Molecular characterization of *Babesia caballi* and *Theileria equi*, the aetiological agents of equine piroplasmosis, in South Africa

By

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Dedicated to the memory of my late father,

Vasantrai Bhoora

DECLARATION

I declare that the thesis, which I hereby submit for the degree Philosophiae Doctor at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Raksha Bhoora
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THESIS SUMMARY

In an attempt to develop quantitative real-time PCR (qPCR) assays for the detection of equine piroplasms, sequence heterogeneity in the V4 hypervariable region of the 18S ribosomal RNA (rRNA) gene sequences within both *Theileria equi* and *Babesia caballi* from South Africa was discovered. A molecular epidemiological survey of the protozoal parasites that cause equine piroplasmosis was therefore carried out using horse and zebra samples from different geographical locations around South Africa.

We evaluated the ability of a recently developed *T. equi*-specific qPCR assay in detecting all *T. equi* 18S rRNA variants identified in South Africa. We further present the first report on the development and application of a TaqMan minor groove binder (MGB™) qPCR assay, targeting the 18S rRNA gene, for the detection of *B. caballi* infections in equine blood samples.

Despite the ability of the 18S rRNA *T. equi*- and *B. caballi*-specific qPCR assays to detect all known 18S rRNA gene sequence variants thus far identified in South Africa, the existence of as yet undetected variants in the field cannot be overlooked. Other qPCR assays targeting alternative genes could be developed which, used in conjunction with the 18S rRNA qPCR assays, may provide better confirmation of test results. A *T. equi*-specific qPCR assay targeting the equi merozoite antigen gene (*ema-1*) was recently developed for the detection of *T. equi* parasites in the midgut of *Rhipicephalus (Boophilus) microplus* nymphs. This assay was not able to detect *T. equi* in all South African samples that were confirmed positive by other molecular and serological assays. Sequence characterization of the *ema-1* gene from South African isolates revealed the existence of variation in the regions where the qPCR primers and probes had been designed. Based on these observations, a conserved region of the *ema-1* gene was selected and targeted in the development of an *ema-1*-specific TaqMan MGB™ qPCR assay, which was shown to have a higher sensitivity than the previously reported *ema-1* qPCR assay.

The rhoptry-associated protein (*rap-1*) gene from South African *B. caballi* isolates was also characterized following the failure of a *B. caballi*-specific competitive-inhibition enzyme-linked immunosorbent assay (cELISA) to detect *B. caballi* antibody in the sera of infected horses from South Africa. The genome walking PCR technique was used to amplify the complete *rap-1* gene sequence from two South African *B. caballi* isolates. Significant heterogeneity in the *rap-1* gene sequences and in the predicted amino acid sequences was found. Marked amino acid sequence differences in the carboxy-terminal region, and therefore the probable absence of the monoclonal antibody binding site, explains the failure of the cELISA to detect antibody to *B. caballi* in sera of infected horses in South Africa.

This is the first comprehensive molecular study of the parasites that cause equine piroplasmosis in South Africa. Our results add further to the existing knowledge of piroplasmosis worldwide and will be invaluable in the development of further molecular or serological diagnostic assays.

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

Acici, M., Umur, S., Guvenc, T., Arslan, H.H., Kurt, M., 2008. Seroprevalence of equine babesiosis in the Black Sea region of Turkey. Parasitology International 57, 198-200.

Alhassan, A., Pumidonming, W., Okamura, M., Hirata, H., Battsetseg, B., Fujisaki, K., Yokoyama, N., Igarashi, I., 2005. Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood. Veterinary Parasitology 129, 43-49.

Alhassan, A., Thekisoe, O.M.M., Yokoyama, N., Inoue, N., Motloang, M.Y., Mbati, P.A., Yin, H., Katayama, Y., Anzai, T., Sugimoto, C., Igarashi, I., 2007. Development of loop-mediated isothermal amplification (LAMP) method for diagnosis of equine piroplasmosis. Veterinary Parasitology 143, 155-160.

Allsopp, B.A., Baylis, H.A., Allsopp, M.T.E.P., Cavalier-Smith, T., Bishop, R.P., Carrington, D.M., Sohanpal, B., Spooner, P., 1993. Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences. Parasitology 107, 157-165.

Allsopp, M.T.E.P., Lewis, B.D., Penzhorn, B.L., 2007. Molecular evidence for transplacental transmission of *Theileria equi* from carrier mares to their apparently healthy foals. Veterinary Parasitology 148, 130-136.

Ambawat, H.K., Malhotra, D.V., Kumar, S., Dhar, S., 1999. Erythrocyte associated haemato-biochemical changes in *Babesia equi* infection experimentally produced in donkeys. Veterinary Parasitology 85, 319-324.

Anonymous, 2008. Equine piroplasmosis. In: OIE Manual of diagnostic tests and vaccines for terrestrial animals. Office International des Epizooties, Paris, France, pp. 884-893.

Asgarali, Z., Coombs, D.K., Mohammed, F., Campbell, M.D., Caesar, E., 2007. A serological study of *Babesia caballi* and *Theileria equi* in Thoroughbreds in Trinidad. Veterinary Parasitology 144, 167-171.

Avarzed, A., de Waal, D.T., Igarashi, I., Saito, A., Oyamada, T., Toyoda, Y., Suzuki, N., 1997. Prevalence of equine piroplasmosis in Central Mongolia. Onderstepoort Journal of Veterinary Research 64, 141-145.

Bashiruddin, J.B., Camma, C., Rebelo, E., 1999. Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. Veterinary Parasitology 84, 75-83.

Battsetseg, B., Lucero, S., Xuan, X., Claveria, F.G., Inoue, N., Alhassan, A., Kanno, T., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K., 2002. Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. Veterinary Parasitology 107, 351-357.

Belloli, C., Crescenzo, G., Lai, O., Carofiglio, V., Marang, O., Ormas, P., 2002. Pharmacokinetics of imidocarb dipropionate in horses after intramuscular administration. Equine Veterinary Journal 34, 625-629.

Boldbaatar, D., Xuan, X., Battsetseg, B., Igarashi, I., Battur, B., Batsukh, Z., Bayambaa, B., Fujisaki, K., 2005. Epidemiological study of equine piroplasmosis in Mongolia. Veterinary Parasitology 127, 29-32.

Böse, R., Jorgensen, W.K., Dalgliesh, R.J., Friedhoff, K.T., de Vos, A.J., 1995. Current state and future trends in the diagnosis of babesiosis. Veterinary Parasitology 57, 61-74.

Brüning, A., 1996. Equine piroplasmosis an update on diagnosis, treatment and prevention. British Veterinary Journal 152, 139-151.

Buling, A., Criado-Fornelio, A., Asenzo, G., Benitez, D., Barba-Carretero, J.C., Florin-Christensen, M., 2007. A quantitative PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina*. Veterinary Parasitology 147, 16-25.

Butler, C.M., Nijhof, A.M., van der Kolk, J.H., de Haseth, O.B., Taoufik, A., Jongejan, F., Houwers, D.J., 2008. Repeated high dose imidocarb dipropionate treatment did not eliminate *Babesia caballi* from naturally infected horses as determined by PCR-reverse line blot hybridization. Veterinary Parasitology 151, 320-322.

Camacho, A.T., Guitian, F.J., Pallas, E., Gestal, J.J., Olmeda, A.S., Habela, M.A., Telford, S.R., III, Spielman, A., 2005. *Theileria* (*Babesia*) *equi* and *Babesia caballi* infections in horses in Galicia, Spain. Tropical Animal Health and Production 37, 293-302.

Carelli, G., Decaro, N., Lorusso, A., Elia, G., Lorusso, E., Mari, V., Ceci, L., Buonavoglia, C., 2007. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. Veterinary Microbiology 124, 107-114.

Criado-Fornelio, A., Gonzalez-del-Rio, M.A., Buling-Sarana, A., Barba-Carretero, J.C., 2004. The "expanding universe" of piroplasms. Veterinary Parasitology 119, 337-345.

d'Oliveira, C., Tijhaar, E.J., Shiels, B.R., van der Weide, M., Jongejan, F., 1996. Expression of genes encoding two major *Theileria annulata* merozoite surface antigens in *Escherichia coli* and a *Salmonella typhimurium aroA* vaccine strain. Gene 172, 33-39.

Dalrymple, B.P., Casu, R.E., Peters, J.M., Dimmock, C.M., Gale, K.R., Boese, R., Wright, I.G., 1993. Characterisation of a family of multi-copy genes encoding rhoptry protein homologues in *Babesia bovis*, *Babesia ovis* and *Babesia canis*. Molecular and Biochemical Parasitology 57, 181-192.

de Waal, D.T., 1990. The transovarial transmission of *Babesia caballi* by *Hyalomma truncatum*. Onderstepoort Journal of Veterinary Research 57, 99-100.

de Waal, D.T., 1992. Equine piroplasmosis: a review. British Veterinary Journal 148, 6-14.

de Waal, D.T., Potgieter, F.T., 1987. The transstadial transmission of *Babesia caballi* by *Rhipicephalus evertsi evertsi*. Onderstepoort Journal of Veterinary Research 54, 655-666.

de Waal, D.T., van Heerden, J., 2004. Equine Piroplasmosis. In: Coetzer, J.A.W., Tustin, R.C. (Eds.), Infectious Diseases of Livestock (Second Edition). Oxford University Press, New York, pp 425-433.

de Waal, D.T., van Heerden, J., Potgieter, F.T., 1987. An investigation into the clinical pathological changes and serological response in horses experimentally infected with *Babesia equi* and *Babesia caballi*. Onderstepoort Journal of Veterinary Research 54, 561-568.

Du Plessis, J.L., Basson, P.A., 1966. Babesiosis in aborted equine foetuses: a report on two cases in South Africa. Journal of the South African Veterinary Medical Association 37, 267-269.

Erbsloh, J.K.E., 1975. Babesiosis in the newborn foal. Journal of Reproduction and Fertility Supplement 23, 725-726.

Friedhoff, K.T., Tenter, A.M., Muller, I., 1990. Haemoparasites of equines: impact on international trade of horses. Revue Scientifique et Technique 9, 1187-1194.

Georges, K., Loria, G.R., Riili, S., Greco, A., Caracappa, S., Jongejan, F., Sparagano, O., 2001. Detection of haemoparasites in cattle by reverse line blot hybridisation with a note on the distribution of ticks in Sicily. Veterinary Parasitology 99, 273-286.

Gerstenberg, C., Allen, W.R., Phipps, L.P., 1998. Mechanical transmission of *Babesia equi* infection in a British herd of horses. In: Wernery, U., Wade, J.F., Mumford, J.A., Kaaden, O.R. (Eds.), Equine infectious diseases VIII, Proceedings of the Eighth International Conference, Dubai, R&W Publications, Newmarket, UK, pp 217-222.

Geysen, D., Delespaux, V., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Veterinary Parasitology 110, 171-180.

Gubbels, J.M., de Vos, A.P., van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. Journal of Clinical Microbiology 37, 1782-1789.

Guimaraes, A.M., Lima, J.D., Ribeiro, M.F., 1998. Sporogony and experimental transmission of *Babesia equi* by *Boophilus microplus*. Parasitology Research 84, 323-327.

Hailat, N.Q., Lafi, S.Q., Al-Darraji, A.M., Al-Ani, F.K., 1997. Equine babesiosis associated with strenuous exercise: clinical and pathological studies in Jordan. Veterinary Parasitology 69, 1-8.

Hanafusa, Y., Cho, K.O., Kanemaru, T., Wada, R., Sugimoto, C., Onuma, M., 1998. Pathogenesis of *Babesia caballi* infection in experimental horses. Journal of Veterinary Medical Science 60, 1127-1132.

Heim, A., Passos, L.M.F., Ribeiro, M.F.B., Costa-Junior, L.M., Bastos, C.V., Cabral, D.D., Hirzmann, J., Pfister, K., 2007. Detection and molecular characterization of *Babesia caballi* and *Theileria equi* isolates from endemic areas of Brazil. Parasitology Research 102, 63-68.

Henning, M.W., 1956. Animal diseases in South Africa. Central News Agency, Ltd, South Africa.

Holman, P.J., Chieves, L., Frerichs, W.M., Olson, D., Wagner, G.G., 1994. *Babesia equi* erythrocytic stage continuously cultured in an enriched medium. Journal of Parasitology 80, 232-236.

Holman, P.J., Frerichs, W.M., Chieves, L., Wagner, G.G., 1993. Culture confirmation of the carrier status of *Babesia caballi*-infected horses. Journal of Clinical Microbiology 31, 698-701.

Homer, M.J., Aguilar-Delfin, I., Telford, S.R., Krause, P.J., Persing, D.H., 2000. Babesiosis. Clinical Microbiology Reviews 13, 451-469.

Hutcheon, D., 1890. Biliary fever or jaundice in the horse. Agricultural Journal of the Cape of Good Hope 2, 361-362.

Ikadai, H., Xuan, X., Igarashi, I., Tanaka, S., Kanemaru, T., Nagasawa, H., Fujisaki, K., Suzuki, N., Mikami, T., 1999. Cloning and expression of a 48-kilodalton *Babesia caballi* merozoite rhoptry protein and potential use of the recombinant antigen in an enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 37, 3475-3480.

Irby, J.R., 2002. Anthrax, screwworms, and equine piroplasmosis-subdued but not eradicated. American Association of Equine Practitioners Proceedings 48, 12-15.

Jefferies, R., Ryan, U.M., Irwin, P.J., 2007. PCR-RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies. Veterinary Parasitology 144, 20-27.

Jeong, W., Kweon, C., Kang, S., Paik, S., 2003. Diagnosis and quantification of *Theileria sergenti* using TaqMan PCR. Veterinary Parasitology 111, 287-295.

Kappmeyer, L.S., Perryman, L.E., Hines, S.A., Baszler, T.V., Katz, J.B., Hennager, S.G., Knowles, D.P., 1999. Detection of equine antibodies to *Babesia caballi* by recombinant *B. caballi* rhoptry-associated protein 1 in a competitive-inhibition enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 37, 2285-2290.

Karatepe, B., Karatepe, M., Çakmak, A., Karaer, Z., Ergün, G., 2009. Investigation of seroprevalence of *Theileria equi* and *Babesia caballi* in horses in Nigde province, Turkey. Tropical Animal Health and Production 41, 109-113.

Katende, J.M., Goddeeris, B.M., Morzaria, S.P., Nkonge, C.G., Musoke, A.J., 1990. Identification of a *Theileria mutans*-specific antigen for use in an antibody and antigen detection ELISA. Parasite Immunology 12, 419-433.

Kawazu, S.I., Sugimoto, C., Kamio, T., Fujisaki, K., 1992. Analysis of the genes encoding immunodominant piroplasm surface proteins of *Theileria sergenti* and *Theileria buffeli* by nucleotide sequencing and polymerase chain reaction. Molecular and Biochemical Parasitology 56, 169-175.

Kim, C.M., Blanco, L.B.C., Alhassan, A., Iseki, H., Yokoyama, N., Xuan, X., Igarashi, I., 2008. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. Veterinary Parasitology 151, 158-163.

Knowles, D.P., Kappmeyer, L.S., Stiller, D., Hennager, S.G., Perryman, L.E., 1992. Antibody to a recombinant merozoite protein epitope identifies horses infected with *Babesia equi*. Journal of Clinical Microbiology 30, 3122-3126.

Knowles, D.P., Perryman, L.E., Goff, W.L., Miller, C.D., Harrington, R.D., Gorham, J.R., 1991. A monoclonal antibody defines a geographically conserved surface protein epitope of *Babesia equi* merozoites. Infection and Immunity 59, 2412-2417.

Koch, R., 1904. Rhodesian investigations. Cape Agricultural Journal 24, 33-45.

Kuttler, K.L., Zaugg, J.L., Gipson, C.A., 1987. Imidocarb and parvaquone in the treatment of piroplasmosis (*Babesia equi*) in equids. American Journal of Veterinary Research 48, 1613-1616.

Laveran, A., 1901. Contribution a' l'etude du Piroplasma equi. Societe de Biologie et de ses Filiales 53, 385-388.

Levine, N.D., 1985. Apicomplexa: The Piroplasms. In: Veterinary Protozoology. Iowa State Universty Press, Ames, Iowa, pp. 291-328.

Lindh, M., Gorander, S., Andersson, E., Horal, P., Mattsby-Balzer, I., Ryd, W., 2007. Real-time Taqman PCR targeting 14 human papilloma virus types. Journal of Clinical Virology 40, 321-324.

Machado, R.Z., McElwain, T.F., Pancracio, H.P., Freschi, C.R., Palmer, G.H., 1999. *Babesia bigemina*: Immunization with purified rhoptries induces protection against acute parasitemia. Experimental Parasitology 93, 105-108.

Martin, R., 1999. Equine piroplasmosis: the temporary importation of seropositive horses into Australia. Australian Veterinary Journal 77, 308-309.

Matsuba, T., Sugimoto, C., Hattori, M., Sako, Y., Fujisaki, K., Onuma, M., 1995. Expression of a 32 kilodalton *Theileria sergenti* piroplasm surface protein by recombinant baculoviruses. International Journal for Parasitology 25, 939-943.

Mehlhorn, H., Schein, E., 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. Parasitology Research 84, 467-475.

Moltmann, U.G., Mehlhorn, H., Schein, E., Voigt, W.P., Friedhoff, K.T., 1983. Ultrastructural study on the development of *Babesia equi* (Coccidia: Piroplasmia) in the salivary glands of its vector ticks. Journal of Protozoology 30, 218-225.

Nagore, D., Garcia-Sanmartin, J., Garcia-Perez, A.L., Juste, R.A., Hurtado, A., 2004a. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: epidemiological survey and phylogenetic analysis. Veterinary Parasitology 123, 41-54.

Nagore, D., Garcia-Sanmartin, J., Garcia-Perez, A.L., Juste, R.A., Hurtado, A., 2004b. Identification, genetic diversity and prevalence of *Theileria* and *Babesia* species in a sheep population from Northern Spain. International Journal for Parasitology 34, 1059-1067.

Nicolaiewsky, T.B., Richter, M.F., Lunge, V.R., Cunha, C.W., Delagostin, O., Ikuta, N., Fonseca, A.S., da Silva, S.S., Ozaki, L.S., 2001. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. Veterinary Parasitology 101, 9-21.

Nijhof, A.M., Pillay, V., Steyl, J., Prozesky, L., Stoltsz, W.H., Lawrence, J.A., Penzhorn, B.L., Jongejan, F., 2005. Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. Journal of Clinical Microbiology 43, 5907-5911.

Norval, R.A.I., Horak, I.G., 2004. Vectors: Ticks. In: Coetzer, J.A.W., Tustin, R.C. (Eds.), Infectious diseases of Livestock (Second Edition). Oxford University Press, New York, pp 3-42.

Nunn, J.A., 1894. Specific fevers of malarial origin in equines. Veterinary Journal 39, 402-405.

Nuttall, G.H.F., Strickland, C., 1910. Die Parasiten des Pferdepiroplasmose resp. der 'Bilary fever'. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten Abteilung I 56, 524-525.

O'Grady, J., Sedano-Balbas, S., Maher, M., Smith, T., Barry, T., 2008. Rapid real-time PCR detection of *Listeria monocytogenes* in enriched food samples based on the ssrA gene, a novel diagnostic target. Food Microbiology 25, 75-84.

Phipps, L.P., Otter, A., 2004. Transplacental transmission of *Theileria equi* in two foals born and reared in the United Kingdom. Veterinary Record 154, 406-408.

Posnett, E.S., Ambrosio, R.E., 1989. Repetitive DNA probes for the detection of *Babesia equi*. Molecular and Biochemical Parasitology 34, 75-78.

