) encoding the equi merozoite antigen-1 (EMA-1) was developed to determine the number of *T. equi* parasites in the midgut of *Rhipicephalus* (*Boophilus*) *microplus* ticks (Ueti et al., 2003). A multiplex assay, using this test and a qPCR based on the rhoptry-associated protein (BC 48) gene of *B. caballi*, was subsequently developed and used to determine the prevalence of both *T. equi* and *B. caballi* parasites in horses in Brazil (Heim et al., 2007).

1.10 Objectives and overview of this study

Improved diagnosis and detection of piroplasmosis carrier animals would be of great benefit to the horse industry both locally and internationally. The main objective of this study was therefore to develop a quantitative real-time PCR assay to complement and improve on the current diagnostic tests. Many molecular diagnostic assays developed for the detection of *Theileria* and *Babesia* species have been based on the amplification of the 18S rRNA gene. A preliminary study in our laboratory, however, provided evidence of sequence heterogeneity in the V4 hypervariable region of the 18S rRNA gene within *Theileria* and *Babesia* parasite species infecting horses in South Africa. In Chapter 2 of this thesis, the extent of genetic heterogeneity within the 18S rRNA genes of *T. equi* and *B. caballi* parasites is explored and conclusions regarding the usefulness of current diagnostic assays employing the V4 hypervariable region in detecting these two piroplasm species in South Africa are made.

The identification of extensive sequence variation in the 18S rRNA gene of *T. equi* and *B. caballi* parasites in South Africa explained the failure of previous molecular assays in detecting these parasites. In Chapter 3, the recently reported *T. equi*-specific TaqMan qPCR assay targeting the 18S rRNA gene (Kim et al., 2008) is evaluated for its ability to detect all *T. equi* 18S rRNA variants that have been shown to occur in South Africa. The development of a TaqMan minor groove binder (MGB™) qPCR assay for the detection of *B. caballi* in equine field blood samples is also described.

Despite the knowledge that piroplasm parasites occur in our zebra populations, the molecular epidemiology and the possible influence through genetic recombination that their existence may have on horse piroplasms, has largely been overlooked. In Chapter 4 zebra samples are screened to identify piroplasm parasites, and 18S rRNA genes of *T. equi*-like piroplasms of zebra are sequenced to further elucidate genetic variation in *T. equi* parasites in South Africa.

Although a qPCR assay based on the 18S rRNA gene has been developed (Kim et al., 2008) and was evaluated in this study, the identification of additional *T. equi* 18S variants in zebras suggests that we cannot rule out the possible existence of as yet undetected 18S gene sequence variants. In chapter 5, a second qPCR assay recently developed for the detection of *T. equi* targeting the equi merozoite antigen-1 gene (*ema-1*) (Ueti et al., 2003) is evaluated. Following its poor performance, the *ema-1* gene from South African *T. equi* samples is characterized and a

more sensitive TaqMan MGB™ qPCR assay that targets a conserved region of the *ema-1* gene is developed.

Finally in Chapter 6 the characterization of *rap-1* gene homologues from South African *B. caballi* isolates is described in an attempt to provide reasons for the failure of the commercial cELISA to detect *B. caballi* antibody in blood samples that tested positive using the IFAT.

1.11 Reference List

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CHAPTER 2

Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa

2.1 Abstract

A molecular epidemiological survey of the protozoal parasites that cause equine piroplasmosis was conducted using samples collected from horses and zebra from different geographical locations in South Africa. A total of 488 samples were tested for the presence of *Theileria equi* and/or *Babesia caballi* using the reverse line blot hybridization assay. Ten percent of the samples hybridized to the *Theileria/Babesia* genus-specific probe and not to the *B. caballi* or *T. equi* species-specific probes, suggesting the presence of a novel species or genotype. The small subunit ribosomal RNA gene (18S; ~1600 bp) was amplified and sequenced from 33 of these 488 samples. Sequences were compared with published sequences from the public sequence databases. Twelve distinct *T. equi* and six *B. caballi* 18S rRNA sequences were identified. Alignments demonstrated extensive sequence variation in the V4 hypervariable region of the 18S rRNA gene within *T. equi*. Sequence variation was also found in *B. caballi* 18S rRNA genes, although there was less variation than observed for *T. equi*. Phylogenetic analysis based on 18S rRNA gene sequences revealed three *T. equi* clades and two *B. caballi* clades in South Africa. The extent of sequence heterogeneity detected within *T. equi* and *B. caballi* 18S rRNA genes was unexpected since concerted evolution is thought to maintain homogeneity within repeated gene families, including rRNA genes, in eukaryotes. The findings reported here show that careful examination of variants of the 18S rRNA gene of *T. equi* and *B. caballi* is required prior to the development of molecular diagnostic tests to detect these parasites in horses. Species-specific probes must be designed in regions of the gene that are both conserved within and unique to each species.