Posnett, E.S., Ambrosio, R.E., 1991. DNA probes for the detection of *Babesia caballi*. Parasitology 102, 357-65.

Posnett, E.S., Fehrsen, J., de Waal, D.T., Ambrosio, R.E., 1991. Detection of *Babesia equi* in infected horses and carrier animals using a DNA probe. Veterinary Parasitology 39, 19-32.

Rampersad, J., Cesar, E., Campbell, M.D., Samlal, M., Ammons, D., 2003. A field evaluation of PCR for the routine detection of *Babesia equi* in horses. Veterinary Parasitology 114, 81-87.

Sahagun-Ruiz, A., Waghela, S.D., Holman, P.J., Chieves, L.P., Wagner, G.G., 1997. Biotin-labeled DNA probe in a PCR-based assay increases detection sensitivity for the equine hemoparasite *Babesia caballi*. Veterinary Parasitology 73, 53-63.

Salim, B.O.M., Hassan, S.M., Bakheit, M.A., Alhassan, A., Igarashi, I., Karanis, P., Abdelrahman, M.B., 2008. Diagnosis of *Babesia caballi* and *Theileria equi* infections in horses in Sudan using ELISA and PCR. Parasitology Research 103, 1145-1150.

Sam-Yellowe, T.Y., 1996. Rhoptry organelles of the apicomplexa: Their role in host cell invasion and intracellular survival. Parasitology Today 12, 308-316.

Sevinc, F., Maden, M., Kumas, C., Sevinc, M., Ekici, O.D., 2008. A comparative study on the prevalence of *Theileria equi* and *Babesia caballi* infections in horse sub-populations in Turkey. Veterinary Parasitology 156, 173-177.

Shaw, M.K., 2003. Cell invasion by *Theileria* sporozoites. Trends in Parasitology 19, 2-6.

Shiels, B.R., d'Oliveira, C., McKellar, S., Ben-Miled, L., Kawazu, S.i., Hide, G., 1995. Selection of diversity at putative glycosylation sites in the immunodominant merozoite/piroplasm surface antigen of *Theileria* parasites. Molecular and Biochemical Parasitology 72, 149-162.

Shkap, V., Cohen, I., Leibovitz, B., Savitsky, Pipano, E., Avni, G., Shofer, S., Giger, U., Kappmeyer, L., Knowles, D.P., 1998. Seroprevalence of *Babesia equi* among horses in Israel using competitive inhibition ELISA and IFA assays. Veterinary Parasitology 76, 251-259.

Sibeko, K.P., Oosthuizen, M.C., Collins, N.E., Geysen, D., Rambritch, N.E., Latif, A.A., Groeneveld, H.T., Potgieter, F.T., Coetzer, J.A.W., 2008. Development and evaluation of a real-time polymerase chain reaction test for the detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer)* and cattle. Veterinary Parasitology 155, 37-48.

Skuce, P.J., Mallon, T.R., Taylor, S.M., 1996. Molecular cloning of a putative rhoptry associated protein homologue from *Babesia divergens*. Molecular and Biochemical Parasitology 77, 99-102.

Sluyter, F.J., 2001. Traceability of Equidae: a population in motion. Revue Scientifique et Technique 20, 500-509.

Suarez, C.E., Palmer, G.H., Florin-Christensen, M., Hines, S.A., Hötzel, I., McElwain, T.F., 2003. Organization, transcription, and expression of rhoptry associated protein genes in the *Babesia bigemina rap-1* locus. Molecular and Biochemical Parasitology 127, 101-112.

Suarez, C.E., Palmer, G.H., Hötzel, I., Hines, S.A., McElwain, T.F., 1998. Sequence and functional analysis of the intergenic regions separating Babesial rhoptry-associated protein-1 (*rap-1*) genes. Experimental Parasitology 90, 189-194.

Theiler, A., 1901. Die Pferde-Malaria. Schweizer Archiv für Tierheilkunde 43, 253-280.

Ueti, M.W., Palmer, G.H., Kappmeyer, L.S., Scoles, G.A., Knowles, D.P., 2003. Expression of equi merozoite antigen 2 during development of *Babesia equi* in the midgut and salivary gland of the vector tick *Boophilus microplus*. Journal of Clinical Microbiology 41, 5803-5809.

Weiland, G., 1986. Species-specific serodiagnosis of equine piroplasma infections by means of complement fixation test (CFT), immunofluorescence (IIF), and enzyme-linked immunosorbent assay (ELISA). Veterinary Parasitology 20, 43-48.

Wengi, N., Willi, B., Boretti, F.S., Cattori, V., Riond, B., Meli, M.L., Reusch, C.E., Lutz, H., Hofmann-Lehmann, R., 2008. Real-time PCR-based prevalence study, infection follow-up and molecular characterization of canine hemotropic mycoplasmas. Veterinary Microbiology 126, 132-141.

Xuan, X., Larsen, A., Ikadai, H., Tanaka, T., Igarashi, I., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., 2001. Expression of *Babesia equi* merozoite antigen 1 in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 39, 705-709.

Yin, H., Lu, W., Luo, J., 1997. Babesiosis in China. Tropical Animal Health and Production 29, 11S-15S.

Yokoyama, N., Okamura, M., Igarashi, I., 2006. Erythrocyte invasion by *Babesia* parasites: Current advances in the elucidation of the molecular interactions between the protozoan ligands and host receptors in the invasion stage. Veterinary Parasitology 138, 22-32.

Young, A.S., Purnell, R.E., 1973. Observations on *Babesia equi* in the salivary glands of *Rhipicephalus evertsi*. Bulletin of Epizootic Diseases of Africa 21, 377-383.

Zweygarth, E., Just, M.C., de Waal, D.T., 1997. *In vitro* cultivation of *Babesia equi:* detection of carrier animals and isolation of parasites. Onderstepoort Journal of Veterinary Research 64, 51-56.

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.

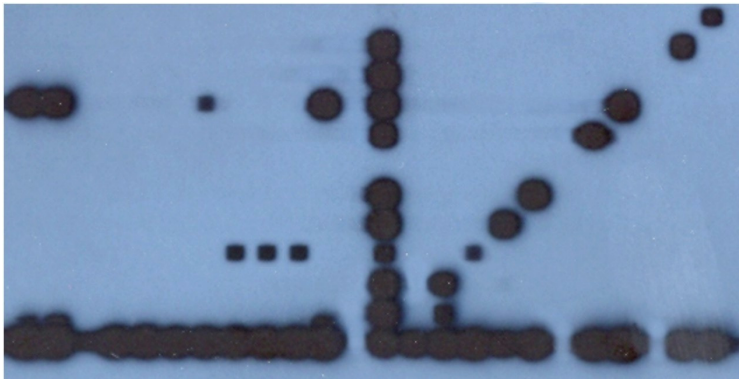


Figure 2.1 Reverse line blot hybridization of *Theileria* and *Babesia* PCR products amplified from genomic DNA extracted from infected equine blood samples. Genus- and species-specific oligonucleotide probes were applied in horizontal rows as indicated. PCR products from samples and control clones were 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).

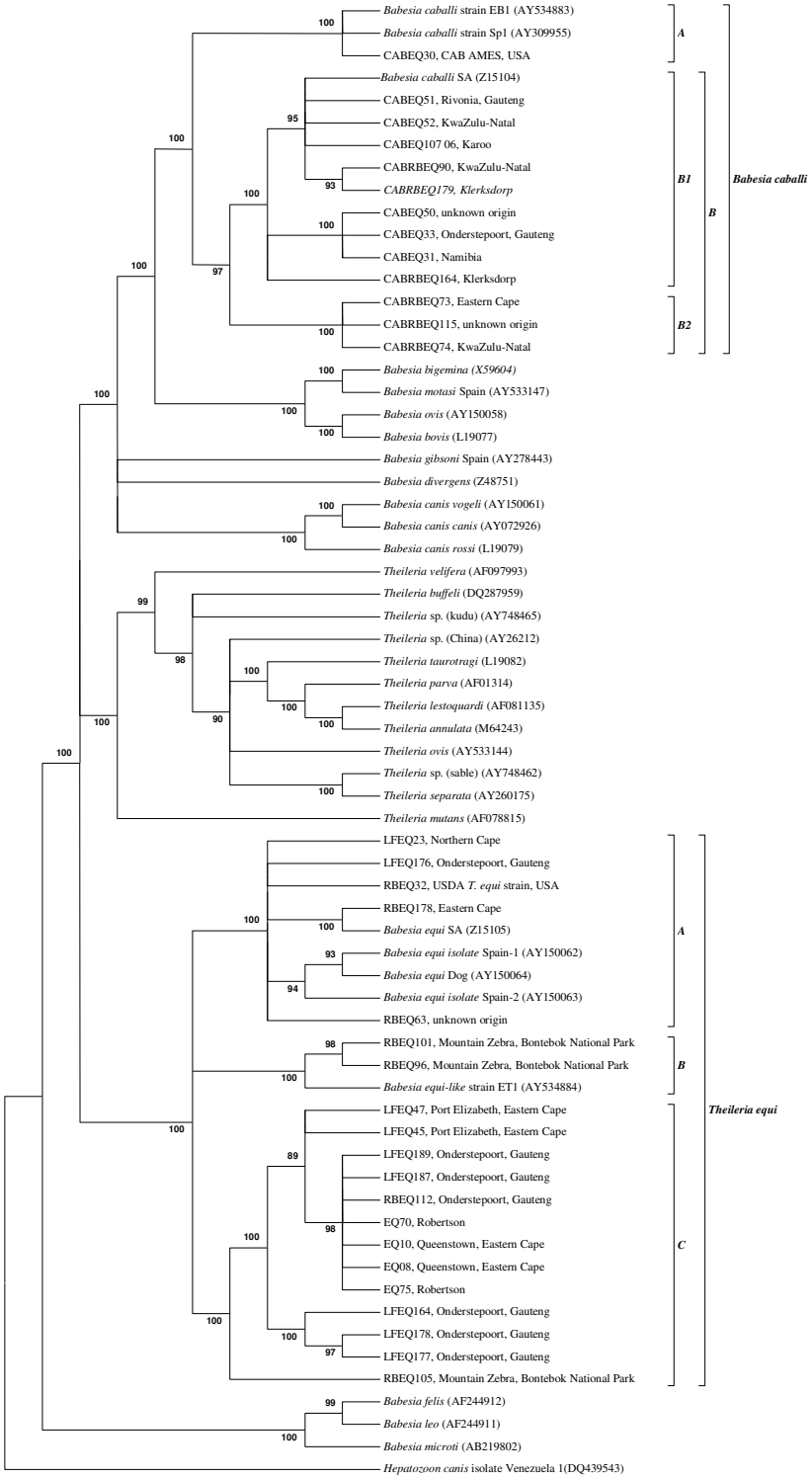


Figure 2.2 Rooted cladogram, determined with Bayesian inference and $5x10^6$ iterations, of the 18S rRNA gene sequences of *T. equi* and *B. caballi* samples identified in this study (square brackets) and *Theileria* and *Babesia* sequences from GenBank (accession numbers are indicated in parentheses).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

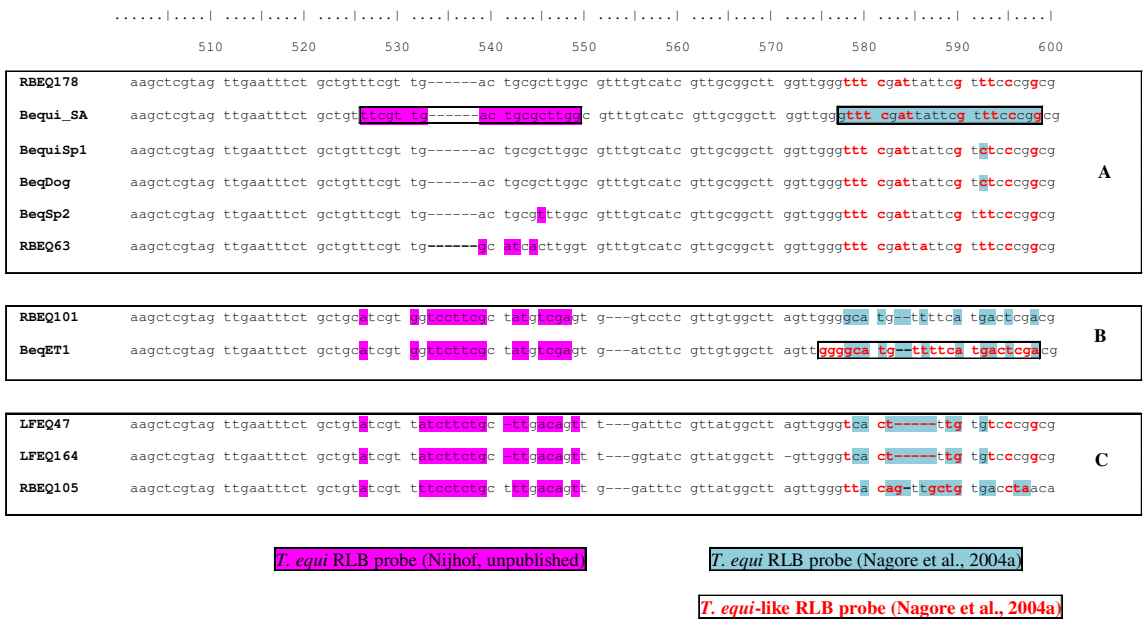


Figure 2.3 Nucleotide alignment of a section of the V4 hypervariable region of the 18S rRNA gene of the *T. equi* isolates examined in this study, to the published *T. equi* sequences (*B. equi* SA, Z15105; *B. equi* isolate Spain-1, AY150062; *B. equi* isolate Spain-2, AY150063; *B. equi* Dog, AY150064; *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

Alhassan, A., Iseki, H., Kim, C., Yokoyama, N., Igarashi, I., 2007. Comparison of polymerase chain reaction methods for the detection of *Theileria equi* infection using whole blood compared with pre-extracted DNA samples as PCR templates. Tropical Animal Health and Production 39, 369-374.

Allsopp, M.T.E.P., Allsopp, B.A., 2006. Molecular sequence evidence for the reclassification of some *Babesia* species. Annals of the New York Academy of Sciences 1081, 509-517.

Allsopp, M.T.E.P., Cavalier-Smith, T., de Waal, D.T., Allsopp. B.A., 1994. Phylogeny and evolution of the piroplasms. Parasitology 108, 147-152.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403-410.

Bashiruddin, J.B., Camma, C., Rebelo, E., 1999. Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. Veterinary Parasitology 84, 75-83.

Battsetseg, B., Lucero, S., Xuan, X., Claveria, F.G., Inoue, N., Alhassan, A., Kanno, T., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K., 2002. Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. Veterinary Parasitology 107, 351-357.

Brayton, K.A., Lau, A.O., Herndon, D.R., Hannick, L., Kappmeyer, L.S., Berens, S.J., Bidwell, S.L., Brown, W.C., Crabtree, J., Fadrosh, D., Feldblum, T., Forberger, H.A., Haas, B.J., Howell, J.M., Khouri, H., Koo, H., Mann, D.J., Norimine, J., Paulsen, I.T., Radune, D., Ren, Q., Smith, R.K. Jr, Suarez, C.E., White, O., Wortman, J.R., Knowles, D.P. Jr, McElwain, T.F., Nene, V.M., 2007. Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. PLoS Pathogens 3, 1401-1413.

Brüning, A., Phipps, P., Posnett, E., Canning, E.U., 1997. Monoclonal antibodies against *Babesia caballi* and *Babesia equi* and their application in serodiagnosis. Veterinary Parasitology 68, 11-26.

Buling, A., Criado-Fornelio, A., Asenzo, G., Benitez, D., Barba-Carretero, J.C., Florin-Christensen, M., 2007. A quantitative PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina*. Veterinary Parasitology 147, 16-25.

Butler, C.M., Nijhof, A.M., Jongejan, F., van der Kolk, J.H., 2008. *Anaplasma phagocytophilum* infection in horses in the Netherlands. Veterinary Record 162, 216-218.

Caccio, S., Camma, C., Onuma, M., Severini, C., 2000. The beta-tubulin gene of *Babesia* and *Theileria* parasites is an informative marker for species discrimination. International Journal for Parasitology 30, 1181-1185.

Calder, J.A.M., Reddy, G.R., Chieves, L., Courtney, C.H., Littell, R., Livengood, J.R., Norval, R.A.I., Smith, C., Dame, J.B., 1996. Monitoring *Babesia bovis* infections in cattle by using PCR-based tests. Journal of Clinical Microbiology 34, 2748-2755.

Chae, J.S., Allsopp, B.A., Waghela, S.D., Park, J.H., Kakuda, T., Sugimoto, C., Allsopp, M.T.E.P., Wagner, G.G., Holman, P.J., 1999. A study of the systematics of *Theileria* spp. based upon small-subunit ribosomal RNA gene sequences. Parasitology Research 85, 877-883.

Chae, J.S., Lee, J.M., Kwon, O.D., Holman, P.J., Waghela, S.D., Wagner, G.G., 1998. Nucleotide sequence heterogeneity in the small subunit ribosomal RNA gene variable (V4) region among and within geographic isolates of *Theileria* from cattle, elk and white-tailed deer. Veterinary Parasitology 75, 41-52.

Collins, N.E., Allsopp, B.A., 1999. *Theileria parva* ribosomal internal transcribed spacer sequences exhibit extensive polymorphism and mosaic evolution: application to characterization of parasites from cattle and buffalo. Parasitology 118, 541-551.

Criado, A., Martinez, J., Buling, A., Barba, J.C., Merino, S., Jefferies, R., Irwin, P.J., 2006. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. Veterinary Parasitology 142, 238-247.

Criado-Fornelio, A., Gonzalez-del-Rio, M.A., Buling-Sarana, A., Barba-Carretero, J.C., 2004. The "expanding universe" of piroplasms. Veterinary Parasitology 119, 337-345.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carretero, J.C., 2003. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part I. Epizootiological aspects. Veterinary Parasitology 113, 189-201.

de Waal, D.T., 1990. The transovarial transmission of *Babesia caballi* by *Hyalomma truncatum*. Onderstepoort Journal of Veterinary Research 57, 99-100.

de Waal, D.T., 1992. Equine piroplasmosis: a review. British Veterinary Journal 148, 6-14.

de Waal, D.T. and van Heerden, J., 2004. Equine piroplasmosis. In: Coetzer, J.A.W and Tustin, R.C. (Eds.), Infectious Diseases of Livestock (Second Edition). Oxford University Press, New York, pp 425-434.

Eickbush, T.H., Eickbush, D.G., 2007. Finely orchestrated movements: evolution of the ribosomal RNA genes. Genetics 175, 477–485.

Georges, K., Loria, G.R., Riili, S., Greco, A., Caracappa, S., Jongejan, F., Sparagano, O., 2001. Detection of haemoparasites in cattle by reverse line blot hybridisation with a note on the distribution of ticks in Sicily. Veterinary Parasitology 99, 273-286.

Geysen, D., Delespaux, V., Geerts, S., 2003. PCR-RFLP using SSU-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Veterinary Parasitology 110, 171-180.

Gubbels, J.M., de Vos, A.P., van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. Journal of Clinical Microbiology 37, 1782-1789.

Gunderson, J.H., Sogin, M.L., Wollet, G., Hollingdale, V.F., de la Cruz, V.F., Waters, A.P., McCutchan, T.F., 1987. Structurally distinct, stage-specific ribosomes occur in *Plasmodium*. Science 238, 933-937.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95-98.