2.2 Introduction

Equine piroplasmosis is a tick-borne disease of equids that is caused by two species of apicomplexan protozoa, *Babesia caballi* and *Theileria equi* (Mehlhorn and Schein, 1998). The disease occurs throughout the tropical and subtropical areas of the world, with endemic areas in many parts of Europe, Asia, Arabia, South and Central America and Africa. *Babesia caballi* and *T. equi* are transmitted by species of ixodid ticks of the genera *Dermacentor*, *Rhipicephalus* and *Hyalomma* (de Waal and van Heerden, 2004). Both species of protozoa cause infections, which can result in an acute or chronic disease, with mortalities of up to 50% (de Waal, 1992). The clinical signs of equine piroplasmosis are often non-specific, complicating diagnosis. Therefore, it is not possible to differentiate between *T. equi* and *B. caballi* infections based on clinical signs alone (Potgieter et al., 1992) and mixed infections occur. Once infected, animals may remain

life-long carriers of *T. equi* infections, while horses may remain carriers of *B. caballi* for up to 4 years (de Waal and van Heerden, 2004).

Traditionally, the microscopic examination of blood smears is the method of choice for the detection and identification of blood parasites infecting horses (de Waal, 1992). However, due to the difficulties experienced in detecting low numbers of parasites by microscopy in sub-clinically infected or carrier animals, serological assays, such as the complement fixation (CF) test, indirect fluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) as well as the competitive-inhibition ELISA (cELISA) have been developed for the diagnosis of equine piroplasmosis (Knowles et al., 1991; Brüning et al., 1997; Kappmeyer et al., 1999; Ikadai et al., 2000). There are limitations with serological assays, however, due to problems with non-specificity, cross-reactivity and antibody detection limits (Brüning et al., 1997).

Advances in molecular biological techniques have resulted in the improved detection, identification and genetic characterization of many haemoparasites (Caccio et al., 2000; Nagore et al., 2004a). The polymerase chain reaction (PCR) has been applied for the detection of many species of *Babesia* and *Theileria* and has been shown to have higher sensitivity and specificity compared with serological assays (Geysen et al., 2003; Buling et al., 2007; Jefferies et al., 2007; Sibeko et al., 2008). A sensitive and specific reverse line blot (RLB) hybridization assay, based on a sequence-specific PCR that targets parasite 18S rRNA genes, has been developed for detection of *Theileria* and *Babesia* infections, including piroplasmosis in horses (Gubbels et al., 1999; Nagore et al., 2004a). This assay enables the identification of mixed infections and has also proven to be a valuable tool in the identification of novel piroplasm species or genotypes (Georges et al., 2001; Criado-Fornelio et al., 2004; Nagore et al., 2004a; Nagore et al., 2004b; Nijhof et al., 2005).

Nuclear ribosomal rRNA genes have been shown to provide appropriate targets to assist in the identification of species (Katzer et al., 1998; Chae et al., 1998; Allsopp and Allsopp, 2006). However, sequence heterogeneity in the 18S rRNA gene has been reported within some species of protozoa (e.g. *Babesia bovis, Cytauxzoon felis, Theileria* sp. (type C)), both within a given isolate and among isolates from different geographical regions (Calder et al., 1996; Chae et al., 1999; Criado-Fornelio et al., 2003). Therefore, although a high degree of 18S rRNA gene sequence conservation has been reported in *Babesia* and *Theileria* species, it has been recommended that the complete 18S rRNA gene of these parasites should be determined,

particularly when dealing with new organisms, to ensure that genetic variation is not overlooked (Hunfeld et al., 2008). Although several PCRs, based on the 18S rRNA gene, have been developed for the detection of the parasites that cause equine piroplasmosis (Bashiruddin et al., 1999; Nicolaiewsky et al., 2001; Battsetseg et al., 2002; Rampersad et al., 2003; Alhassan et al., 2007), the 18S rRNA gene sequence has been determined for only one isolate each of *T. equi* and *B. caballi* in South Africa. A preliminary study in our laboratory on the development of a real-time PCR assay for the specific detection of *T. equi* and *B. caballi* provided evidence of sequence heterogeneity in the V4 hypervariable region of the 18S rRNA gene sequences within each of these two species of piroplasm (unpublished findings). Therefore, the main purpose of this study was to explore levels of genetic heterogeneity within the *Theileria* and *Babesia* parasite species infecting horses in South Africa and make conclusions regarding the usefulness of diagnostic assays for equine piroplasmosis, which employ this V4 hypervariable region as a genetic marker.

2.3 Materials and methods

2.3.1 Field samples and tissue-culture stabilates

A total of 488 samples were investigated (Table 2.1). Blood samples, collected from 148 yearlings on six different stud farms in different locations in South Africa (Table 2.2), were obtained from the blood bank of the Equine Research Centre, Faculty of Veterinary Science, University of Pretoria. Also, 39 blood samples were collected from South African horses housed in the premises of this Faculty. Serum samples were collected at the South African National Two-year Sale (2005) and the National Yearling Sale (2006) and sent to the Agricultural Research Council-Onderstepoort Veterinary Institute where they were tested using IFAT. Based on these results, 211 horses, which tested positive serologically for *T. equi* and/or *B. caballi*, were identified and whole-blood samples were collected from each of them.