Hanafusa, Y., Cho, K.O., Kanemaru, T., Wada, R., Sugimoto, C., Onuma, M., 1998. Pathogenesis of *Babesia caballi* infection in experimental horses. Journal of Veterinary Medical Science 60, 1127-1132.

Heim, A., Passos, L.M.F., Ribeiro, M.F.B., Costa-Junior, L.M., Bastos, C.V., Cabral, D.D., Hirzmann, J., Pfister, K., 2007. Detection and molecular characterization of *Babesia caballi* and *Theileria equi* isolates from endemic areas of Brazil. Parasitology Research 102, 63-68.

Hunfeld, K.P., Hildebrandt, A., Gray, J.S., 2008. Babesiosis: recent insights into an ancient disease. International Journal for Parasitology 38, 1219-1237.

Ikadai, H., Osorio, C.R., Xuan, X., Igarashi, I., Kanemaru, T., Nagasawa, H., Fujisaki, K., Suzuki, N., Mikami, T., 2000. Detection of *Babesia caballi* infection by enzyme-linked immunosorbent assay using recombinant 48-kDa merozoite rhoptry protein. International Journal for Parasitology 30, 633-635.

Jefferies, R., Ryan, U.M., Irwin, P.J., 2007. PCR-RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies. Veterinary Parasitology 144, 20-27.

Kappmeyer, L.S., Perryman, L.E., Hines, S.A., Baszler, T.V., Katz, J.B., Hennager, S.G., Knowles, D.P., 1999. Detection of equine antibodies to *Babesia caballi* by recombinant *B. caballi* rhoptry-associated protein 1 in a competitive-inhibition enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 37, 2285-2290.

Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Research 33, 511-518.

Katzer, F., McKellar, S., Kirvar, E., Shiels, B., 1998. Phylogenetic analysis of *Theileria* and *Babesia equi* in relation to the establishment of parasite populations within novel host species and the development of diagnostic tests. Molecular and Biochemical Parasitology 95, 33-44.

Kim, C.M., Blanco, L.B.C., Alhassan, A., Iseki, H., Yokoyama, N., Xuan, X., Igarashi, I., 2008. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. Veterinary Parasitology 151, 158-163.

Knowles, D.P., Perryman, L.E., Kappmeyer, L.S., Hennager, S.G., 1991. Detection of equine antibody to *Babesia equi* merozoite proteins by a monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 29, 2056-2058.

Laughery, J.M., Lau, A.O.T., White, S.N., Howell, J.M., Suarez, C.E., 2009. *Babesia bovis*: Transcriptional analysis of rRNA gene unit expression. Experimental Parasitology 123, 45-50.

Matjila, P.T., Leisewitz, A.L., Oosthuizen, M.C., Jongejan, F., Penzhorn, B.L., 2008. Detection of a *Theileria* species in dogs in South Africa. Veterinary Parasitology 157, 34-40.

Mehlhorn, H., Schein, E., 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. Parasitology Research 84, 467-475.

Nagore, D., Garcia-Sanmartin, J., Garcia-Perez, A.L., Juste, R.A., Hurtado, A., 2004a. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: epidemiological survey and phylogenetic analysis. Veterinary Parasitology 123, 41-54.

Nagore, D., Garcia-Sanmartin, J., Garcia-Perez, A.L., Juste, R.A., Hurtado, A., 2004b. Identification, genetic diversity and prevalence of *Theileria* and *Babesia* species in a sheep population from Northern Spain. International Journal for Parasitology 34, 1059-1067.

Nicolaiewsky, T.B., Richter, M.F., Lunge, V.R., Cunha, C.W., Delagostin, O., Ikuta, N., Fonseca, A.S., da Silva, S.S., Ozaki, L.S., 2001. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. Veterinary Parasitology 101, 9-21.

Nijhof, A.M., Pillay, V., Steyl, J., Prozesky, L., Stoltsz, W.H., Lawrence, J.A., Penzhorn, B.L., Jongejan, F., 2005. Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. Journal of Clinical Microbiology 43, 5907-5911.

Oosthuizen, M.C., Zweygarth, E., Collins, N.E., Troskie, M., Penzhorn, B.L., 2008. Identification of a novel *Babesia* sp. from a sable antelope (*Hippotragus niger* Harris, 1838). Journal of Clinical Microbiology 46, 2247-2251.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Potgieter, F.T., de Waal, D.T., Posnett, E.S., 1992. Transmission and diagnosis of equine babesiosis in South Africa. Memórias do Instituto Oswaldo Cruz 87, 139-142.

Rampersad, J., Cesar, E., Campbell, M.D., Samlal, M., Ammons, D., 2003. A field evaluation of PCR for the routine detection of *Babesia equi* in horses. Veterinary Parasitology 114, 81-87.

Reddy, R.G., Chakrabarti, D., Yowell, C.A., Dame, J.B., 1991. Sequence microheterogeneity of the three small subunit ribosomal RNA genes of *Babesia bigemina*: expression in eukaryotic culture. Nucleic Acids Research 19, 3641-3645.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

Sibeko, K.P., Oosthuizen, M.C., Collins, N.E., Geysen, D., Rambritch, N.E., Latif, A.A., Groeneveld, H.T., Potgieter, F.T., Coetzer, J.A.W., 2008. Development and evaluation of a real-time polymerase chain reaction test for the detection of *Theileiria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle. Veterinary Parasitology 155, 37-48.

Staden, R., 1996. The Staden sequence analysis package. Molecular Biotechnology 5, 233-241.

Swofford, D. L., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4b10. Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution 24, 1596-1599.

Zweygarth, E., Just, M.C., de Waal, D.T., 1997. *In vitro* cultivation of *Babesia equi*: detection of carrier animals and isolation of parasites. Onderstepoort Journal of Veterinary Research 64, 51-56.

Zweygarth, E., Lopez-Rebollar, L.M., Meyer, P., 2002. *In vitro* isolation of equine piroplasms derived from Cape Mountain zebra (*Equus zebra zebra*) in South Africa. Onderstepoort Journal of Veterinary Research 69, 197-200.

CHAPTER 3

Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa

3.1 Abstract

A quantitative real-time polymerase chain reaction (qPCR) assay using a TaqMan minor groove binder (MGB™) probe was developed for the detection of *Babesia caballi* infection in equids from South Africa. Nine previously published sequences of the V4 hypervariable region of the *B. caballi* 18S rRNA gene were used to design primers and probes to target unique, conserved regions. The *B. caballi* TaqMan MGB™ qPCR assay was shown to be efficient and specific. The detection limit, defined as the concentration at which 95% of positive samples can be detected, was determined to be 1.14 x 10^{-4}% parasitized erythrocytes (PE). We further evaluated a previously reported *Theileria equi*-specific qPCR assay and showed that it was able to detect the 12 *T. equi* 18S rRNA sequence variants previously identified in South Africa. Both qPCR assays were tested on samples from two ponies experimentally infected with either *T. equi* or *B. caballi*. The qPCR assays were more sensitive than the indirect fluorescent antibody test (IFAT) and the reverse-line blot (RLB) during the early onset of the disease. The assays were subsequently tested on field samples collected from 41 horses, resident on three stud farms in the Northern Cape Province, South Africa. The IFAT detected circulating *T. equi* and *B. caballi* antibody in, respectively, 83% and 70% of the samples. The RLB detected *T. equi* parasite DNA in 73% of the samples, but none of the samples was positive for *B. caballi*, although 19 *T. equi*-positive samples also hybridized to the *Babesia* genus-specific probe. This could indicate a mixed *T. equi* and *B. caballi* infection in these samples, with either the *B. caballi* parasitaemia at a level below the detection limit of the *B. caballi* RLB probe, or the occurrence of a novel *Babesia* genotype or species. In contrast, the qPCR assays correlated fairly well with the IFAT. The *B. caballi* TaqMan MGB™ qPCR assay detected *B. caballi* parasite DNA in 78% of the samples. The *T. equi*-specific qPCR assay detected *T. equi* DNA in 80% of the samples. These results suggest that the qPCR assays are more sensitive than the RLB assay for the detection of *T. equi* and *B. caballi* infections in field samples.

3.2 Introduction

Babesia caballi and *Theileria equi* are haemoprotozoan parasites that cause equine piroplasmosis (Mehlhorn and Schein, 1998). The disease is of worldwide importance and occurs throughout the tropical and subtropical parts of the world with its prevalence being related to the distribution of its tick vectors (de Waal, 1992). Fourteen species of ixodid ticks of the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus* have been identified worldwide as vectors of either *T. equi* or *B. caballi* (de Waal, 1992).

Both parasites cause disease in equids, which may be either acute or chronic with mortalities ranging from less than 10% up to 50%. The disease is generally characterized by fever and anaemia. The clinical signs are often variable and non-specific, making it easy to confuse the disease with other conditions, therefore complicating diagnosis. It is also not possible to differentiate between *B. caballi* and *T. equi* infections based on clinical signs alone. Once infected, horses may remain life-long carriers of *T. equi* infections whereas with *B. caballi* infections, which are self-limiting, horses remain carriers for up to four years (de Waal and van Heerden, 2004).

The global transport of horses has led to the spread of equine piroplasmosis from its endemic tropical and subtropical zones to more temperate regions. Stringent regulatory import restrictions are in place in some countries to prevent the entrance of horses that are carriers of *B. caballi* and *T. equi* as they may act as reservoirs of infection (Friedhoff et al., 1990; Sluyter, 2001). Regulations often require the serological testing of horses in order to confirm seronegativity and to identify seropositive animals whose movement is restricted (Böse et al., 1995; Brüning, 1996). A variety of serological methods, which include the complement fixation (CF) test, the indirect fluorescent antibody test (IFAT) and the competitive-inhibition ELISA (cELISA), have been developed for the detection of specific antibodies (Donnelly et al., 1982; Weiland et al., 1984; Knowles et al., 1991; Böse et al., 1995; Brüning et al., 1997; Kappmeyer et al., 1999). The reliability of these serological assays is, however, restricted by antibody detection limits and cross-reactivity (Brüning et al., 1997).

Molecular-based diagnostic tests, which have higher sensitivities and specificities than serological tests, and which can detect samples with very low parasitaemias, have been developed. *Babesia caballi*- and *T. equi*-specific oligonucleotide probes based on sequence differences in the small subunit (18S) ribosomal RNA (rRNA) genes (Allsopp et al., 1994) were used in a preliminary study to demonstrate transplacental transmission of *T. equi* (Lewis et al., 1999). Further developments using 18S rRNA genes as target sequences include species-specific nested polymerase chain reaction (PCR) assays (Bashiruddin et al., 1999; Rampersad et al., 2003) and the reverse-line blot (RLB) assay, which allows for the identification of novel genotypes or species and also allows for the detection of mixed infections (Gubbels et al., 1999; Nagore et al., 2004; Bhoora et al., 2009; Chapter 2).

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 quantitative real-time polymerase chain reaction (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 mammalian host and in *Rhipicephalus (Boophilus) microplus* ticks (Ueti et al., 2003). A multiplex assay, using the *ema-1* qPCR assay 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).

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 *T. equi* DNA in infected equine samples. In the present study, we describe the development and application of a TaqMan minor groove binder (MGB™) qPCR assay, also based on the 18S rRNA gene, for detection of *B. caballi* in equine field blood samples. Furthermore, we evaluate the ability of the *T. equi*-specific qPCR assay to detect all *T. equi* 18S rRNA variants that have been shown to occur in South Africa (Bhoora et al., 2009; Chapter 2).

3.3 Materials and Methods

3.3.1 In vitro culture of South African B. caballi and T. equi isolates

The South African *B. caballi* 502 culture was initiated from blood samples collected from an infected horse at the National Yearling Sale in March 2000 (Zweygarth et al., 2002). In February 2006, blood collected from a *T. equi*-infected horse resident on a farm in Vastfontein, Onderstepoort, was used to culture the *T. equi* WL isolate used in this study. The *B. caballi* 502 and *T. equi* WL isolates were propagated in purified equine red blood cells using established culture systems previously described (Zweygarth et al., 1995; Zweygarth et al., 2002). The *in vitro* culture was performed at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI).

3.3.2 Infection trial

Experimental infections were approved by the Committee of Animal Welfare of the Faculty of Veterinary Medicine, Utrecht University. Blood stabilates of *T. equi* (strain Zaria) and the USDA strain of *B. caballi* (Kappmeyer et al., 1999) were used in the infection trial. *Theileria equi* (strain Zaria) was isolated in Nigeria in 1973. A blood stabilate was prepared from an infected horse, frozen and transported to the Faculty of Veterinary Medicine at Utrecht University in the Netherlands, where pony "Nico" was infected subcutaneously with the stabilate. Blood was collected and frozen 18 days later. Five years later (1978) Pony 187 was infected both subcutaneously and intramuscularly with the blood stabilate from pony "Nico". Blood was collected and frozen ten days post infection (p.i.) and stored in liquid nitrogen. This stabilate was used in the current investigation. A blood stabilate of the USDA strain of *B. caballi* was kindly supplied by Prof. Friedhoff from the Faculty of Veterinary Medicine, Hannover, Germany.

Two three-year-old, male Shetland ponies were housed together under tick-free conditions in the large-animal experimental facility, Utrecht University. Prior to infection both ponies were tested negative for both *T. equi* and *B. caballi* by PCR/RLB hybridization and IFAT. Before infection the ponies were immuno-suppressed by the administration of dexamethasone (Dexadresone, Intervet®, Boxmeer, The Netherlands) at a dose of 0.04 mg/kg body weight (BW) by intramuscular injection, three times: four and two days before infection and on the day of infection. Pony A, a stallion weighing 150 kg, was infected with *T. equi* (Zaria) blood stabilate by 2 ml intravenous and 2 ml subcutaneous inoculation. Pony B, a gelding weighing 104 kg, was infected with 1 ml of *B. caballi* (USDA) blood stabilate administered subcutaneously and 1 ml intravenously. The horses were monitored daily for clinical responses. General physical appearance, early morning rectal temperatures, pulse and respiratory rates were monitored, mucosal membranes were inspected and lymph nodes were checked for possible enlargement. Serum and EDTA blood samples were taken daily from the jugular vein of each pony from day 3 until day 52 p.i. Haematocrit readings were taken daily, and between 3000 and 5000 erythrocytes were examined in Giemsa-stained blood smears for the presence of parasites. Two hundred µl of blood was spotted in duplicate on FTA filter paper (Whatman®). Filter papers were sent to South Africa where DNA was extracted. On day 60 p.i. both ponies were euthanized using 20 ml pentobarbital (30%) intravenously.

3.3.3 Field samples

Serum and EDTA blood samples were collected from 41 horses of unknown piroplasm status, resident on three stud farms in the Northern Cape Province, South Africa, where tick-control measures were only implemented when high tick burdens were observed.

3.3.4 Indirect fluorescent antibody test (IFAT)

The IFAT was conducted at the ARC-OVI. A standard IFAT protocol, as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2004 (Anonymous, 2008) was used; antigens were locally produced in the Netherlands or South Africa. Bound equine antibodies were detected with fluorescein isothiocyanate-conjugated rabbit anti-horse immunoglobulin (RAHo/IgG(H+L)FITC, Nordic Immunology, Tilburg, the Netherlands) and examined in a wet mount by fluorescence microscopy.

3.3.5 DNA extraction

DNA was extracted from 200 µl of *in vitro* culture material or EDTA-anticoagulated blood using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that DNA was eluted in 100 µl. Six round holes, 3 mm in diameter, were punched out of a single blood spot stored on FTA paper (Whatman®), and DNA was extracted using the dried blood spot protocol from the QIAamp DNA mini kit (Qiagen, Hilden, Germany).

3.3.6 PCR amplification and reverse-line blot (RLB) hybridization

Primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3'), which are specific for *Theileria* and *Babesia* species, were used to amplify the V4 hypervariable region of the parasite 18S rRNA gene, as described by Nijhof et al. (2005). Samples were subjected to RLB hybridization as described previously (Bhoora et al., 2009; Chapter 2).

3.3.7 Design of a B. caballi-specific TaqMan® MGB™ qPCR assay

Six 18S rRNA gene sequences (GenBank Accession numbers: EU642512, EU642513, EU642514, EU888900, EU888901, EU888904), obtained from *B. caballi*-positive field samples in a previous study (Bhoora et al., 2009), along with three other previously published *B. caballi* 18S rRNA sequences, Z15104 (Allsopp et al., 1994), AY309955 (Criado-Fornelio et al., 2004) and AY534883 (Nagore et al., 2004), were used to develop the assay. A TaqMan minor groove binder (MGB™) probe qPCR assay was designed using the Primer express software v2.0 (Applied Biosystems). A primer pair, [Bc_18SF402: 5'-GTA ATT GGA ATG ATG GCG ACT TAA-3' and Bc_18SR496: 5'-CGC TAT TGG AGC TGG AAT TAC C-3' (IDT)], and a TaqMan® MGB™ probe [Bc_18SP: 5'-6-FAM-CCT CGC CAG AGT AA-MGB-3' (Applied Biosystems)], were designed to amplify and detect a 95 bp fragment in the V4 hypervariable region of the 18S rRNA gene. The TaqMan MGB™ probe was labeled with the fluorescent dye 6-carboxyfluorescein at the 5′ end and a non-fluorescent quencher at the 3′ end (Applied Biosystems). The forward primer and the probe are specific for *B. caballi*, but the reverse primer is not.

Real-time quantitative PCR was performed in MicroAmp optical 96-well reaction plates using the StepOnePlus™ Real-time PCR instrument (v. 2.0, Applied Biosystems). All qPCR assays were run in a total reaction volume of 20 µl comprising 1x TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 0.9 µM of each forward and reverse primer, 0.25 µM TaqMan MGB™ probe and 5 µl of target DNA. The qPCR cycling conditions were as follows: activation of the FastStart DNA polymerase at 95°C for 20 seconds, then 40 cycles of 1 second at 95 °C and 20 seconds at 60 °C.

3.3.8 Theileria equi-specific TaqMan qPCR assay

Primers Be18SF (5'-GCG GTG TTT CGG TGA TTC ATA-3') and Be18SR (5'-TGA TAG GTC AGA AAC TTG AAT GAT ACA TC-3') and the TaqMan probe, Be18SP (5'-6-VIC-AAA TTA GCG AAT CGC ATG GCT T-3'), previously designed for a *T. equi*-specific qPCR assay (Kim et al., 2008) were used. The qPCR was performed as described above.

3.3.9 *Efficiency, sensitivity and specificity of the qPCR assays*

The *in vitro*-cultured South African *B. caballi* 502 and *T. equi* WL isolates with percentage parasitized erythrocytes (PE) of approximately 8.1 (~7.29 x 10^5 parasites/µl) and 13.8 (~1.24 x 10^6 parasites/µl), respectively, were used in the generation of standard curves from which the efficiencies of the qPCR assays were determined. A ten-fold dilution series (10^0 to 10^{-7}) from each of the *in vitro*-cultured equine parasites was prepared in duplicate using a suspension of uninfected equine red blood cells. DNA was extracted from all diluted samples as described above and qPCR amplifications of both standard dilution series were repeated in triplicate and on ten separate occasions. The data generated from each of the runs were used to calculate linear regression equations of quantification cycle (C_q) (Bustin et al., 2009) against log copy number. The efficiency of each assay was determined from the regression equations. SigmaPlot® (ver. 11) was used to plot a graph from which the sensitivity by concentration, for each assay, could be determined. The estimated sensitivity and 95% confidence intervals for the true sensitivity for each group of dilutions prepared were calculated using the standard error of the estimated sensitivity of each dilution group (Sibeko et al., 2008).

The analytical specificity of each assay was evaluated using DNA extracted from other protozoal parasites expected to occur in equids, including *Trypanosoma brucei evansi*, *Trypanosoma brucei equiperdum*, *Trypanosoma vivax* and either *T. equi* for the *B. caballi* qPCR assay or *B. caballi* for the *T. equi* assay. DNA extracted from blood from a piroplasm-free horse was included in each assay as a negative control.

3.3.10 *Comparison of the qPCR assays with other tests for the detection of B. caballi and T. equi*

The qPCR assays were used to detect parasites in daily blood samples taken from two experimentally infected ponies as well as from 41 field samples of unknown piroplasm status. The results were compared with serological detection by IFAT and detection of parasites by RLB hybridization.

3.4 Results

3.4.1 Specific detection of B. caballi and T. equi using the qPCR assays

The *B. caballi* TaqMan MGB™ qPCR assay proved to be efficient in the amplification of a 95 bp fragment of the V4 hypervariable region of the 18S rRNA gene from an *in vitro*-cultured *B. caballi* isolate (Figure 3.1). No amplification signal was detected from negative control DNA extracted from blood from a horse free from piroplasms or from DNA extracted from an *in vitro*-cultured *T. equi* isolate (Figure 3.1). Furthermore, no amplification signals were observed from DNA extracted from *T. b. evansi*, *T. b. equiperdum* and *T. vivax*, other protozoal parasites expected to occur in horses (Figure 3.1).

Similarly, the *T. equi*-specific qPCR assay recently developed by Kim et al. (2008) was shown to be successful in the amplification of a fragment of the *T. equi* 18S rRNA gene from DNA extracted from an *in vitro*-cultured *T. equi* isolate (results not shown). No amplification signal was obtained from negative control DNA, DNA extracted from an *in vitro*-cultured *B. caballi* isolate, or from *T. b. evansi*, *T. b. equiperdum* and *T. vivax* DNA (results not shown). The latter result agrees with the findings of Kim et al. (2008) who showed that the *T. equi* qPCR assay did not detect *B. caballi* and *Trypanosoma evansi* DNA.

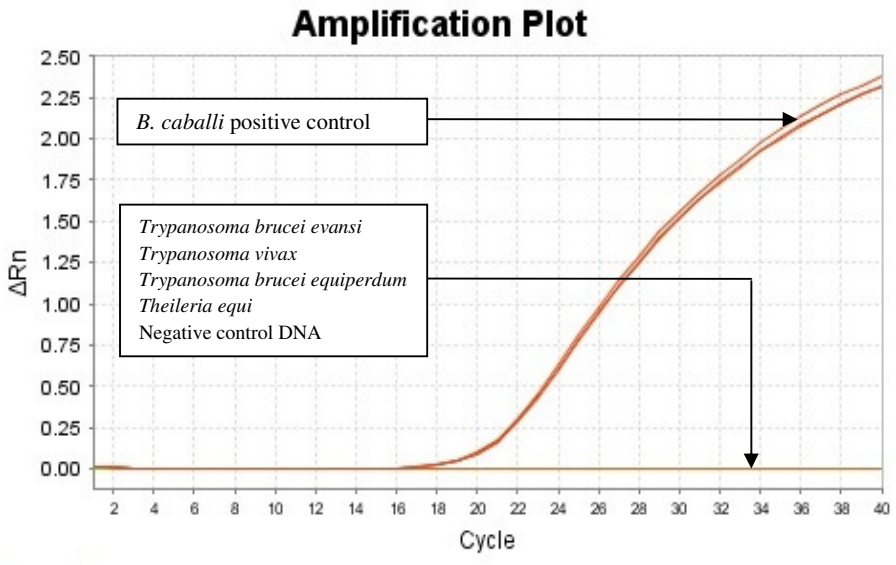
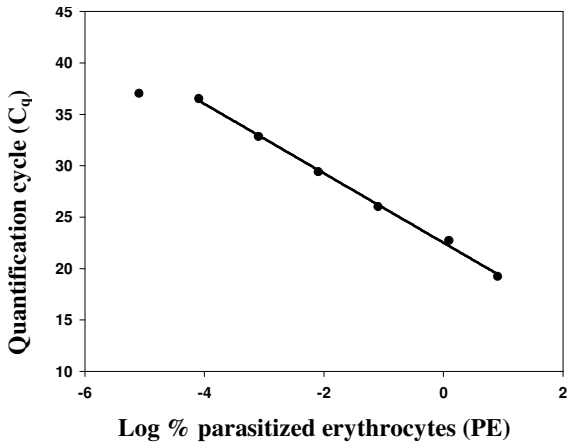


Figure 3.1 Detection of *B. caballi* positive control DNA using the *B. caballi* TaqMan MGB™ qPCR assay, indicated by an increase in the fluorescence signal. No increase in fluorescence was observed in the negative control sample (DNA extracted from blood from a piroplasm-free horse), or from *T. equi* control DNA or DNA from other protozoal parasites expected to occur in equids. The threshold (ΔRn) value was set at 0.05.

The efficiencies of the qPCR assays were determined from linear regression equations, generated from ten-fold serial dilutions of genomic DNA extracted from *B. caballi*- and *T. equi*-infected equine erythrocytes (Figure 3.2; Appendix A). For *B. caballi*, the efficiency of the TaqMan MGB™ qPCR assay was determined to be 97.8% and for the *T. equi*-specific qPCR assay, an efficiency of 99.7% was calculated.

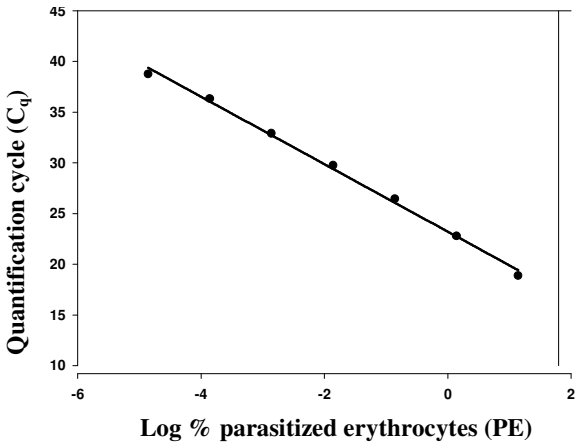
(A) *B. caballi* MGB™ qPCR standard curve



y = -3.374x + 22.52

R^2= 0.998

(B) *T. equi* MGB™ qPCR standard curve



y = -3.329x + 23.21

R^2 = 0.998

Figure 3.2 Standard curve for the quantification of **(A)** the *B. caballi* 18S rRNA gene and **(B)** the *T. equi* 18S rRNA gene. C_q values were plotted against the Log % parasitized erythrocytes (PE) of the initial ten-fold dilution series of parasite DNA equivalent to 8.1% PE to 8.1 x 10^{-7}% PE for *B. caballi* and 13.8% PE to 1.38 x 10^{-6}% PE for *T. equi*.

The qPCR assays were further tested for their ability to detect parasite DNA in field samples representative of the *T. equi* and *B. caballi* 18S rRNA genotypes previously identified in South Africa (Bhoora et al., 2009). The *B. caballi* TaqMan MGB™ qPCR assay successfully detected *B. caballi* parasite DNA in field samples representative of each of the groups of *B. caballi* 18S rRNA genotypes (groups A, B1 and B2) (Table 3.1). Similarly, the *T. equi*-specific qPCR assay could detect parasite DNA in field samples representative of each of the previously identified groups of *T. equi* 18S rRNA genotypes (groups A, B and C) (Table 3.1). Mixed infections are likely to occur in field samples and the use of the qPCR assays allowed for the identification of samples with dual *T. equi* and *B. caballi* infections.

Table 3.1 Ability of the qPCR assays to detect previously identified South African *B. caballi* and *T. equi* 18S rRNA genotypes from field samples containing single and mixed infections.

Sample name	Species and 18S rRNA genotype[a]	*B. caballi*-specific qPCR result (C_q)[b]	*T. equi*-specific qPCR result (C_q)
LFEQ23	*T. equi* group A	Negative	26.42
RBEQ32[c]	*T. equi* group A	35.85	20.88
RBEQ63[c]	*T. equi* group A	34.24	21.90
RBEQ178[c]	*T. equi* group A	39.62	28.65
RBEQ96	*T. equi* group B	Negative	23.33
RBEQ101	*T. equi* group B	Negative	24.81
EQ08	*T. equi* group C	Negative	28.73
EQ10	*T. equi* group C	Negative	29.79
EQ70	*T. equi* group C	Negative	32.38
EQ75	*T. equi* group C	Negative	32.81
LFEQ45	*T. equi* group C	Negative	35.43
LFEQ47	*T. equi* group C	Negative	36.09
LFEQ177	*T. equi* group C	Negative	33.33
RBEQ105	*T. equi* group C	Negative	27.41
RBEQ112	*T. equi* group C	Negative	31.69
CABEQ30[c]	*B. caballi* group A	18.31	34.99
CABEQ31[c]	*B. caballi* group B1	18.38	34.76
CABEQ33[c]	*B. caballi* group B1	17.37	35.11
CABEQ50	*B. caballi* group B1	17.70	Negative
CABEQ51[c]	*B. caballi* group B1	19.91	29.53
CABEQ52[c]	*B. caballi* group B1	18.52	36.78
CABEQ107[c]	*B. caballi* group B1	35.83	38.07
CABRBEQ164	*B. caballi* group B1	32.72	Negative
CABRBEQ179	*B. caballi* group B1	33.91	Negative
CABRBEQ73	*B. caballi* group B2	21.09	Negative
CABRBEQ74	*B. caballi* group B2	19.66	Negative
CABRBEQ115	*B. caballi* group B2	19.46	Negative

[a] The sequence of the 18S rRNA genotype in each sample was obtained in a previous study in which phylogenetic analyses indicated that the *T. equi* 18S rRNA sequences could be grouped into three main clades and the *B. caballi* sequences could be divided into two clades (Bhoora et al., 2009; Chapter 2).

[b] Quantification cycle value.

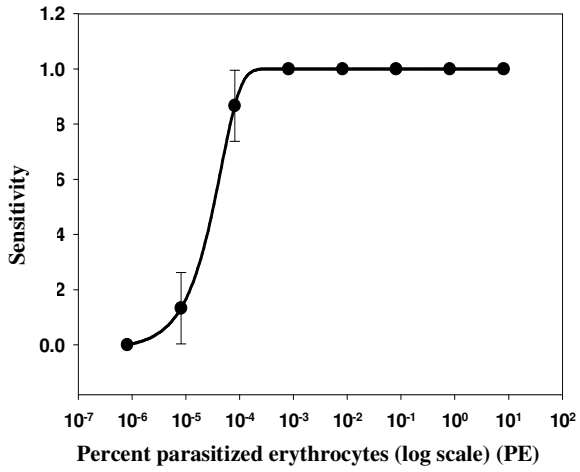
[c] Field samples with dual *T. equi* and *B. caballi* infections.

3.4.2 Analytical sensitivity of the qPCR assays

B. caballi DNA was detected in all 30 replicates of the dilutions of the cultured *B. caballi* 502 isolate ranging from undiluted (8.1% PE) to 8.1 x 10^{-4}% PE. The detection limit of the assay, defined as the concentration at which 95% of positive samples are detected (Bustin et al., 2009), was determined from the sensitivity curve (Figure 3.3A; Appendix A) to be 1.14 x 10^{-4} % PE,

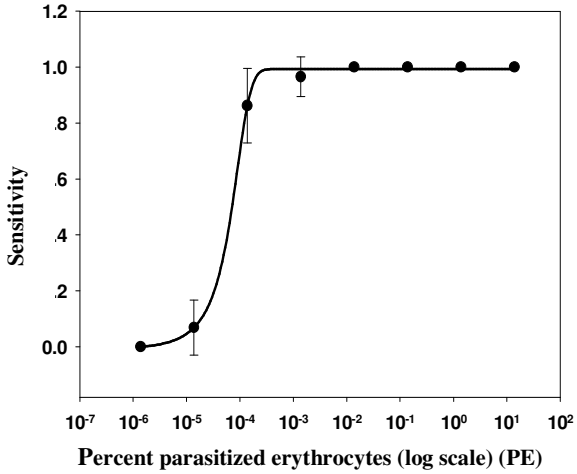
equating to a quantification cycle (C_q) of 35.82. The sensitivity of the assay decreased at higher dilutions. At 8.1 x 10^{-5}% PE, the sensitivity decreased to 86.7% with a 95% confidence interval (CI) of 74.5-98.8%, and at 8.1 x 10^{-6}% PE (C_q of 39.69), the sensitivity was 13.3% with a 95% CI of 1.16-25.4% (Figure 3A). Similarly, *T. equi* DNA was detected in all 29 replicates of the dilutions ranging from 13.8% PE to 1.38 x 10^{-2}% PE. The detection limit (95% sensitivity) of the *T. equi* qPCR assay was determined to be 1.9 x 10^{-4}% PE at a C_q value of 35.63. The sensitivity decreased from 96.5% (95% CI 89.9-100%) at 1.38 x 10^{-3}% PE (C_q of 39.38) to 6.8% (95% CI 0-16.1%) at 1.38 x 10^{-5}% PE (Figure 3.3B; Appendix A). The reportable range of qPCR assays refers to the lowest and highest results reliably produced by the test method (Sloan, 2007). For the *B. caballi*-specific MGB™ qPCR assay, C_q values greater than 39.69 were regarded as negative. Similarly, for *T. equi*, the highest reportable C_q value was determined to be 39.38.

(A) *B. caballi* MGB™ qPCR assay sensitivity



$$y = -1.6224 + \frac{2.6224}{1 + e^{\frac{-(x-(-1.507\times10^{-5}))}{3.282\times10^{-5}}}}$$

(B) *T. equi* qPCR assay sensitivity



$$y = -0.2430 + \frac{1.2361}{1 + e^{\frac{-(x-5.571\times10^{-5})}{3.359\times10^{-5}}}}$$

Figure 3.3 The sensitivity and 95% confidence intervals for the qPCR assays. **(A)** *B. caballi* TaqMan MGB™ qPCR sensitivity assay determined using a ten-fold dilution series from 10^0 to 10^{-7} prepared from a *B. caballi in vitro* culture with approximately 8.1% PE. **(B)** *T. equi* qPCR sensitivity assay determined using a 10-fold dilution series from 10^0 to 10^{-7} prepared from a *T. equi in vitro* culture with approximately 13.8% PE.

3.4.3 Comparison of the qPCR assays with other tests for the detection of B. caballi and T. equi

The newly developed *B. caballi*-specific TaqMan MGB™ qPCR assay and the previously reported *T. equi*-specific qPCR assay were compared with IFAT and RLB by testing the ability of the different tests to detect parasites or antibody in the blood of two experimentally infected ponies. Pony A, which was infected with *T. equi*, had a temperature rise (≥ 40°C), that was observed on day 8 p.i. and remained high until day 10 p.i. During this period of increased temperature the pony was lethargic, but showed no other clinical signs. On day 12 p.i. parasites were seen in blood smears but the parasitaemia never became high enough to calculate reliably (Table 3.2). From day 14 p.i. no further parasites were found in any of the blood smears. The haematocrit started to decline after infection and was less than 36% from day 6 p.i., with the lowest haematocrit value of 25% recorded on day 15 p.i. The haematocrit remained under the normal level of 36-40% until day 53 p.i. when it reached 37%. The first sample that was IFAT positive was taken on day 9 p.i. (Table 3.2) and Pony A remained IFAT positive until the end of the experiment. IFAT was not performed on all samples, since antibody titres were not expected to vary much from day to day. The first positive result of the RLB test was on day 8 p.i. and remained positive until the last test was performed on day 52 p.i., with five exceptions (days 16-18, 34 and 45) (Table 3.2). The qPCR test appeared to be the most sensitive of the three tests as it was able to detect *T. equi* in Pony A from day 7 p.i. and remained positive until day 52 p.i.

Pony B, infected with *B. caballi,* had an increased temperature (≥ 40°C) from day 6 p.i. until day 15 p.i. During this time the animal suffered from general malaise; it moved slowly, laid down frequently and had a decreased appetite. Parasites were seen in the blood smear from day 9 p.i. (Table 3.2). From day 19 p.i. no parasites were found, except for day 22 p.i. when a single parasite was seen. After infection, the haematocrit value declined to a low of 20% on day 19 p.i. and then increased again, reaching a normal haematocrit value (36%) on day 46 p.i. Pony B tested IFAT positive on day 10 p.i. and remained IFAT positive until the last test was performed on day 52 p.i. (Table 3.2). According to the RLB test, the first positive sample was on day 9 p.i., and samples remained positive until day 51 p.i. The RLB was negative on day 52 p.i. when the last test was performed (Table 3.2). The TaqMan MGB™ qPCR test was able to detect *B. caballi* from day 7 until day 52 p.i.

The PCR-based DNA detection tests used in this study were more sensitive than IFAT during the early onset of the disease, since they detected *T. equi* and *B. caballi* DNA in the circulation prior to antibody production. After day 9 p.i. for Pony A and day 10 p.i. for Pony B all three tests were in full agreement, with the exception of five days when the RLB was negative for *T. equi* (Pony A) and one day when it was negative for *B. caballi* (Pony B). On most days when the RLB was negative for *T. equi*, the qPCR C_q values were very high (Table 3.2). The variations in the qPCR C_q values over time likely reflect the concomitant fluctuations in the circulating parasitaemia. The negative RLB results were thus probably as a result of periods when the parasitaemia dropped below the detection limit of the RLB. The qPCR assays were more sensitive than the RLB as they were able to detect parasite DNA in both ponies earlier than the RLB hybridization assay, and qPCR results remained positive until the last day.

Table 3.2 Results of microscopic examination of blood smears, IFAT, RLB and qPCR tests for Pony A infected with *T. equi* and Pony B infected with *B. caballi*.

Days	Pony A (infected with *T. equi*)				Pony B (infected with *B. caballi*)			
p.i.	Blood	IFAT	RLB	*T. equi*-	Blood	IFAT	RLB	*B. caballi*-
3	-	0	-	-	-	0	-	-
4	-		-	-	-		-	-
5	-		-	-	-		-	-
6	-		-	-	-		-	-
7	-	0	-	35.11	-	0	-	36.30
8	-	0	+	34.12	-	0	-	34.43
9	-	160	+	31.89	+	0	+	31.79
10	-	640	+	30.85	+	160	+	29.63
11	-	1280	+	30.72	+	320	+	28.48
12	+		+	30.27	+		+	26.47
13	+		+	30.98	+		+	26.53
14	-		+	35.18	+		+	31.67
15	-	>2560	+	32.93	+	320	+	27.34
16	-		-	35.35	+		+	29.93
17	-	5120	-	37.77	+	2560	+	29.24
18	-		-	38.30	+		+	31.08
19	-	2560	+	35.57	-	640	+	31.17
20	-		+	32.99	-		+	31.17
21	-		+	33.04	-		+	31.16
22	-	2560	+	31.65	+	1280	+	32.85
24	-		+	31.40	-		+	31.08
26	-	>2560	+	33.62	-	640	+	30.50
28	-		+	37.32	-		+	30.50
31	-	2560	+	31.54	-	640	+	30.44
34	-		-	34.31	-		+	32.81
38	-	>2560	+	33.05	-	1280	+	32.50
45	-	>2560	-	36.17	-	1280	+	32.95
48	-	5120	+	32.39	-		+	32.76
52	-	>1280	+	33.40	-	640	-	32.75

[a] *T. equi* C_q values greater than 39.38 are considered negative.

[b] *B. caballi* C_q values greater than 39.69 are considered negative.