In addition, 90 tissue-culture samples, which included 17 Cape mountain zebra (*Equus zebra zebra*) samples from the Bontebok National Park, Western Cape province, South Africa (Zweygarth et al., 1997; Zweygarth et al., 2002) were investigated.

Table 2.1 Origin and number of samples tested.

Origin	Number of samples
Stud farms[a]	148
Faculty of Veterinary Science, Onderstepoort	39
Field samples[b]	211
Tissue-culture samples	90
Total	**488**

[a] For locations of the stud farms in South Africa see Table 2.2

[b] Field samples collected from horses at the South African National Two-year Sale (2005) and National Yearling Sale (2006).

Table 2.2 Geographic location of stud farms in South Africa.

Farm	Place	Co-ordinates	Province	Number of samples
SA1	Port Elizabeth	S33.98 E25.51	Eastern Cape	18
SA2	Port Elizabeth	S34.02 E25.51	Eastern Cape	8
SA3	Port Elizabeth	S34.02 E25.50	Eastern Cape	3
SA4	Ceres	S33.36 E19.45	Western Cape	21
SA5	Colesberg	S30.65 E25.32	Northern Cape	64
SA6	Nottingham Road	S29.46 E29.91	KwaZulu-Natal	32
Total				**148**

2.3.2 DNA extraction

Genomic DNA was extracted from 200 µl of citrate-buffered or EDTA-treated blood or tissue-culture stabilate samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.3.3 PCR amplification and reverse line blot (RLB) hybridization

Primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (Biotin-5'-CTA AGA ATT TCA CCT CTG ACA GT-3') specific for *Theileria* and *Babesia* species, were used to amplify the V4 hypervariable region of the 18S rRNA genes of the parasites present in the samples, as described previously (Nijhof et al., 2005). PCR products were subjected to RLB hybridization as described by Nijhof et al. (2005). The oligonucleotide probes used for the detection of *T. equi* and *B. caballi* are listed in Table 2.3.

Table 2.3 Oligonucleotide probes used in the RLB hybridization assay.

Species	Probe Sequence (5'-3')	Reference
Theileria/Babesia catch-all	TAATGGTTAATAGGARCRGTTG	Gubbels et al. (1999)
Theileria catch-all	ATTAGAGTGCTCAAAGCAGGC	Nijhof* (unpublished)
Theileria equi	TTCGTTGACTGCGYTTGG	Butler et al. (2008)
Babesia catch-all 1	ATTAGAGTGTTTCAAGCAGAC	Nijhof (unpublished)
Babesia catch-all 2	ACTAGAGTGTTTCAAACAGGC	Nijhof (unpublished)
Babesia caballi	GTTGCGTTGTTCTTGCTTTT	Nijhof (unpublished)

Ambiguity codes: R = A/G; Y = C/T

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2.3.4 Sequencing and analyses

The complete 18S rRNA genes of parasites with novel genotypes were amplified using three nested PCRs. As it was not possible to amplify the 18S rRNA gene from samples with low piroplasm parasitaemia, the GenomiPhi DNA amplification kit (Amersham BioSciences) was used to amplify genomic DNA as described by the manufacturer, prior to performing nested PCR reactions.

Primers NBabesia1F and 18SRev-TB (Table 2.4) were used in a primary PCR to amplify a fragment of ~1600 bp. Reactions were performed in a final volume of 25 µl with High Fidelity PCR Master mix (Roche Diagnostics, Mannheim, Germany), 0.2 µM of each primer and 30 ng of genomic DNA. The cycling conditions were: an initial denaturation of 2 min at 94°C, followed by 40 cycles of 30s at 94°C, 45s at 60°C and 1 min at 72°C, and a final extension of 7 min at 72°C. It was not possible to obtain sufficient primary PCR product to sequence the full-length 18S rRNA gene from any of the samples directly. Therefore, three nested PCR reactions were subsequently performed to obtain enough amplicon for sequence analysis. Primers NBabesia1F and BT18S3R (Table 2.4) were used to amplify an 800 bp PCR product at the 5'-end of the gene. The amplification program was the same as employed for the first PCR, except that an annealing step of 58°C for 1 min was used. The amplification of the 3'-end of the 18S rRNA gene was accomplished using primers BT18S3F and 18SRev-TB (Table 2.4) with an annealing step of 55°C for 1 min. An internal 800 bp product, which overlaps both the 5' and 3' fragments by ~400 bp, was amplified using primers BT18S2F and BT18S2R using an annealing step of 55°C for 1 min.

Nested PCR products were purified using the Qiagen PCR purification kit (Qiagen, Hilden, Germany). Samples were sequenced using BigDye chemistry (v.3.1, Applied Biosystems) in a 3130XL sequencer (Applied Biosystems).

Table 2.4 Oligonucleotide primers used to amplify and sequence parasite 18S rRNA genes.