In order to further evaluate the *T. equi* and *B. caballi* qPCR assays, we tested 41 field samples of unknown piroplasm status (Table 3.3). Due to the low prevalence of *B. caballi* in field blood samples in South Africa, we targeted three stud farms in the Northern Cape Province, where tick vectors for both *T. equi* and *B. caballi* occur, namely, *Hyalomma truncatum* and *Rhipicephalus evertsi evertsi* (de Waal and van Heerden, 2004). Tick-control strategies were only implemented on the selected stud farms when high tick burdens were observed and therefore horses were almost certainly exposed several times to the tick vectors and parasites, making them an ideal target population for detecting natural *T. equi* and *B. caballi* infections. The *B. caballi* TaqMan MGB™ qPCR assay detected *B. caballi* parasite DNA in 32 of the 41 field samples with C_q

values that ranged between 23.62 and 37.41. The *T. equi* qPCR assay detected *T. equi* parasite DNA in 33 of the 41 samples with C_q values between 22.37 and 34.49.

The qPCR results were compared to those obtained by IFAT and RLB (Table 3.3). The IFAT detected circulating *T. equi* and *B. caballi* antibody in, respectively, 83% and 70% of the samples tested (Table 3.3). The RLB detected *T. equi* parasite DNA in 73% of samples tested, while none of the samples was positive for *B. caballi*. However, 19 of the *T. equi* positive samples also hybridized to a *Babesia* genus-specific probe. This could indicate a mixed *T. equi* and *B. caballi* infection with either the *B. caballi* parasitaemia at a level below the detection limit of the *B. caballi* RLB probe, or the occurrence of a novel *Babesia* genotype or species. In a molecular epidemiological survey of equine piroplasmosis conducted in Spain, Nagore et al. (2004) showed that of the 243 equine blood samples tested using the RLB, *B. caballi* was detected in only one sample and *B. caballi-like* parasites in a further eight samples. *Babesia caballi* infections have been reported to occur at very low parasitaemias that rarely exceed 1% (Hanafusa et al., 1998). Previous reports indicate that *B. caballi* is extremely difficult to detect in blood smears at any stage of the disease except the early acute phase, and once a carrier status is established, there may be complete absence of circulating parasites (Frerichs et al., 1969; Holman et al., 1993). The occurrence of such low parasitaemias could possibly explain the inability of the RLB to detect all positive *B. caballi* infections. Alternatively, the presence of sequence variation, which has previously been reported to occur in the region of the 18S rRNA gene where the RLB primers and probes were designed (Bhoora et al., 2009; Chapter 2), could explain the discrepant results. Despite the apparently low parasitemias, or the possible presence of sequence variants, the *B. caballi* TaqMan MGB™ qPCR assay proved to be efficient in detecting infected animals. The assay detected *B. caballi* parasite DNA in 78% of the samples tested, 34% (17) of which were from samples co-infected with *T. equi* as shown by the *T. equi*-specific qPCR assay. The *T. equi*-specific qPCR assay was also shown to be more sensitive than the RLB, and could detect parasite DNA in 80% of the samples tested.

Table 3.3 IFAT, RLB and qPCR results for 41 equine field blood samples of unknown piroplasm status, obtained from three stud farms in the Northern Cape Province, South Africa, where limited tick-control measures were implemented.

Sample no.	*T. equi* IFAT	*B. caballi* IFAT	RLB	*T. equi* qPCR (C_q)	*B. caballi* qPCR (C_q)
1	Positive	Positive	*T. equi*, B. catch all[a]	25.06	32.76
2	Positive	Positive	*T. equi*, B. catch all	25.14	32.43
3	Positive	Positive	*T. equi*	34.49	37.41
4	Positive	Positive	*T. equi*, B. catch all	24.18	32.49
5	Positive	Positive	*T. equi*	29.99	34.85
6	Positive	Positive	*T. equi*, B. catch all	25.53	32.83
7	Positive	Positive	T/B. catch all[b]	25.07	32.72
8	Positive	Positive	Negative	34.13	30.72
9	Positive	Positive	*T. equi*, B. catch all	23.86	28.43
10	Positive	Positive	*T. equi*, B. catch all	24.19	29.36
11	Positive	Positive	*T. equi*, B. catch all	Negative	Negative
12	Positive	Positive	*T. equi*, B. catch all	22.37	29.76
13	Positive	Negative	*T. equi*, B. catch all	25.09	36.69
14	Positive	Positive	*T. equi*, B. catch all	24.09	28.29
15	Positive	Positive	*T. equi*, B. catch all	24.72	31.58
16	Positive	Positive	*T. equi*, B. catch all	26.04	36.43
17	Positive	Positive	*T. equi*, B. catch all	26.18	31.70
18	Negative	Positive	Negative	30.13	37.29
19	Positive	Positive	*T. equi*, B. catch all	25.39	29.95
20	Positive	Positive	*T. equi*, B. catch all	Negative	26.41
21	Positive	Positive	*T. equi*, B. catch all	23.24	29.70
22	Positive	Positive	*T. equi*, B. catch all	25.02	36.39
23	Positive	Positive	*T. equi*	30.00	33.49
24	Positive	Positive	*T. equi*	22.87	34.84
25	Positive	Positive	*T. equi*	24.50	29.64
26	Positive	Positive	T/B. catch all	32.16	36.51
27	Positive	Negative	*T. equi*	22.48	33.90
28	Positive	Positive	*T. equi*, B. catch all	22.86	23.62
29	Positive	Positive	*T. equi*, B. catch all	31.06	Negative
30	Positive	Positive	*T. equi*	23.17	36.18
31	Negative	Negative	Negative	Negative	Negative
32	Positive	Negative	*T. equi*	29.08	Negative
33	Negative	Negative	Negative	Negative	36.53
34	Negative	Negative	Negative	Negative	Negative
35	Negative	Negative	Negative	Negative	Negative
36	Negative	Positive	Negative	Negative	Negative
37	Positive	Negative	*T. equi*	30.10	36.83
38	Positive	Negative	*T. equi*	30.43	Negative
39	Positive	Negative	*T. equi*	28.64	36.40
40	Negative	Negative	Negative	Negative	Negative
41	Positive	Negative	Negative	34.12	32.98

[a] B. catch all – *Babesia* genus-specific probe

[b] T/B catch all – *Theileria/Babesia* genus-specific probe

3.5 Discussion

In the present study we describe the development and application of a TaqMan MGB™ qPCR assay targeting the *B. caballi* 18S rRNA gene, for the quantitative detection of the parasite from equine blood samples, as well as the evaluation of a qPCR assay for the detection of *T. equi* infections in South Africa. Molecular tests previously developed for the detection and differentiation of equine parasite species were based on conventional PCR and probe-based assays, which are relatively sensitive, but involve complex procedures which are time-consuming (Allsopp et al., 1993; Bashiruddin et al., 1999; Nicolaiewsky et al., 2001; Rampersad et al., 2003; Alhassan et al., 2005). Quantitative PCR technology has recently been applied to the 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). This technology provides several advantages over the use of conventional PCR assays. Detection and quantification of a PCR 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 been developed for a number of haemoparasitic diseases 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. While a qPCR assay for the detection of *T. equi* (Kim et al., 2008), based on the amplification of the 18S rRNA gene has been developed, there is no report on the application of a qPCR test for the quantitative diagnosis of equine *Babesia* parasites using this gene.

Although sequence heterogeneity has been demonstrated in the 18S rRNA gene of *B. caballi* in South Africa (Bhoora et al., 2009), we chose this gene as the target for development of a *B. caballi*-specific qPCR assay. Other genes previously targeted for the development of molecular diagnostic assays encode outer membrane proteins (Nicolaiewsky et al., 2001; Ueti et al., 2003; Alhassan et al., 2005; Alhassan et al., 2007; Heim et al., 2007) which are under intense selection pressure, and sequence heterogeneity in the genes encoding these proteins is therefore likely to be even greater than in the 18S rRNA gene. Prior to the development of the primers and probes, the *B. caballi* 18S rRNA sequence variants were carefully examined for conserved regions. TaqMan MGB™ probes have previously been demonstrated to be helpful in the case of variable nucleotide sequences, since they allow for the use of smaller probes that are capable of detecting shorter conserved regions and with lower fluorescent background signals, as the 3' end of the probe is labelled with a non-fluorescent quencher (Kutyavin et al., 2000). Therefore, for a

robust and quantitative assay, a TaqMan MGB™ probe was designed in a conserved target sequence of the V4 hypervariable region of the *B. caballi* 18S rRNA gene.

The *T. equi*-specific qPCR assay designed by Kim et al. (2008) targets a region of the 18S rRNA gene that occurs outside of the V4 hypervariable region. Inspection of the 18S rRNA sequences obtained from the twelve distinct South African *T. equi* variants (Bhoora et al., 2009; Chapter 2), indicated the occurrence of a single nucleotide difference in the forward primer target sequence and no differences in the regions where the reverse primer and probe had been designed. These differences did not appear to affect the sensitivity of the qPCR assay developed by Kim et al. (2008) as it was able to detect all *T. equi* variants thus far identified.

The *B. caballi*-specific TaqMan MGB™ qPCR assay was highly sensitive proving to be able to detect as few as 1.14 x 10^{-4}% PE. The sensitivity of the *B. caballi* TaqMan MGB™ qPCR assay is comparable to that reported for the qPCR assay developed for the detection of *T. parva* (8.79 x 10^{-4}% parasitaemia) in cattle and buffalo in South Africa (Sibeko et al., 2008). Kim et al. (2008) determined the detection limit of their *T. equi* qPCR to be 1.5 parasites per µl per sample, which equates to 1.5 x 10^{-5}% PE. In our hands, the detection limit (95% sensitivity) of the *T. equi* qPCR assay was 1.9 x 10^{-4}% PE. Both assays were shown to be specific for the target organism and no amplification signals were observed from DNA of other protozoal parasites expected to occur in equids. *Babesia caballi* infections generally tend to occur at extremely low parasitaemias, often due to the early elimination of the parasite after a short period of infection, making diagnosis almost impossible (Frerichs et al., 1969). The development of a highly sensitive and specific qPCR assay provides a major advantage in the detection of *B. caballi* infections in field blood samples.

The high seroprevalence of *T. equi* and *B. caballi* on three stud farms in the Northern Cape Province where limited tick-control measures were implemented confirmed the occurrence of both *T. equi* and *B. caballi* infections on the selected farms. Although there was significant correlation between the qPCR and IFAT results in the detection of *B. caballi* and *T. equi* in infected horses, minor differences between the results were observed. Three samples were IFAT-positive for *B. caballi* but the qPCR results were negative. In addition, *B. caballi* DNA could be detected in six samples that were reported to be *B. caballi* IFAT-negative. Similarly for *T. equi*, two IFAT-positive samples were negative when tested using the qPCR, while *T. equi* parasite DNA could be detected in one sample that was reported to be IFAT-negative.

Quantitative PCR tests detect the presence of parasite DNA, whereas IFAT detects antibodies, which can be present in the absence of parasites (Holman et al., 1993). Depending on the tick vectors, the prepatent period of *B. caballi* infections is between 10-20 days, (de Waal and Potgieter, 1987; de Waal, 1990) and that of *T. equi* infections is reported to be between 12-14 days (Mehlhorn and Schein, 1998). The sampling time thus plays a critical role in the detection of circulating parasites. The occurrence of IFAT-negative but qPCR-positive results may be explained by the observation that parasites can be detected in newly infected animals prior to the development of antibodies. This can be observed in the results obtained from our experimental infection trial (Table 3.2), where both ponies remained IFAT-negative during the first few days of infection even though parasite DNA was detected by the qPCR assays. Allsopp et al. (2007) reported the detection of *T. equi* parasite DNA in a four month old aborted foetus, which indicated that transplacental transmission takes place long before the foetal immune system is sufficiently developed to recognize these parasites as foreign. Foals born to carrier mares in endemic areas, however, usually acquire maternal antibodies in the colostrum, which can be detected up to five months of age (Donnelly et al., 1982). However, if sample collection occurred prior to colostrum ingestion, new-born naïve foals may test serologically negative, but PCR positive (Allsopp et al., 2007; Kumar et al., 2008).

Babesia caballi infections are self-limiting, usually lasting one to three years, and horses are generally able to eliminate the infection naturally or drugs can be used to sterilize the infection (Friedhoff and Soule, 1996; Brüning et al., 1996), although a recent study has shown that even high-dose treatment with imidocarb may not be capable of eliminating *B. caballi* infections from healthy carriers (Butler et al., 2008). It is however possible that *B. caballi* antibody titres remain at detectable levels for some period after the parasite has been transiently cleared, either naturally or by the use of drugs, resulting in animals testing IFAT-positive but negative using PCR-based methods. *Theileria equi* infections are not self-limiting and, once infected, horses remain life-long carriers of the parasite. Samples that test positive for *T. equi* by IFAT should therefore be detectable by qPCR, unless the parasitaemia is below the detection limit of the assay. However, IFAT-positive and qPCR-negative results could also be explained either by the existence of parasite 18S rRNA gene sequence variants that have not yet been identified and can therefore not be detected by the qPCR tests or the presence of PCR inhibitors.

To our knowledge, this is the first report on the development of a quantitative TaqMan MGB™ qPCR assay, based on the 18S rRNA gene, for the detection of *B. caballi* infections in equine blood samples. Accurate diagnosis of equine piroplasmosis is essential for effective control measures. Previous assays proved to be limited in their ability to detect *B. caballi* infections in field blood samples due to the extremely low or undetectable parasitaemias observed. We have demonstrated rapid and accurate quantification of *B. caballi* from sub-clinically infected or carrier animals, using the TaqMan MGB™ qPCR assay on a StepOnePlus real-time PCR instrument. We envisage that application of this assay, along with the *T. equi*-specific qPCR assay developed by Kim et al. (2008), will provide better confirmation of diagnosis of equine piroplasmosis, particularly in cases where symptoms are non-specific. However, while we were able to show that the *B. caballi* and *T. equi* qPCR assays were able to detect all known 18S rRNA sequence variants that have previously been identified in South Africa, we do not know whether other variants exist in the field. It would therefore be prudent to develop a multiplex qPCR assay, including a "catch-all" TaqMan probe, similar to the *Theileria/Babesia* genus-specific probe used in the RLB, to ensure that if a piroplasm parasite is present in a sample, it will be detected by the qPCR test. Once validated, the tests could be incorporated as required tests by the OIE for the import and export of horses and for checking whether attempts at sterilizing equine piroplasmosis infections have been successful.

3.6 Reference List

Alhassan, A., Pumidonming, W., Okamura, M., Hirata, H., Battsetseg, B., Fujisaki, K., Yokoyama, N., Igarashi, I., 2005. Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood. Veterinary Parasitology 129, 43-49.

Alhassan, A., Thekisoe, O.M.M., Yokoyama, N., Inoue, N., Motloang, M.Y., Mbati, P.A., Yin, H., Katayama, Y., Anzai, T., Sugimoto, C., Igarashi, I., 2007. Development of loop-mediated isothermal amplification (LAMP) method for diagnosis of equine piroplasmosis. Veterinary Parasitology 143, 155-160.

Allsopp, B.A., Baylis, H.A., Allsopp, M.T.E.P., Cavalier-Smith, T., Bishop, R.P., Carrington, D.M., Sohanpal, B., Spooner, P., 1993. Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences. Parasitology 107, 157-165.

Allsopp, M.T.E.P., Cavalier-Smith, T., de Waal, D.T., Allsopp, B.A., 1994. Phylogeny and evolution of the piroplasms. Parasitology 108, 147-152.

Allsopp, M.T.E.P., Lewis, B.D., Penzhorn, B.L., 2007. Molecular evidence for transplacental transmission of *Theileria equi* from carrier mares to their apparently healthy foals. Veterinary Parasitology 148, 130-136.

Anonymous, 2008. Equine piroplasmosis. In: OIE Manual of diagnostic tests and vaccines for terrestrial animals. Office International des Epizooties, Paris, France, pp. 884-893.

Bashiruddin, J.B., Camma, C., Rebelo, E., 1999. Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. Veterinary Parasitology 84, 75-83.

Bhoora, R., Franssen, L., Oosthuizen, M.C., Guthrie, A.J., Zweygarth, E., Penzhorn, B.L., Jongejan, F., Collins, N.E., 2009. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Veterinary Parasitology 159, 112-120.

Böse, R., Jorgensen, W.K., Dalgliesh, R.J., Friedhoff, K.T., de Vos, A.J., 1995. Current state and future trends in the diagnosis of babesiosis. Veterinary Parasitology 57, 61-74.

Brüning, A., 1996. Equine piroplasmosis an update on diagnosis, treatment and prevention. British Veterinary Journal 152, 139-151.

Brüning, A., Phipps, P., Posnett, E., Canning, E.U., 1997. Monoclonal antibodies against *Babesia caballi* and *Babesia equi* and their application in serodiagnosis. Veterinary Parasitology 68, 11-26.

Buling, A., Criado-Fornelio, A., Asenzo, G., Benitez, D., Barba-Carretero, J.C., Florin-Christensen, M., 2007. A quantitative PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina*. Veterinary Parasitology 147, 16-25.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 55, 611-622.

Butler, C.M., Nijhof, A.M., van der Kolk, J.H., de Haseth, O.B., Taoufik, A., Jongejan, F., Houwers, D.J., 2008. Repeated high dose imidocarb dipropionate treatment did not eliminate *Babesia caballi* from naturally infected horses as determined by PCR-reverse line blot hybridization. Veterinary Parasitology 151, 320-322.

Carelli, G., Decaro, N., Lorusso, A., Elia, G., Lorusso, E., Mari, V., Ceci, L., Buonavoglia, C., 2007. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. Veterinary Microbiology 124, 107-114.

Criado-Fornelio, A., Gonzalez-del-Rio, M.A., Buling-Sarana, A., Barba-Carretero, J.C., 2004. The "expanding universe" of piroplasms. Veterinary Parasitology 119, 337-345.

de Waal, D.T., 1990. The transovarial transmission of *Babesia caballi* by *Hyalomma truncatum.* Onderstepoort Journal of Veterinary Research 57, 99-100.

de Waal, D.T., 1992. Equine piroplasmosis: a review. British Veterinary Journal 148, 6-14.

de Waal, D.T., Potgieter, F.T., 1987. The transstadial transmission of *Babesia caballi* by *Rhipicephalus evertsi evertsi*. Onderstepoort Journal of Veterinary Research 54, 655-656.

de Waal, D.T., van Heerden, J., 2004. Equine piroplasmosis. In: Coetzer, J.A.W., Tustin, R.C. (Eds.), Infectious Diseases of Livestock (Second Edition). Oxford University Press, New York, pp 425-434.

Donnelly, J., Phipps, L.P., Watkins, K.L., 1982. Evidence of maternal antibodies to *Babesia equi* and *B. caballi* in foals of seropositive mares. Equine Veterinary Journal 14, 126-128.

Frerichs, W.M., Holbrook, A.A., Johnson, A.J., 1969. Equine piroplasmosis: complement-fixation titers of horses infected with *Babesia caballi*. American Journal of Veterinary Research 30, 697-702.

Friedhoff, K.T., Soule, C., 1996. An account on equine babesioses. Revue Scientifique et Technique 15, 1191-1201.

Friedhoff, K.T., Tenter, A.M., Müller, I., 1990. Haemoparasites of equines: impact on international trade of horses. Revue Scientifique et Technique 9, 1187-1194.

Gubbels, J.M., de Vos, A.P., van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. Journal of Clinical Microbiology 37, 1782-1789.

Hanafusa, Y., Cho, K.O., Kanemaru, T., Wada, R., Sugimoto, C., Onuma, M., 1998. Pathogenesis of *Babesia caballi* infection in experimental horses. Journal of Veterinary Medical Science 60, 1127-1132.