Primer	Size (bp)	Sequence 5'-3'	Tm (°C)	Reference
NBabesia1F	29	AAGCCATGCATGTCTAAGTATAAGCTTTT	57.0	Oosthuizen et al. (2008)
18SRev-TB	22	GAATAATTCACCGGATCACTCG	61.0	Matjila et al. (2008)
BT18S2F	20	GGGTTCGATTCCGGAGAGGG	60.3	Oosthuizen et al. (2008)
BT18S3F	26	GGGCATTCGTATTTAACTGTCAGAGG	59.2	Oosthuizen et al. (2008)
BT18S2R	24	CCCGTGTTGAGTCAAATTAAGCCG	60.1	Matjila et al. (2008)
BT18S3R	26	CCTCTGACAGTTAAATACGAATGCCC	59.2	This study

Sequences were assembled and edited using the Staden software suite (Staden, 1996) and have been deposited in GenBank under accession numbers EU642507, EU642508, EU642509, EU642510, EU642511, EU642512, EU642513, EU642514, EU888900, EU888901, EU888902, EU888903, EU888904, EU888905 and EU888906. Multiple sequence alignments were performed using MAFFT (Multiple Sequence Alignment) employing the FFT-NS-1 algorithm (Katoh et al., 2005). The alignments were adjusted manually using BioEdit (version 7.0.5.2) (Hall, 1999). Searches of databases for orthologous sequences were performed using BLASTN (Altschul et al., 1990). Modeltest v.3.7 (Posada and Crandall, 1998) was used to select a TrN+I+G substitution model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites. Phylogenetic analyses using distance and maximum-likelihood methods were carried out using the program PAUP* v4b10 (Swofford, 2003) using 1473 characters for *T. equi* and 1406 characters for *B. caballi*. Trees were constructed (1000 replicates) using the 18S rRNA gene sequence of *Hepatozoon canis* (DQ439543) as an outgroup. Analysis by Bayesian inference was performed using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003) accessible via the Computational Biology Service Unit at Cornell University (http://cbsuapps.tc.cornell.edu/mrbayes.aspx). Four simultaneous Markov chain Monte Carlo (MCMC) chains were run for 5,000,000 generations, with sampling every 100 generations for a total of 50,000 samples per run. All consensus trees generated were edited using MEGA4 (Tamura et al., 2007).

2.4 Results

RLB results for the total number (488) of samples tested indicated that 50% of the samples hybridized to the *T. equi* probe, 3% to the *B. caballi* probe, and 37% tested negative. Ten percent of the samples hybridized to the *Theileria/Babesia* genus-specific probe alone. Representative RLB results, showing reactivity of some samples with the *Theileria/Babesia* genus-specific probe but not with the species-specific probes, are shown in Figure 2.1. Of the 48 *Theileria/Babesia* genus positive samples, the full-length 18S rRNA gene could be amplified and sequenced from only 33. BLAST analysis revealed that 20 of these sequences were most closely related to *T. equi* 18S rRNA sequences. Eight new *T. equi* 18S rRNA sequences were identified which had between 96.1 and 99.9 % identity to the previously published *T. equi* sequence from South Africa (accession number: Z15105) (Allsopp et al., 1994). The remaining thirteen sequences were most similar to other *B. caballi* sequences. Four new *B. caballi* 18S rRNA sequences with 96.9-99.9% identity to the previously published *B. caballi* sequence from South Africa (accession number: Z15104) (Allsopp et al., 1994) were identified. Sequence alignments demonstrated extensive sequence variation in the V4 hypervariable region.

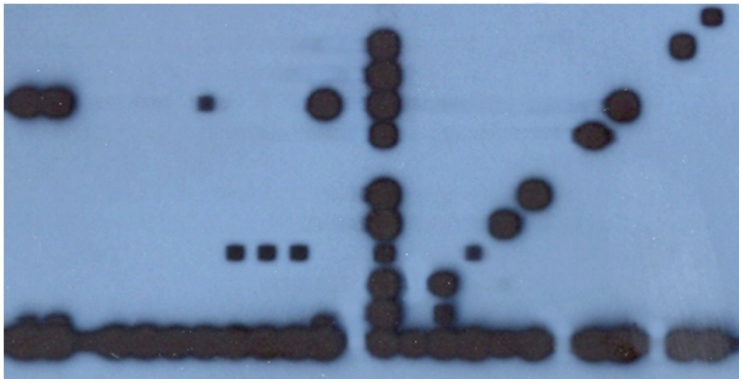

Figure 2.1 Reverse line blot hybridization of *Theileria* and *Babesia* PCR products amplified from genomic DNA extracted from infected equine blood samples. Genus- and species-specific oligonucleotide probes were applied in horizontal rows as indicated. PCR products from samples and control clones were applied in vertical lanes. Lanes 1-11: PCR products from equine field samples. Lanes 12-21: PCR products from clones containing control DNA, specifically; lane 12: RLB plasmid control; lane 13: *Theileria/Babesia* genus-specific catch-all; lane 14: *B. felis* and *B. microti*; lane 15: *B. caballi*; lane 16: *B. vogeli*; lane 17: *B. rossi*; lane 18: *T. buffeli*; lane 19: *T. equi*; lane 20: *T. taurotragi*; lane 21: *T. separata*. Note that *T. equi* could be identified in samples 1, 2, 7 and 11, and *B. caballi* was present in samples 8-10, but samples 3-6 hybridized only to the *Theileria/Babesia* genus-specific probe.

Phylogenetic analyses using the neighbor-joining, maximum-likelihood and Bayesian inference, all yielded trees with almost identical topologies and high bootstrap or nodal support values. The analyses showed that the *T. equi* 18S rRNA gene sequences fell into three main groups,

designated A, B and C (Figure 2.2). Group A contained the previously published *T. equi* sequences from South Africa (accession number: Z15105) (Allsopp et al., 1994) and from Spain (accession numbers: AY150062, AY150063 and AY150064) (Criado-Fornelio et al., 2003). Group B contained sequences from two Cape mountain zebra samples from the Western Cape Province of South Africa that were derived in this study, and which grouped together with a *T. equi*-like isolate from a horse from Spain (strain ET1, AY534882), reported previously (Nagore et al., 2004a). Sequences representing this genotype were not detected in any of the South African samples from horses. The third group (C) contained a number of new South African *T. equi* 18S sequences determined in this study, which were distinct from all previously published 18S rRNA sequences from *T. equi*. *Theileria equi* samples from horses and/or zebras from different localities within South Africa were present in all three groups.

While there was less sequence variation amongst *B. caballi* samples, the *B. caballi* 18S rRNA gene sequences could be divided into two groups (Figure 2.2). Group A contained the published *B. caballi* sequences from Spain (AY309955 and AY534883) (Criado-Fornelio et al., 2004; Nagore et al., 2004a) as well as a sequence obtained from a *B. caballi* USDA reference strain (*B. caballi* Ames, Iowa) (Kappmeyer et al., 1999). Sequences of the South African samples investigated in this study, fell into the second group (B), which could be sub-divided into sub-groups B1 and B2, the former sub-group containing the original South African *B. caballi* sequence (Z15104) (Allsopp et al., 1994).

The *T. equi* RLB probe used in this study could detect genotypes within group A, but not those within groups B and C. There were 15 nucleotide changes in the probe region between *T. equi* group A and B sequences, whereas there were 16 nucleotide differences for sequences within groups A and C (Figure 2.3). The *B. caballi* RLB probe used in this study could detect most samples within group B, but not those within group A. The sequence of the RLB probe for *B. caballi* used in this study was the same in sequence to samples from group B1, but there was a point mutation in this region in group B2 (and in one sample, two point mutations) and six nucleotide differences in sequences in group A (Figure 2.4).