Heim, A., Passos, L.M.F., Ribeiro, M.F.B., Costa-Junior, L.M., Bastos, C.V., Cabral, D.D., Hirzmann, J., Pfister, K., 2007. Detection and molecular characterization of *Babesia caballi* and *Theileria equi* isolates from endemic areas of Brazil. Parasitology Research 102, 63-68.

Holman, P.J., Frerichs, W.M., Chieves, L., Wagner, G.G., 1993. Culture confirmation of the carrier status of *Babesia caballi*-infected horses. Journal of Clinical Microbiology 31, 698-701.

Jeong, W., Kweon, C., Kang, S., Paik, S., 2003. Diagnosis and quantification of *Theileria sergenti* using TaqMan PCR. Veterianry Parasitology 111, 287-295.

Kappmeyer, L.S., Perryman, L.E., Hines, S.A., Baszler, T.V., Katz, J.B., Hennager, S.G., Knowles, D.P., 1999. Detection of equine antibodies to *Babesia caballi* by recombinant *B. caballi* rhoptry-associated protein 1 in a competitive-inhibition enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 37, 2285-2290.

Kim, C.M., Blanco, L.B.C., Alhassan, A., Iseki, H., Yokoyama, N., Xuan, X., Igarashi, I., 2008. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. Veterinary Parasitology 151, 158-163.

Knowles, D.P., Perryman, L.E., Goff, W.L., Miller, C.D., Harrington, R.D., Gorham, J.R., 1991. A monoclonal antibody defines a geographically conserved surface protein epitope of *Babesia equi* merozoites. Infection and Immunity 59, 2412-2417.

Kumar, S., Kumar, R., Gupta, A.K., Dwivedi, S.K., 2008. Passive transfer of *Theileria equi* antibodies to neonate foals of immune tolerant mares. Veterinary Parasitology 151, 80-85.

Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousor, E.S., Singer, M.J., Walburger, D.K., Lokhov, G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B., Hedgpeth, J., 2000. 3'-Minor groove binder probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Research 28, 655-656.

Lewis, B.D., Penzhorn, B.L., Volkmann, D.H., 1999. Could treatment of pregnant mares prevent abortions due to equine piroplasmosis? Journal of the South African Veterinary Association 70, 90-91.

Lindh, M., Gorander, S., Andersson, E., Horal, P., Mattsby-Balzer, I., Ryd, W., 2007. Real-time Taqman PCR targeting 14 human papilloma virus types. Journal of Clinical Virology 40, 321-324.

Mehlhorn, H., Schein, E., 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. Parasitology Research 84, 467-475.

Nagore, D., Garcia-Sanmartin, J., Garcia-Perez, A.L., Juste, R.A., Hurtado, A., 2004. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: epidemiological survey and phylogenetic analysis. Veterinary Parasitology 123, 41-54.

Nicolaiewsky, T.B., Richter, M.F., Lunge, V.R., Cunha, C.W., Delagostin, O., Ikuta, N., Fonseca, A.S., da Silva, S.S., Ozaki, L.S., 2001. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. Veterinary Parasitology 101, 9-21.

Nijhof, A.M., Pillay, V., Steyl, J., Prozesky, L., Stoltsz, W.H., Lawrence, J.A., Penzhorn, B.L., Jongejan, F., 2005. Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. Journal of Clinical Microbiology 43, 5907-5911.

O'Grady, J., Sedano-Balbas, S., Maher, M., Smith, T., Barry, T., 2008. Rapid real-time PCR detection of *Listeria monocytogenes* in enriched food samples based on the ssrA gene, a novel diagnostic target. Food Microbiology 25, 75-84.

Rampersad, J., Cesar, E., Campbell, M.D., Samlal, M., Ammons, D., 2003. A field evaluation of PCR for the routine detection of *Babesia equi* in horses. Veterinary Parasitology 114, 81-87.

Sibeko, K.P., Oosthuizen, M.C., Collins, N.E., Geysen, D., Rambritch, N.E., Latif, A.A., Groeneveld, H.T., Potgieter, F.T., Coetzer, J.A.W., 2008. Development and evaluation of a real-time polymerase chain reaction test for the detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle. Veterinary Parasitology 155, 37-48.

Sloan, L.M., 2007. Real-time PCR in clinical microbiology: verification, validation, and contamination control. Clinical Microbiology Newsletter 29, 87-95.

Sluyter, F.J., 2001. Traceability of Equidae: a population in motion. Revue Scientifique et Technique 20, 500-509.

Ueti, M.W., Palmer, G.H., Kappmeyer, L.S., Scoles, G.A., Knowles, D.P., 2003. Expression of equi merozoite antigen 2 during development of *Babesia equi* in the midgut and salivary gland of the vector tick *Boophilus microplus*. Journal of Clinical Microbiology 41, 5803-5809.

Weiland, G., Aicher, B.M., Boch, J., 1984. Serodiagnosis and therapy control of equine piroplasmosis by CFT and IFAT. Berliner und Münchener Tierärztliche Wochenschrift 97, 34134-9.

Wengi, N., Willi, B., Boretti, F.S., Cattori, V., Riond, B., Meli, M.L., Reusch, C.E., Lutz, H., Hofmann-Lehmann, R., 2008. Real-time PCR-based prevalence study, infection follow-up and molecular characterization of canine hemotropic mycoplasmas. Veterinary Microbiology 126, 132-141.

Zweygarth, E., Just, M.C., de Waal, D.T., 1995. Continuous *in vitro* cultivation of erythrocytic stages of *Babesia equi*. Parasitology Research 81, 355-358.

Zweygarth, E., Lopez-Rebollar, L.M., Nurton, J.P., Guthrie, A.J., 2002. Culture, isolation and propagation of *Babesia caballi* from naturally infected horses. Parasitology Research 88, 460-462.

CHAPTER 4

Genetic diversity of piroplasms in plains zebra (*Equus quagga burchellii*) and Cape mountain zebra (*Equus zebra zebra*) in South Africa

4.1 Abstract

Seventy EDTA blood samples collected from plains zebra (*Equus quagga burchellii*) and Cape mountain zebra (*Equus zebra zebra*) were screened for the presence of piroplasm DNA using the reverse line blot (RLB) hybridization assay, and quantitative *T. equi*-specific and *B. caballi*-specific TaqMan real-time PCR (qPCR) tests. RLB results indicated that 17 samples were positive for *T. equi*, while no samples hybridized to the *B. caballi* probe. Eight samples hybridized only to the *Theileria/Babesia* genus-specific probe and 45 samples were negative for the presence of piroplasms. In contrast, the *T. equi*-specific qPCR assay detected *T. equi* parasite DNA in 59 samples and the *B. caballi*-specific qPCR was able to detect *B. caballi* parasite DNA in 19 samples. The complete 18S rRNA gene was amplified and sequenced from 17 samples, while the V4 hypervariable region of the 18S rRNA gene could be amplified, cloned and sequenced from 31 samples. BLASTN analysis revealed that all of the sequences obtained were most similar to *T. equi* genotypes and not *B. caballi* genotypes. Although *Babesia* parasites were present in some of these samples, as indicated by qPCR, the parasitaemia may have been too low to allow detection by cloning of PCR products from a mixed infection. Based on these findings and our previous results obtained for parasites from horses, we conclude that there are three groups of *T. equi* 18S rRNA sequences in South Africa, designated A, B and C. Sequence analyses of both the full-length and the V4 hypervariable region of the *T. equi* 18S rRNA gene suggest the existence of 13 new *T. equi* sequences from zebra, including a novel sequence in clade A, seven new sequences in clade B and five novel sequences in clade C. These results confirm the existence of sequence heterogeneity in the rRNA genes of the parasites that cause equine piroplasmosis, and suggest that there may be additional, as yet unidentified, *T. equi* and *B. caballi* 18S rRNA sequences present in the horse and zebra populations in South Africa. The occurrence of previously unrecognized sequence variation could pose a potential problem in the implementation of diagnostic tests targeting the 18S rRNA gene.

4.2 Introduction

Equine piroplasmosis is caused by the intra-erythrocytic, tick-transmitted protozoan parasites *Theileria equi* (Mehlhorn and Schein, 1998) and *Babesia caballi* (Nuttal and Strickland, 1910), which affect horses, mules, donkeys and zebras. Twelve species of ixodid ticks in the genera *Dermacentor, Rhipicephalus* and *Hyalomma* have been identified worldwide as vectors of the disease (de Waal and van Heerden, 2004). Piroplasmosis of horses has an economic impact on the international movement of horses from endemic to disease-free areas (de Waal, 1992).

Various serological and molecular assays have thus been developed to detect *T. equi* and *B. caballi* in infected horses and to identify and allow the movement of negative horses (Brüning et al., 1997; Avarzed et al., 1998; Ikadai et al., 2000; Nagore et al., 2004; Heim et al., 2007; Kim et al., 2008).

A recent study in our laboratory on the molecular characterization of the small subunit (18S) ribosomal RNA (rRNA) genes of equine piroplasms revealed that several molecular assays based on this gene failed to detect the parasites because of sequence variation in the target gene (Bhoora et al., 2009a; Chapter 2). Furthermore, we found that the nucleotide sequences of two zebra samples showed a high degree of identity to a previously published *T. equi*-like strain (*Babesia equi* ET1, accession number AY534884) identified from a horse in Spain (Nagore et al., 2004), which was not detected in any of the South African horse samples, suggesting that there may be more variation in *T. equi* 18S rRNA genotypes in zebra in South Africa.

The African continent is home to the largest number of species in the genus *Equus*. Three species of zebra survive in sub-Saharan Africa. Plains zebra (*Equus quagga*) are the most common and geographically widespread. Historically, the species occurred from the south of Ethiopia through East Africa to Angola and South Africa. Its range has been much reduced by human activities, but it remains common in game reserves. Six subspecies are recognized (Groves and Bell, 2004), of which Burchell's zebra (*Equus quagga burchellii*) occurs in South Africa. The Cape mountain zebra (*Equus zebra zebra*), a subspecies of mountain zebra (*Equus zebra*), once inhabited all the mountain ranges of the Western and Eastern Cape Provinces of South Africa, but today its distribution is limited to small pockets in game reserves (Penzhorn, 1988). Grévy's zebras (*Equus grevyi*) are found in Kenya and Ethiopia. Small populations of wild asses, from which donkeys are descended (Beja-Pereira et al., 2004), remain in the deserts of Ethiopia and Somalia (Blench, 2000). All of these wild equids are likely to carry piroplasms, which will have co-evolved with their hosts and vectors before the introduction of horses and donkeys into South Africa in the mid 17th century (Thom, 1952; Starkey, 2000).

Piroplasm infections in zebra were first described early in the twentieth century, from an East African plains zebra (Koch, 1905). The inoculation of zebra blood into a susceptible horse led to an increase in the animal's temperature and the appearance of parasites. These observations thus suggested that zebras are also carriers of piroplasms. *Theileria equi* parasites were subsequently found incidentally in the blood of plains zebras from Umfolozi in KwaZulu-Natal,

South Africa (Neitz, 1931) and parasites were also reported to occur in Cape mountain zebras in South Africa (Young et al., 1973; Zweygarth et al., 2002). *Babesia caballi* parasites have also been reported from both plains and Grévy's zebras in East Africa (Neitz, 1965; Zweygarth et al., 2002). Despite the knowledge that these parasites occur in our zebra populations, the molecular epidemiology and the possible influence through genetic recombination that their existence may have on horse piroplasms, which are thought to have originated in Asia (de Waal and van Heerden, 2004), has largely been overlooked. We therefore screened zebra samples to identify piroplasm parasites, and sequenced the 18S rRNA gene of *T. equi*-like piroplasms of zebra to further elucidate genetic variation in *T. equi* parasites in South Africa.

4.3 Materials and Methods

4.3.1 Samples and DNA extraction

Seventy EDTA blood samples were obtained from the Kruger National Park (n=20), the Wildlife Biological Resource Centre (n=14) and the Equine Research Centre (n= 36) (Table 4.1). These samples were collected from plains (*E. quagga burchellii*) and Cape mountain zebra (*E. zebra zebra*). Genomic DNA was extracted from 1 ml of EDTA blood using the QiaAmp Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Table 4.1 Origin and number of zebra samples tested.

Origin	Zebra species	Geographical location	No.
Equine Research Center, University of Pretoria	*E. quagga burchellii*	Western Cape	23
	E. quagga burchellii	Northern Cape	13
Kruger National Park	*E. quagga burchellii*	Limpopo and Mpumalanga	10
Bontebok National Park	*E. zebra zebra*	Western Cape	10
Wildlife Breeding Research Centre			
Rietfontein farm	*E. quagga burchellii*	KwaZulu-Natal	2
Atherstone Nature Reserve	*E. quagga burchellii*	North West Province	12
Total			**70**

4.3.2 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 CTA 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 previously (Bhoora et al., 2009a; Chapter 2). Since the RLB is likely to result in many false-negatives, either if the parasitaemia in the sample is very low or due to the occurrence of novel genotypes, the presence of *T. equi* and *B. caballi* parasite DNA was confirmed using a *T. equi*-specific TaqMan qPCR assay (Kim et al., 2008) and a TaqMan MGB™ qPCR for *B. caballi* (Bhoora et al., 2009b; Chapter 3).

4.3.3 Cloning and sequencing

It was not possible to obtain sufficient PCR product to directly sequence the full-length 18S rRNA gene from any of the samples that were positive by RLB and/or qPCR assays, nor was there sufficient DNA to clone the amplicons. Three overlapping nested PCRs were therefore used to amplify ~1480 bp of the complete 18S rRNA gene and nested PCR products were sequenced. The Genomiphi DNA amplification kit (Amersham Biosciences) was used to exponentially amplify genomic DNA from samples with low parasitaemia, prior to performing nested PCRs for amplification and direct sequencing of the complete 18S rRNA gene as described previously (Bhoora et al., 2009a; Chapter 2). Briefly, primers NBabesia1F (5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3') and 18SRev-TB (5'-GAA TAA TTC ACC GGA TCA CTC G-3') were used in a primary PCR to amplify a ~1600 bp 18S rRNA fragment, using the cycling conditions previously reported (Bhoora et al., 2009a; Chapter 2). Three nested PCR reactions were subsequently performed, using the primary amplicon as template and primer pairs NBabesia1F and BT18S3R (5'-CCT CTG ACA GTT AAA TAC GAA TGC CC-3'), BT18S2F (5'-GGG TTC GAT TCC GGA GAG GG-3') and BT18S2R (5'-CCC GTG TTG AGT CAA ATT AAG CCG-3'), and BT18S3F (5'-GGG CAT TCG TAT TTA ACT GTC AGA GG-3') and 18SRev-TB. PCR products were purified using a QIAquick® PCR Purification Kit (Qiagen) and sequenced directly using BigDye chemistry (v.3.1, Applied Biosystems) in a 3130XL sequencer (Applied Biosystems).

Nested PCR products containing the V4 hypervariable region of the 18S rRNA gene, obtained by amplification with primers BT18S2F (5'-GGG TTC GAT TCC GGA GAG GG-3') and BT18S2R (5'-CCC GTG TTG AGT CAA ATT AAG CCG-3'), were cloned using the pGEM®-T Easy Vector System II (Promega), according to the manufacturer's instructions. Transformants were screened using ImMedia™ Amp Blue (Invitrogen) and recombinant colonies were selected and grown in ImMedia™ Amp Liquid (Invitrogen). Plasmid DNA was isolated by means of a High Pure Plasmid Purification kit (Roche). In an attempt to minimize the effects of PCR artifacts introduced during nested PCRs, a consensus sequence, using T7 and SP6 primers, was obtained from six clones from each sample.

Sequences were assembled and edited using gap4 of the Staden software suite (Staden, 1996; Staden et al., 2000). BLASTN (Altschul et al., 1990) was used to search the public sequence databases for orthologous sequences.

4.3.4 Phylogenetic analyses

Multiple sequence alignments were performed using the Mafft sequence alignment tool (Multiple Sequence Alignment) employing the FFT-NS-1 algorithm (Katoh et al., 2002; Katoh et al., 2005) and alignments were manually edited using BioEdit version 7.0.5.2 (Hall, 1999). 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 PAUP* v4b10 (Swofford, 2003) with 1481 positions for the complete *T. equi* 18S rRNA gene and 673 positions for the V4 hypervariable region. The 18S rRNA gene sequence of *Hepatozoon canis* (DQ439543) was used as an outgroup in the construction of phylogenetic trees (1000 replicates). Analysis by Bayesian inference was performed using MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001; 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 1,000,000 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).

4.4 Results

RLB results for the 70 zebra samples tested showed that 25 samples (35.71%) were infected with a piroplasm parasite as indicated by hybridization to the *Theileria/Babesia* genus-specific probe (Table 4.2). Of these, 17 samples (24.3%) hybridized to the *T. equi* probe while none of the samples hybridized to the *B. caballi* probe. Eight samples (11.4%) hybridized only to the *Theileria/Babesia* genus-specific probe and not to any of the species-specific probes. Forty-five samples (64.3%) tested negative on RLB for the presence of piroplasms.

A linear standard curve generated in our laboratory using a *T. equi*-specific qPCR assay developed by Kim et al. (2008) revealed that the detection limit of the TaqMan assay (95% sensitivity) was 1.9 x 10^{-4}% parasitised erythrocytes at a quantification cycle (C_q) of 35.89 (Bhoora et al., 2009b; Chapter 3). The upper limit of detection of the *T. equi*-specific qPCR, calculated from the sensitivity curve, equated to a C_q value of 39.74 (Bhoora et al., 2009b; Chapter 3). The assay was able to detect *T. equi* parasite DNA in 59 of the 70 zebra samples with C_q values that ranged between 20.98 and 34.48, and in one sample, below the 95% sensitivity of the assay, at a C_q value of 39.14 (Table 4.2). The *T. equi* qPCR assay detected parasite DNA in all 17 *T. equi* RLB positive samples, as well as in 35 of the samples that tested negative on RLB and in the eight samples that hybridized to the *Theileria/Babesia* genus-specific probe only.

The detection limit (95% sensitivity) of the *B. caballi*-specific TaqMan MGB™ qPCR assay was shown to be 1.14 x 10^{-4}% parasiatemia at a C_q of 35.82 (Bhoora et al., 2009b; Chapter 3). For the *B. caballi*-specific qPCR assay, the upper limit of detection equated to a C_q value of 39.69 (Bhoora et al., 2009b). The *B. caballi* qPCR assay could detect *B. caballi* parasite DNA in 13 of the 70 zebra samples with C_q values that ranged between 29.85 and 35.74 (Table 4.2) and in 6 samples, below the 95% sensitivity of the assay, with C_q values between 36.08 and 39.40. Of these positive samples, *B. caballi* parasite DNA could be detected in seven samples that were positive for *T. equi* on RLB, four samples that hybridized to the *Theileria/Babesia* genus-specific probe only and in eight of the 45 negative RLB samples. All 19 *B. caballi* positive samples were also co-infected with *T. equi* as indicated by the *T. equi* qPCR assay.