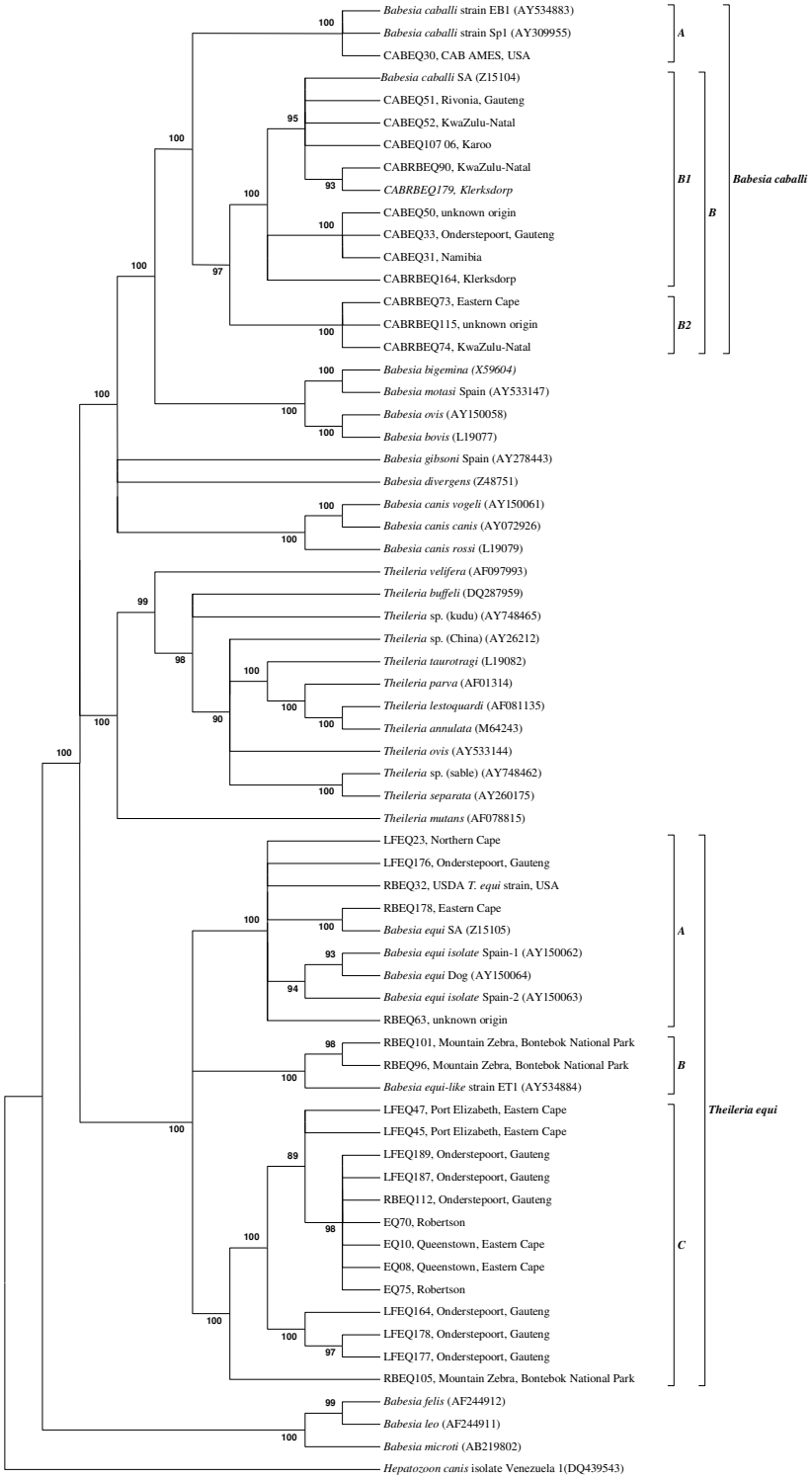

Figure 2.2 Rooted cladogram, determined with Bayesian inference and $5x10^6$ iterations, of the 18S rRNA gene sequences of *T. equi* and *B. caballi* samples identified in this study (square brackets) and *Theileria* and *Babesia* sequences from GenBank (accession numbers are indicated in parentheses). Posterior probabilities are indicated on the nodes of the tree.

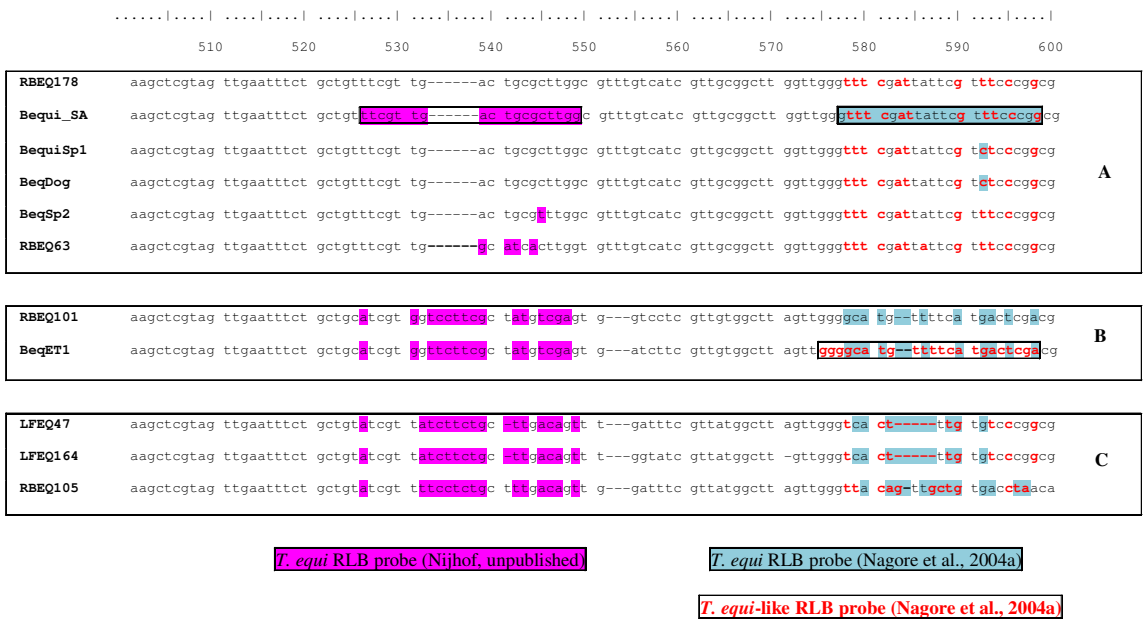

Figure 2.3 Nucleotide alignment of a section of the V4 hypervariable region of the 18S rRNA gene of the *T. equi* isolates examined in this study, to the published *T. equi* sequences (*B. equi* SA, Z15105; *B. equi* isolate Spain-1, AY150062; *B. equi* isolate Spain-2, AY150063; *B. equi* Dog, AY150064; *T. equi*-like strain ET1, AY534882). The sequence of the *T. equi* RLB probe used in the present study is highlighted in pink. The sequences of the RLB probes designed by Nagore et al. (2004a) are either highlighted in blue or indicated in bold red font. Nucleotide differences between isolates in the probe regions are either highlighted in the same colours used to indicate the oligonucleotides, or represented in bold red font. A, B and C respresent the three *T. equi* 18S rRNA groups identified in this study.

Figure 2.4 Nucleotide alignment of a section of the V4 hypervariable region of the 18S rRNA gene of the *B. caballi* isolates used in this study, to the published *B. caballi* 18S sequences (*B. caballi* SA, Z15104; *B. caballi* isolate Spain-1, AY309955; *B. caballi*-like strain EB1, AY534883). The sequence of the *B. caballi* RLB probe used in the present study is highlighted in pink. The sequences of the RLB probes designed by Nagore et al. (2004a) are either highlighted in blue or indicated in bold red font. Nucleotide differences between isolates in the probe regions are highlighted in the same colours used to indicate the oligonucleotides or represented in bold red font. A, B1 and B2 represent the different *B. caballi* 18S rRNA groups identified in this study.

2.5 Discussion

The RLB indicated the existence of novel genetic variants of *Theileria* and *Babesia* in blood samples from horses from South Africa. Sequence analysis confirmed the existence of twelve distinct 18S rRNA sequences for *T. equi*, which belonged to three main groups. In addition, six different *B. caballi* 18S sequences could be divided into two groups. These findings supported our previous unpublished results showing that real-time PCR probes designed to be specific were not able to detect target DNA in a large proportion of serologically positive *T. equi* or *B. caballi* samples. In addition to poor amplification curves, the melting peaks observed for many of these samples were ill defined. The detection of significant variation in the 18S rRNA genes of *T. equi* and *B. caballi* explains why many IFAT-positive field samples were not detected by the species-specific RLB probes and/or the real-time PCR assays.

The real-time PCR primers and probes (unpublished data) and the RLB probe used in the present study were designed using one 18S rRNA sequence for each *T. equi* and *B. caballi* from South Africa (accession numbers Z15105 and Z15104, respectively) as reference sequences. Subsequently, other studies led to the identification of two genetically distinct *Theileria* and two other *Babesia* genotypes infecting Spanish horses (Criado-Fornelio et al., 2004; Nagore et al., 2004a). Another study (Criado et al., 2006) showed genetically distinct *T. equi*-like isolates. Nagore et al. (2004a) designed RLB probes to detect both *T. equi* and *T. equi*-like 18S genotypes identified in their study. Their *T. equi* probe was designed to almost the same sequence region as our real-time PCR probe and was identical to most group A *T. equi* sequences (Figure 2.3), but it was significantly different from sequences of members of group C identified in this study. The probe designed to detect *T. equi*-like parasites (Nagore et al., 2004a) would have been able to detect *T. equi* samples in group B, but in groups A and C there are twelve and thirteen nucleotide differences in the region of this probe. These differences would almost certainly prevent hybridization in the RLB assay, although this was not tested.

The nucleotide sequences of parasites from two Cape mountain zebra samples (RBEQ96 and RBEQ101), from the Bontebok National Park in the Western Cape, were similar (99% identity) to the *T. equi*-like (*B. equi*-like strain ET1, AY534882) sequence (group B), but this genotype was not detected in any of the samples from horses in South Africa. A parasite 18S rRNA gene sequence from a third Cape mountain zebra grouped in clade C, suggesting that there is more variation in *T. equi* genotypes in zebra in South Africa, but a study of a larger group of samples from zebra is required to substantiate these findings.

The RLB probe designed by Nagore et al. (2004a) to detect the *B. caballi* 18S genotype is five nucleotides longer at the 3'-end (Figure 2.4), than the *B. caballi* RLB probe used in the present study. Sequence alignments indicated that both *B. caballi* RLB probes should have detected infections in most positive samples originating from South Africa, since these samples belonged to *B. caballi* groups B1 and B2 (although there are one or two nucleotide difference in the region of the RLB probe sequence within group B2 samples). In some cases, however, positive *B. caballi* samples were not detected in the RLB assay, but sequence analysis of some of these samples revealed *B. caballi* group B1 sequences. The inability of the RLB to detect all positive *B. caballi* infections is possibly due to very low parasitaemia in some samples, since it has been reported that the parasitaemia in *B. caballi* infections generally tends to be very low, rarely exceeding 1% (Hanafusa et al., 1998). The starting concentration of target DNA in the PCR

affects the final amount of PCR product obtained (Sibeko et al., 2008), and in samples where the parasitaemia was very low, there may not have been enough target DNA to yield sufficient PCR product to allow detection by the RLB probes. The group A *B. caballi* sequences differed by six and eight nucleotides in the region of these two RLB probes (Figure 2.4), suggesting that neither probe would have been able to detect samples in group A, which include *B. caballi* isolate Spain-1 (AY309955), *B. caballi*-like strain EB1 (AY534883) and the USDA isolate (CABEQ30, *B. caballi* AMES). On the other hand, the sequence of the *B. caballi*-like RLB probe, also reported by Nagore et al. (2004a), is identical to the Spanish and American isolates, but differs from the South African group B1 and B2 *B. caballi* sequences at seven positions (Figure 2.4).