Three overlapping nested PCRs resulted in the amplification of ~1480 bp of the complete 18S rRNA gene and nested PCR products were sequenced from 17 of the 70 zebra samples. Nine of the almost full-length 18S rRNA gene sequences were obtained from samples with single *T. equi* infections, and, although eight of the samples contained mixed *T. equi* and *B. caballi* infections, single 18S rRNA sequences were obtained from each of them. BLAST analysis revealed that all sequences obtained were most similar to previously published *T. equi* and not *B. caballi* 18S rRNA sequences. Phylogenetic analyses of the complete *T. equi* 18S rRNA gene sequences using the neighbor-joining, maximum-likelihood and Bayesian inference methods, yielded trees with almost identical topologies and high bootstrap or nodal support values. The phylogenetic analyses indicated the occurrence of the same three phylogenetic groups identified previously (Bhoora et al., 2009a; Chapter 2), designated A, B and C (Figure 4.1). While none of the complete 18S rRNA sequences from zebra grouped in clade A, twelve sequences grouped together with a *T. equi*-like isolate from a horse [strain ET1, accession number AY534882 (Nagore et al., 2004)] in clade B. Sequences grouping in clade B showed 98.6 – 98.9% identity to the *T. equi*-like strain ET1. Sequences representing this genotype were not detected previously in any South African horse samples (Bhoora et al., 2009a; Chapter 2), although similar sequences were identified in Cape mountain zebra from South Africa [*T. equi* RBEQ101, accession number EU642507; *T. equi* RBEQ96, unpublished (Bhoora et al., 2009a; Chapter 2)]. None of the new sequences from zebra were identical to EU642507, and five novel sequences were identified, with 99.5 – 99.8 % identity to EU642507. The remaining five *T. equi* 18S sequences occurred in clade C together with five previously published South African *T. equi* 18S sequences from horses [accession numbers EU888903, EU888905, EU642509, EU642510 and EU642511 (Bhoora et al., 2009a; Chapter 2)]. Three of the *T. equi* sequences from zebra (ZB29, ZB44 and ZB56) were identical to each other as well as to EU888903, while two novel sequences (ZB11 and ZB13), with 99.3 – 99.9% identity to EU888903, were identified (Figure 4.1).

Table 4.2 RLB and qPCR results obtained from zebra samples.

Sample ID	Species	RLB result	*B. caballi*-specific qPCR result	*T. equi*-specific qPCR result
ZB1	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	32.99	23.95
ZB2	*E. quagga burchellii*	Negative	Negative	26.25
ZB3	*E. quagga burchellii*	Negative	34.87	30.59
ZB4	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	Negative	31.07
ZB5	*E. quagga burchellii*	Negative	34.09	24.35
ZB6	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	Negative	30.87
ZB7	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	36.13	27.81
ZB8	*E. quagga burchellii*	Negative	Negative	29.21
ZB9	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	29.85	20.98
ZB10	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	Negative	26.71
ZB11	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	37.08	28.03
ZB12	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	38.26	28.47
ZB13	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	27.92
ZB14	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	31.59
ZB15	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	28.55
ZB16	*E. zebra zebra*	Negative	Negative	31.79
ZB17	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	27.15
ZB18	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	39.40	31.37
ZB20	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	28.13
ZB21	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	33.74	24.25
ZB22	*E. quagga burchellii*	Negative	Negative	26.86
ZB23	*E. quagga burchellii*	Negative	Negative	27.81
ZB24	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	35.47	22.37
ZB25	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	23.40
ZB26	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	34.45	24.77
ZB27	*E. quagga burchellii*	Negative	Negative	23.70
ZB28	*E. quagga burchellii*	*Theileria/Babesia* catch all; *T. equi*	Negative	30.78
ZB29	*E. quagga burchellii*	Negative	Negative	25.89
ZB30	*E. quagga burchellii*	Negative	Negative	28.85
ZB31	*E. quagga burchellii*	*Theileria/Babesia* catch all; *T. equi*	Negative	24.96

Sample ID	Species	RLB result	*B. caballi*-specific qPCR result	*T. equi*-specific qPCR result
ZB32	*E. quagga burchellii*	*Theileria/Babesia* catch all; *T. equi*	Negative	24.81
ZB33	*E. quagga burchellii*	*Theileria/Babesia* catch all; *T. equi*	Negative	32.19
ZB34	*E. quagga burchellii*	Negative	Negative	21.00
ZB35	*E. quagga burchellii*	Negative	Negative	26.91
ZB36	*E. quagga burchellii*	Negative	Negative	Negative
ZB37	*E. quagga burchellii*	Negative	Negative	Negative
ZB38	*E. quagga burchellii*	Negative	Negative	33.38
ZB39	*E. quagga burchellii*	Negative	Negative	30.38
ZB40	*E. quagga burchellii*	Negative	Negative	26.96
ZB41	*E. quagga burchellii*	Negative	34.15	21.71
ZB42	*E. quagga burchellii*	Negative	Negative	26.01
ZB43	*E. quagga burchellii*	Negative	Negative	Negative
ZB44	*E. quagga burchellii*	Negative	Negative	27.14
ZB45	*E. quagga burchellii*	Negative	32.14	25.77
EQ46	*E. quagga burchellii*	Negative	Negative	Negative
ZB47	*E. quagga burchellii*	Negative	Negative	29.13
ZB48	*E. quagga burchellii*	Negative	Negative	25.36
ZB49	*E. quagga burchellii*	Negative	Negative	25.57
ZB50	*E. quagga burchellii*	Negative	Negative	25.95
ZB51	*E. quagga burchellii*	Negative	Negative	30.38
ZB52	*E. quagga burchellii*	Negative	Negative	29.21
ZB53	*E. quagga burchellii*	Negative	Negative	32.54
ZB54	*E. quagga burchellii*	Negative	Negative	34.48
ZB55	*E. quagga burchellii*	Negative	35.74	28.95
ZB56	*E. quagga burchellii*	Negative	36.08	29.80
ZB57	*E. quagga burchellii*	Negative	32.92	26.52
ZB58	*E. quagga burchellii*	Negative	Negative	Negative
ZB59	*E. quagga burchellii*	Negative	Negative	39.14
ZB60	*E. quagga burchellii*	Negative	Negative	26.61
ZB61	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	Negative	28.72
ZB62	*E. quagga burchellii*	Negative	Negative	Negative
ZB63	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	37.31	24.51
ZB64	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	34.97	23.84
ZB65	*E. quagga burchellii*	Negative	Negative	32.33
ZB66	*E. quagga burchellii*	Negative	Negative	Negative
ZB67	*E. quagga burchellii*	Negative	Negative	Negative
ZB68	*E. quagga burchellii*	Negative	Negative	30.61
ZB69	*E. quagga burchellii*	Negative	Negative	Negative
ZB70	*E. quagga burchellii*	Negative	35.22	23.33
ZB71	*E. quagga burchellii*	Negative	Negative	Negative

Figure 4.1 Rooted phylogram determined with Bayesian inference and 5 x 10^6 iterations using ~1480 bp of the full-length 18S rRNA sequences of *T. equi* samples identified in this study and *Theileria* and *Babesia* sequences from GenBank (accession numbers are indicated in parentheses). Posterior probabilities are indicated on the nodes of the tree. Where more than one identical sequence was obtained, one representative sequence is shown with the number of identical sequences obtained indicated in square brackets. ZB6 is representative of sequences obtained from ZB3, ZB30, ZB31, ZB32, ZB33, ZB34 and ZB64. Sequences obtained from ZB29, ZB44 and ZB56 are represented by *T. equi* LFEQ189 (EU888903).

Since it was not possible to directly sequence the full-length primary PCR product from all of the samples, the nested PCR product of the V4 hypervariable region (~670 bp) of the 18S rRNA gene was cloned and sequenced in order to obtain sequence data from more samples. In order to minimize the effects of PCR artifacts introduced during nested PCRs, a consensus sequence was obtained from six clones from each sample. A total of 33 18S rRNA V4 hypervariable region sequences were obtained from 31 samples. Seventeen sequences were obtained from 15 samples with mixed *T. equi* and *B. caballi* infections and 16 sequences from 16 samples with single *T. equi* infections. Once again, BLAST analysis revealed that all of the sequences were most similar to previously published *T. equi* 18S rRNA sequences. Phylogenetic analyses of the *T. equi* V4 hypervariable region sequences using neighbour-joining, maximum-likelihood and Bayesian inference methods again yielded trees with almost identical topologies and high bootstrap or nodal support values, and again, the same three phylogenetic groups were identified (Figure 4.2).

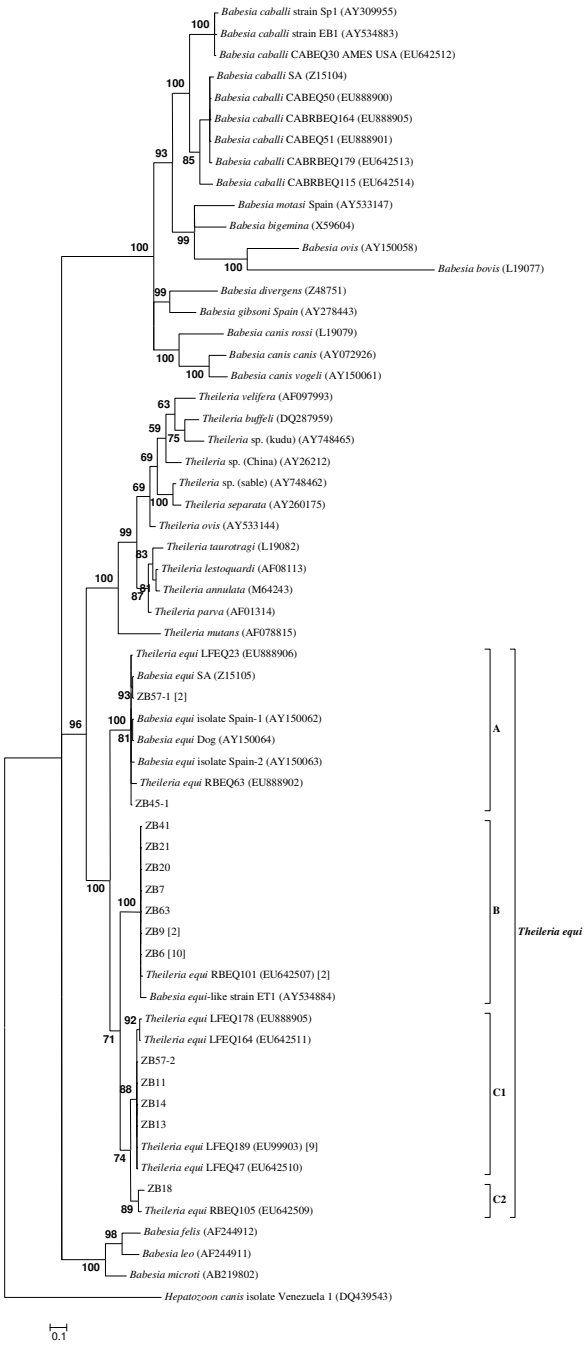


Figure 4.2 Rooted phylogram, determined with Bayesian inference and 5 x 10^6 iterations, of the V4 hypervariable region of the 18S rRNA gene sequences of *T. equi* samples identified in this study and *Theileria* and *Babesia* sequences from GenBank (accession numbers are indicated in parentheses). Posterior probabilities are indicated on the nodes of the tree. Where more than one identical sequence was obtained, one representative sequence is shown with the number of identical sequences obtained indicated in square brackets. One of the sequences obtained from sample ZB57 (ZB57-1) was identical to the published sequence *T. equi* RBEQ178 (EU642508). Identical sequences were obtained from ZB9 and ZB61. ZB6 is representative of sequences obtained from ZB1, ZB3, ZB10, ZB30, ZB31, ZB32, ZB33, ZB34 and ZB64. The sequence obtained from ZB24 was identical to *T. equi* RBEQ101 (EU642507). Similarly, *T. equi* LFEQ189 (EU888903) represents sequences obtained from ZB12, ZB15, ZB16, ZB29, ZB44, ZB45-2, ZB54 and ZB56.

Two zebra samples, ZB45 and ZB57, each contained *T. equi* parasites with two different V4 hypervariable 18S rRNA sequences; in each case the two different sequences grouped in different clades. Two of the *T. equi* 18S rRNA V4 hypervariable region sequences from zebra, ZB45-1 and ZB57-1, clustered within clade A, which contained previously published *T. equi* sequences from horses from South Africa [accession numbers: Z15105 (Allsopp et al., 1994), EU888906, EU642508, EU888902 (Bhoora et al., 2009a; Chapter 2)] and from Spain [accession numbers: AY150062; AY150063 and AY150064 (Criado-Fornelio et al., 2003)]. The ZB57-1 sequence was identical to two of the previously published South African *T. equi* 18S sequences, Z15105 and EU642508, while ZB45-1 showed 99.7% identity to these two sequences. Clade B contained 18 of the *T. equi* sequences from zebra, which showed 99.0% identity to *T. equi*-like strain ET1 [AY534882 (Nagore et al., 2004)], and between 99.5 and 100% identity to EU642507, previously identified in Cape mountain zebra from South Africa (Bhoora et al., 2009a; Chapter 2). Only one sequence (ZB24) was identical to EU642507. In addition to the five novel sequences identified by full-length 18S rRNA sequence analysis, two novel *T. equi* 18S rRNA V4 hypervariable sequences were identified. The remaining 13 *T. equi* sequences from zebra fell into the third phylogenetic group (clade C) which could be sub-divided into clades C1 and C2. Clade C1 contained 12 *T. equi* sequences from zebra, which grouped together with four recently published *T. equi* sequences from horses [accession numbers: EU888905, EU642510, EU642511 and EU888903 (Bhoora et al., 2009a; Chapter 2)]. Eight of the new sequences were identical to EU888903 while the remaining four sequences showed between 99.2 and 99.8% identity to the four published *T. equi* 18S rRNA sequences in clade C1. Clade C2 contained one new *T. equi* sequence from zebra, ZB18, which grouped together with a *T. equi* sequence from zebra that was characterized in a previous study [*T. equi* RBEQ105, accession number EU642509 (Bhoora et al., 2009a; Chapter 2)]. ZB18 showed 98.1% identity to EU642509.

4.5 Discussion

Molecular diagnostic assays developed for the detection of parasites that cause equine piroplasmosis are largely based on the 18S rRNA gene as a genetic marker (Bashiruddin et al., 1999; Battsetseg et al., 2001; Nicolaiewsky et al., 2001; Rampersad et al., 2003; Alhassan et al., 2007). Although nuclear ribosomal rRNA genes have been shown to provide appropriate targets to assist in the identification of species (Chae et al., 1998; Katzer et al., 1998; Allsopp and Allsopp, 2006), sequence heterogeneity has been reported within this gene in several species of

protozoa (Calder et al., 1996; Criado-Fornelio et al., 2003), including *T. equi* and *B. caballi* from horses in South Africa (Bhoora et al., 2009a; Chapter 2). Preliminary sequence data from three Cape mountain zebra samples further suggested that there may be more variation in *T. equi* genotypes in zebra in South Africa (Bhoora et al., 2009a; Chapter 2). In this study, we present sequence data from a larger group of zebra samples which substantiate our earlier findings.

RLB analysis indicated that 35% of the 70 zebra samples tested were positive for *Theileria* and/or *Babesia* piroplasm parasites. In contrast, the prevalence of piroplasm infection in South African horses as indicated by RLB analysis was shown previously to be approximately 63% based on a total of 488 samples collected from various geographical locations around the country (Bhoora et al., 2009a; Chapter 2). These results might suggest that equine piroplasmosis is not as widespread in zebra as it is in horses. However, further analysis using the recently developed TaqMan qPCR assay for *T. equi* (Kim et al., 2008) and the TaqMan MGB™ qPCR assay for *B. caballi* (Bhoora et al., 2009b; Chapter 3), enabled the detection of *T. equi* parasite DNA in 86% of samples and *B. caballi* in 27% of zebra samples tested. The high C_q values obtained by the qPCR assays for many of the samples indicate that the parasites are present at very low parasitaemia. We chose to use the RLB assay as it can detect novel genotypes and identify mixed infections; however, these advantages may be limited in equine piroplasmosis studies by the lack of sensitivity of the test. Both the RLB and the qPCR assays suggest that *T. equi* is more commonly found in zebra than *B. caballi*. This is similar to the observation in horses in South Africa, in which fewer cases of *B. caballi* infections are reported to occur (de Waal, 1990).

Although the RLB did not detect *Babesia* species-specific hybridization signals, nineteen samples were positive for *B. caballi* when tested using the *B. caballi*-specific TaqMan MGB™ qPCR assay. The C_q values reported were much higher than those obtained when samples were tested using the *T. equi*-specific qPCR assay, thus suggesting that the *B. caballi* parasitaemia was much lower than the *T. equi* parasitaemia in the zebra samples. This was further confirmed by cloning and sequencing of PCR products from mixed infections, which yielded only *T. equi* sequences. These results indicate that *B. caballi* infections in zebra may be similar to *B. caballi* infections in horses, in which the parasitaemia generally tends to be very low, rarely exceeding 1% (Hanafusa et al., 1998). It was therefore not possible to determine the level of intraspecific sequence variation in *B. caballi* genotypes that infect zebra in South Africa. In our previous study, however, we found that while there was less sequence variation amongst the *B. caballi* samples from horses, the *B. caballi* 18S rRNA gene sequences could be divided into two main

groups. We can therefore only speculate that there may also be less variation in the 18S rRNA gene sequences of *B. caballi* parasites infecting South African zebra than is observed for *T. equi* parasites of zebra.

Sequence analyses of both the full-length and the V4 hypervariable region of the *T. equi* 18S rRNA gene suggest the existence of 13 new *T. equi* and/or *T. equi*-like sequences from zebra. In many cases there were very few differences between the sequences, which could suggest that errors were introduced during nested PCRs. These differences are unlikely to be artifacts, however, as they were frequently present in sequences obtained from more than one sample. An additional sequence was identified in clade A, seven new sequences were identified in clade B and 5 in clade C. Thus, including the 12 *T. equi* 18S rRNA sequences that were previously identified in South African horse samples (Bhoora et al., 2009a; Chapter 2), a total of 25 *T. equi* 18S rRNA sequences have been identified, which belong to three main phylogenetic groups (Figure 4.1, Figure 4.2).

While the sequence differences in most cases were minimal, the results reported in this study indicate that there may be additional, as yet unidentified *T. equi* 18S rRNA sequences present in the horse and zebra populations in South Africa. The occurrence of previously unrecognized sequence variation could pose a potential problem in the implementation of the *T. equi* qPCR assay, as variation in the regions where the *T. equi* real-time primers and probe have been developed would render the assay non-specific. The *T. equi* TaqMan qPCR assay was, however, developed using a more conserved region of the 18S rRNA gene that occurs outside of the V4 hypervariable region, and this test has been able to detect all genotypes identified to date. Nonetheless, single nucleotide variations in the *T. equi* real-time primer regions and/or in the probe region were observed in two of the full-length sequences obtained in this study which grouped in clade B (ZB7 and ZB64), and in all sequences occurring in clade C, except *Theileria equi* RBEQ105 (accession number EU642509) (Figure 4.3).