Based on the sequence variation detected within each species of piroplasm, we determined whether primers and probes described by other authors would have amplified and detected the 18S rRNA gene from our newly identified South African *T. equi* and *B. caballi* genotypes. We were able to conclude that primers BEQF and BEQR, designed to amplify a 664 bp fragment from the 18S rRNA gene of *T. equi* (Bashiruddin et al., 1999), would not have been able to amplify all *T. equi* genotypes identified in this study. In addition, there were four nucleotide differences in the region where the *B. caballi* primer, BCAF, reported by the same authors, was designed. The reverse primer, BCAR, however, showed no nucleotide differences to other sequences in that region. These primers could therefore possibly amplify *B. caballi* DNA from all groups. This is consistent with the observation that sequence variation between the *B. caballi* samples investigated in the present study was not as extensive as that observed for *T. equi*. Rampersad et al. (2003) designed a nested PCR for the detection of *T. equi* based on the primers designed by Bashiruddin et al. (1999). Substantial sequence variation in groups B and C occurred in the region where their primers for the nested PCR (BEQF1 and BEQR1) were designed. More recently, a quantitative TaqMan assay, based on an 18S rRNA sequence outside of the V4 hypervariable region, has been developed for the detection of *T. equi* infections in horses (Kim et al., 2008). The 18S rRNA gene sequences obtained in the present study were identical in the regions used to design the primers and probe for this *T. equi* TaqMan assay.

The extent of sequence heterogeneity detected here within *T. equi* and *B. caballi* 18S rRNA genes was unexpected. Multiple copies of rRNA genes are present in most eukaryotes and are thought to be highly homogenized owing to concerted evolution (reviewed by Eickbush and Eickbush, 2007). Divergence from the concerted evolution model has been identified in *Plasmodium* species, which have functionally distinct rRNA 'types' that differ in their

expression frequencies (Gunderson et al., 1987). Similarly, *B. bigemina* and *B. bovis* parasites have been reported to contain distinct rRNA genes, which are preferentially expressed depending on the environmental and/or developmental conditions (Reddy et al., 1991; Brayton et al., 2007; Laughery et al., 2009).. *Theileria parva* is recognised as a genetically and antigenically diverse parasite (Collins and Allsopp, 1999), and variants of this parasite can cause clinically quite distinct diseases in cattle (East Coast fever and Corridor disease). Despite the existence of such diversity in this parasite, a real-time PCR test specific for the detection of *T. parva* infections in buffalo has been developed using the 18S rRNA gene as a target (Sibeko et al., 2008) and to date, has proved to be a reliable test to detect *T. parva* (Sibeko, personal communication). While genetic variants within *T. equi* and *B. caballi* have been identified in Spanish horses based on 18S sequence data (Criado-Fornelio et al., 2004; Nagore et al., 2004a), a recent study (Heim et al., 2007) did not reveal sequence variation in the 18S rRNA gene within either *T. equi* or *B. caballi* from horses in Brazil. In South Africa, the diverse equid species present are likely to carry closely related parasites that could be transmitted by a wide range of tick vectors. It is possible that genetic recombination during sexual reproduction in the tick vectors has led to the greater degree of sequence diversity of the 18S rRNA gene in equine parasites within this geographical region.

While the 18S rRNA gene sequences we have obtained in this study group most closely together with other *T. equi* and *B. caballi* sequences, some distinct and well-characterized species (e.g. *T. parva* and *T. annulata*) show higher levels of identity across their 18S rRNA sequences than the range for the two single equine species reported in this paper. We cannot rule out the possibility that the different groups that we have identified here represent different parasite species, although it is not possible to use 18S rRNA gene sequence variation alone to classify organisms as different species (Chae et al., 1999). However, all the field samples for which sequence data was obtained in this study were seropositive for either *T. equi* or *B. caballi*, although it is possible that this was due to previous infection or co-infection or cross-reactivity between closely related species.

The existence of sequence variation in the rRNA genes of the parasites that cause equine piroplasmosis serves as a note of caution to researchers wishing to use rRNA genes for diagnostic purposes. Prior to the development of a molecular diagnostic test, it is recommended that a thorough survey should be carried out to assess levels of intraspecific sequence divergence in the genetic marker/s employed in the assay.

In conclusion, we have discovered extensive sequence variation in the 18S rRNA gene within *T. equi*, and have identified three distinct genetic groups of *T. equi* in South Africa. There was less sequence variation within *B. caballi* in this gene although fewer *B. caballi* samples were examined as there are fewer cases of *B. caballi* infections in South Africa (de Waal, 1990). The findings reported here show that careful examination of variants of the 18S rRNA gene of *T. equi* and *B. caballi* is required in order to identify regions that are both conserved within and unique to each species, before this gene can be considered as a target for the development of molecular diagnostic tests to detect these parasites in horses.

2.6 Reference List

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