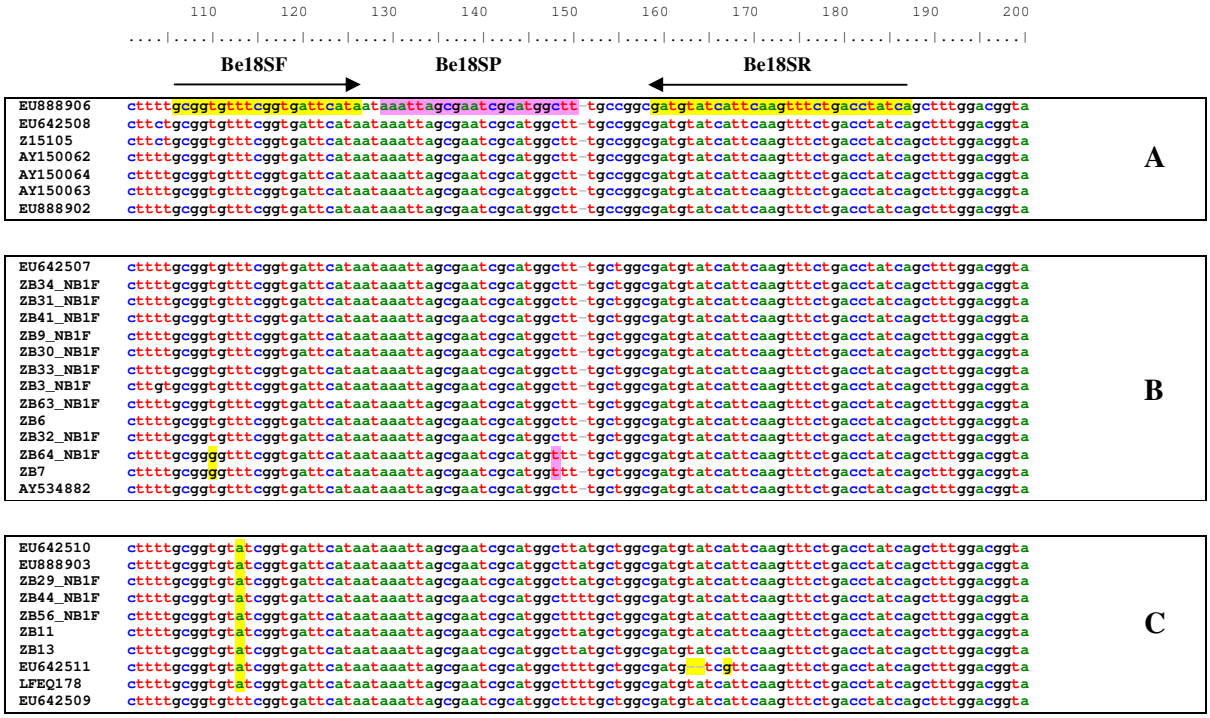


Figure 4.3 Nucleotide sequence alignment of a section of the 18S rRNA gene of the *T. equi* isolates examined in this study to published *T. equi* sequences (*B. equi* SA, Z151505; *B. equi* isolate Spain-1, AY150062; *B. equi* isolate Spain-2, AY150063; *B. equi* Dog, AY150064; *T. equi*-like strain ET1, AY534882; *T. equi* LFEQ23, EU888906; *T. equi* LFEQ178, EU888905; *T. equi* LFEQ189, EU88903; *T. equi* RBEQ63, EU888902; *T. equi* LFEQ164, EU642511; *T. equi* LFEQ47, EU642510; *T. equi* RBEQ105, EU642509; *T. equi* RBEQ178, EU642508; *T. equi* RBEQ101, EU642507). The *T. equi* real-time primers (Be18SF and Be18SR) and probe (Be18SP) sequences designed by Kim et al. (2008) are highlighted in yellow and pink, respectively. Nucleotide differences between sequences in the primer and probe regions are also highlighted in the same colours used to indicate the oligonucleotides. A, B and C represent the three *T. equi* 18S rRNA groups identified in this study.

Less than 10,000 years ago, horses became extinct in the Americas and the only survivors were horses in Asia (www.irishhorsesociety.com/horsedata/horseorigin.htm). Piroplasmosis of horses is therefore thought to be indigenous to Asia, but with the global movement of horses, both *T. equi* and *B. caballi* parasites have become distributed worldwide. When horses were introduced into Africa from Asia, they were almost certainly introduced along with their Asian piroplasm species. The diverse equid species indigenous to the African continent, including several species of zebra, probably carried closely related parasites, which evolved in Africa from a common ancestor of equid piroplasms and which could be transmitted by a wide range of tick vectors. Genetic recombination between the horse and zebra parasites, during sexual reproduction in the tick vectors, possibly led to the greater degree of genetic diversity of the 18S rRNA gene in equine parasites in this geographic region. As previously found for *T. equi* in horses, we have identified three groups of *T. equi* 18S rRNA sequences in zebra. Only two sequences from zebra were identified in group A, which contains sequences that are similar to

previously published *T. equi* sequences from horses in South Africa, Spain and the USA. The second group, group B, contains *T. equi*-like sequences identified in horses in Spain, but thus far only contains *T. equi*-like 18S rRNA sequences from zebra in South Africa. The third group contains new *T. equi* 18S sequences from both zebra and horses in South Africa. It appears that group A *T. equi* parasites occur more frequently in horses in South Africa while group B parasites appear to be more common in South African zebra, but unfortunately the number of zebra samples used in this study was not sufficient to infer an association between a specific parasite genotype and a particular zebra species. The results presented in this study therefore suggest that there exists an assortment of *T. equi* and *T. equi*-like 18S genotypes in all equid species. Examination of a larger sample population, containing samples from equal numbers of plains (*E. quagga*) and Cape mountain zebra (*E. zebra zebra*) species, may allow us to identify whether there is a prevalence of one particular genotype over the other in the different zebra species and might therefore indicate the original parasite for that equid species.

In conclusion, this study presents the first report on the molecular epidemiology of piroplasms infecting zebra in South Africa. The identification of 13 new *T. equi*-like 18S rRNA gene sequences confirms our earlier hypothesis that there is more variation in the *T. equi* genotypes in zebra in South Africa than in horses. Although we were able to confirm the presence of *B. caballi* infections in zebra using the recently developed *B. caballi*-specific MGB qPCR assay (Bhoora et al., 2009b; Chapter 3), sequence variation in the *B. caballi* 18S rRNA gene could not be determined due to the existence of extremely low circulating *B. caballi* parasitaemias in zebra.

4.6 Reference List

Alhassan, A., Thekisoe, O.M.M., Yokoyama, N., Inoue, N., Motloang, M.Y., Mbati, P.A., Yin, H., Katayama, Y., Anzai, T., Sugimoto, C., Igarashi, I., 2007. Development of loop-mediated isothermal amplification (LAMP) method for diagnosis of equine piroplasmosis. Veterinary Parasitology 143, 155-160.

Allsopp, M.T.E.P., Allsopp, B.A., 2006. Molecular sequence evidence for the reclassification of some *Babesia* species. Annals of the New York Academy of Sciences 1081, 509-517.

Allsopp, M.T.E.P., Cavalier-Smith, T., de Waal, D.T., Allsopp, B.A., 1994. Phylogeny and evolution of the piroplasms. Parasitology 108, 147-152.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403-410.

Avarzed, A., Igarashi, I., de Waal, D.T., Kawai, S., Oomori, Y., Inoue, N., Maki, Y., Omata, Y., Saito, A., Nagasawa, H., Toyoda, Y., Suzuki, N., 1998. Monoclonal antibody against *Babesia equi*: characterization and potential application of antigen for serodiagnosis. Journal of Clinical Microbiology 36, 1835-1839.

Bashiruddin, J.B., Camma, C., Rebelo, E., 1999. Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. Veterinary Parasitology 84, 75-83.

Battsetseg, B., Xuan, X., Ikadai, H., Bautista, J.L., Byambaa, B., Boldbaatar, D., Battur, B., Battsetseg, G., Batsukh, Z., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K., 2001. Detection of *Babesia caballi* and *Babesia equi* in *Dermacentor nuttalli* adult ticks. International Journal for Parasitology 31, 384-386.

Beja-Pereira, A., England, P.R., Ferrand, N., Jordan, S., Bakhiet, A.O., Abdalla, M.A., Mashkour, Jordana, J., Taberlet, P., Luikart, G., 2004. African origins of the domestic donkey. Science 304, 1781.

Bhoora, R., Franssen, L., Oosthuizen, M.C., Guthrie, A.J., Zweygarth, E., Penzhorn, B.L., Jongejan, F., Collins, N.E., 2009a. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Veterinary Parasitology 159, 112-120.

Bhoora, R., Quan, M., Franssen, L., Butler, C.M., van der Kolk, J.H., Guthrie, A.J., Zweygarth, E., Jongejan, F., Collins, N.E., 2009b. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. Veterinary Parasitology doi:10.1016/j.vetpar.2009.11.011.

Blench, R.M., 2000. A history of donkeys, wild asses and mules in Africa. In: Roger M.Blench, Kevin C.MacDonald (Eds.), The origins and development of African livestock. Archaeology, genetics, linguistics and ethnography. UCL Press, pp. 339-354.

Brüning, A., Phipps, P., Posnett, E., Canning, E.U., 1997. Monoclonal antibodies against *Babesia caballi* and *Babesia equi* and their application in serodiagnosis. Veterinary Parasitology 68, 11-26.

Calder, J.A.M., Reddy, G.R., Chieves, L., Courtney, C.H., Littell, R., Livengood, J.R., Norval, R.A.I., Smith, C., Dame, J.B., 1996. Monitoring *Babesia bovis* infections in cattle by using PCR-based tests. Journal of Clinical Microbiology 34, 2748-2755.

Chae, J.S., Lee, J.M., Kwon, O.D., Holman, P.J., Waghela, S.D., Wagner, G.G., 1998. Nucleotide sequence heterogeneity in the small subunit ribosomal RNA gene variable (V4) region among and within geographic isolates of *Theileria* from cattle, elk and white-tailed deer. Veterinary Parasitology 75, 41-52.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carretero, J.C., 2003. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part I. Epizootiological aspects. Veterinary Parasitology 113, 189-201.

de Waal, D.T., 1990. The transovarial transmission of *Babesia caballi* by *Hyalomma truncatum*. Onderstepoort Journal of Veterinary Research 57, 99-100.

de Waal, D.T., 1992. Equine piroplasmosis: a review. British Veterinary Journal 148, 6-14.

de Waal, D.T., van Heerden, J., 2004. Equine piroplasmosis. In: Coetzer, J.A.W., Tustin, R.C. (Eds.), Infectious Diseases of Livestock (Second Edition). Oxford University Press, New York, pp 425-434.

Groves, C.P., Bell, H.B., 2004. New investigations on the taxonomy of the zebra genus *Equus*, subgenus *Hippotigris*. Mammalian Biology 69, 182-196.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95-98.

Hanafusa, Y., Cho, K.O., Kanemaru, T., Wada, R., Sugimoto, C., Onuma, M., 1998. Pathogenesis of *Babesia caballi* infection in experimental horses. Journal of Veterinary Medical Science 60, 1127-1132.

Heim, A., Passos, L.M.F., Ribeiro, M.F.B., Costa-Junior, L.M., Bastos, C.V., Cabral, D.D., Hirzmann, J., Pfister, K., 2007. Detection and molecular characterization of *Babesia caballi* and *Theileria equi* isolates from endemic areas of Brazil. Parasitology Research 102, 63-68.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754-755.

Ikadai, H., Osorio, C.R., Xuan, X., Igarashi, I., Kanemaru, T., Nagasawa, H., Fujisaki, K., Suzuki, N., Mikami, T., 2000. Detection of *Babesia caballi* infection by enzyme-linked immunosorbent assay using recombinant 48-kDa merozoite rhoptry protein. International Journal for Parasitology 30, 633-635.

Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Research 33, 511-518.

Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30, 3059-3066.

Katzer, F., McKellar, S., Kirvar, E., Shiels, B., 1998. Phylogenetic analysis of *Theileria* and *Babesia equi* in relation to the establishment of parasite populations within novel host species and the development of diagnostic tests. Molecular and Biochemical Parasitology 95, 33-44.

Kim, C.M., Blanco, L.B.C., Alhassan, A., Iseki, H., Yokoyama, N., Xuan, X., Igarashi, I., 2008. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. Veterinary Parasitology 151, 158-163.

Koch, R., 1905. Vorläufige Mitteilungen über die Ergebnisse einer Forschungsreise nach Ostafrika. Deutsche medizinische Wochenschrift 31, 1865-1869.

Mehlhorn, H., Schein, E., 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. Parasitology Research 84, 467-475.

Nagore, D., Garcia-Sanmartin, J., Garcia-Perez, A.L., Juste, R.A., Hurtado, A., 2004. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: epidemiological survey and phylogenetic analysis. Veterinary Parasitology 123, 41-54.

Neitz, W.O., 1931. Blood parasites of game in Zululand. Report of the Director of Veterinary Services and Animal Industry, South Africa 17, 45-60.

Neitz, W.O., 1965. Classification, transmision, and biology of piroplasms of domestic animals. Annals of the New York Academy of Sciences 64, 56-111.

Nicolaiewsky, T.B., Richter, M.F., Lunge, V.R., Cunha, C.W., Delagostin, O., Ikuta, N., Fonseca, A.S., da Silva, S.S., Ozaki, L.S., 2001. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. Veterinary Parasitology 101, 9-21.

Nijhof, A.M., Pillay, V., Steyl, J., Prozesky, L., Stoltsz, W.H., Lawrence, J.A., Penzhorn, B.L., Jongejan, F., 2005. Molecular characterization of *Theileria species* associated with mortality in four species of African antelopes. Journal of Clinical Microbiology 43, 5907-5911.

Nuttall, G.H.F., Strickland, C., 1910. Die Parasiten des Pferdepiroplasmose resp. der 'Bilary fever'. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten Abteilung I 56, 524-525.

Penzhorn, B.L., 1988. *Equus zebra*. Mammalian Species 314, 1-7.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Rampersad, J., Cesar, E., Campbell, M.D., Samlal, M., Ammons, D., 2003. A field evaluation of PCR for the routine detection of *Babesia equi* in horses. Veterinary Parasitology 114, 81-87.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

Staden, R., 1996. The Staden sequence analysis package. Molecular Biotechnology 5, 233-241.

Staden, R., Beal, K.F., Bonfield, J.K., 2000. The Staden package, 1998. Methods in Molecular Biology 132, 115-130.

Starkey, P., 2000. The history of working animals in Africa. In: The origins and development of African livestock: archaeology, genetics, linguistics, ethnography. University College London Press, London, UK, pp. 478-502.

Swofford, D.L., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4b10. Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular biology and evolution 24, 1596-1599.

Thom, H.B., 1952. Journal of Jan van Riebeeck, vol. 1 (1651-1655). A.A. Balkema, Cape Town/Amsterdam, p. 25.

Young, E., Zumpt, F., Boomker, J., Penzhorn, B.L., Erasmus, B., 1973. Parasites and diseases of Cape mountain zebra, black wildebeest, mountain reedbuck and blesbok in the Mountain Zebra National Park. Koedoe 16, 77-81.

Zweygarth, E., Lopez-Rebollar, L.M., Meyer, P., 2002. *In vitro* isolation of equine piroplasms derived from Cape Mountain zebra (*Equus zebra zebra*) in South Africa. Onderstepoort Journal of Veterinary Research 69, 197-200.

CHAPTER 5

Sequence heterogeneity in the equi merozoite antigen gene (*ema-1*) of *Theileria equi* and development of an *ema-1*-specific TaqMan MGB™ assay for the detection of *T. equi*

5.1 Abstract

A *Theileria equi ema-1*-specific quantitative real-time PCR assay (qPCR) (Ueti et al., 2003) was tested on 107 South African field samples, 90 of which tested positive for *T. equi* antibody using the immuno-fluorescent antibody test (IFAT). The qPCR assay performed poorly, as *T. equi* was detected in only 67 of the 90 IFAT-positive field samples at quantification cycle (C_q) values ranging from 27 to 39.95. Furthermore, a high C_q value of 36.18 was obtained from DNA extracted from a South African *in vitro*-cultured *T. equi* WL isolate [1.38% parasitized erythrocytes (PE)] when a low C_q value (high *T. equi* concentration) was expected. Approximately 600 bp of the *ema-1* gene from 38 samples were sequenced and BLASTN analysis confirmed all sequences to be merozoite surface protein genes, with an identity of 87.1 - 100% to previously published *T. equi ema-1* gene sequences. Alignment of the sequences revealed extensive sequence variations in the target regions of the primers and probes (Ueti et al., 2003), explaining the poor performance of the qPCR assay. Based on these observations, we developed a new TaqMan minor groove binder (MGB™) probe-based qPCR assay, targeting a more conserved region of the *ema-1* gene. This assay was shown to be efficient and specific, and the detection limit, defined as the concentration at which 95% of *T. equi* positive samples are detected, was determined to be 0,00014% PE. The two *ema-1* assays were compared by testing 41 South African field samples in parallel. The results suggested that the new assay was more sensitive than the original assay, as *T. equi* was detected in more samples and at lower C_q values when the new assay was used. Phylogenetic analyses of the 18S rRNA gene sequences and *ema-1* amino acid sequences from the same samples showed inconsistencies between the clades, indicating that the *T. equi* 18S rRNA genetic groups identified in South Africa may not represent distinct *T. equi* lineages and that genetic recombination most probably occurs within *T. equi* parasite populations in South Africa, explaining the sequence diversity observed.

5.2 Introduction

The pathogenesis of haemoprotozoan diseases is orchestrated by merozoite surface proteins, which play pivotal roles in the recognition of, attachment to and penetration of host erythrocytes by parasites (Knowles et al., 1991a). 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., 1991a). 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 orthologous 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). A competitive-inhibition ELISA (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).

The recent application of molecular biological techniques has resulted in the improved detection, identification and genetic characterization of many haemoprotozoan parasites (Caccio et al., 2000; Nagore et al., 2004). The polymerase chain reaction (PCR) has been applied for the detection of many *Theileria* and *Babesia* species and has been reported to have higher sensitivities and specificities when compared to serological assays (Geysen et al., 2003; Buling et al., 2007; Jefferies et al., 2007). Many current molecular assays target nuclear rRNA genes specifically for species identification (e.g. Allsopp et al., 1993), and a number of such PCR tests have been designed to detect *T. equi* (Bashiruddin et al., 1999; Gubbels et al., 1999; Nagore et al., 2004). Many of these assays will not reliably detect all *T. equi* variants, as a previous study in our laboratory revealed extensive sequence variation in the 18S rRNA gene in South African *T. equi* parasites, where three distinct genetic groups were identified (Bhoora et al., 2009a; Chapter 2). Although conserved regions in the 18S rRNA gene exist, and a sensitive and specific qPCR assay for detection of *T. equi* has been developed and evaluated (Kim et al., 2008; Bhoora et al., 2009b; Chapter 3), the existence of as yet undetected 18S rRNA gene sequence variants cannot be ruled out. It would therefore be advantageous to develop a qPCR assay targeting a different gene, which could be used to confirm results obtained from the *T. equi* 18S rRNA qPCR assay.

Genes encoding outer membrane proteins have previously also been targeted for the development of molecular diagnostic assays (Nicolaiewsky et al., 2001; Ueti et al., 2003; Alhassan et al., 2005; Alhassan et al., 2007; Heim et al., 2007). Due to their surface location, however, they are likely to be under intense selection pressure, and sequence heterogeneity in the genes encoding these proteins is expected to occur (Allsopp and Allsopp, 2006). Preliminary studies in our laboratory revealed some discrepencies between the IFAT and cELISA, with the IFAT performing slightly better than the commercially available cELISA in the detection of *T. equi* antibody in the sera of infected horses (Bhoora et al., 2010; Chapter 6). Sequence differences in the EMA-1 antigen within South African *T. equi* isolates may have prevented the detection of *T. equi* antibodies by the cELISA assay in some samples.

Despite the possibility of heterogeneity within the *T. equi ema-1* gene, it has been used as a target in the development of a TaqMan qPCR assay (Ueti *ema-1* qPCR assay) for the quantification of parasite load in *Rhipicephalus* (*Boophilus*) *microplus* ticks, as well as in the peripheral blood of a splenectomized pony experimentally infected with the Florida strain of *T. equi* (Ueti et al., 2003). In this study, the Ueti *ema-1* qPCR assay was tested on South African field samples, and following the poor performance of the test, the *ema-1* gene was sequenced to determine the extent of sequence heterogeneity. This was followed by the development of a sensitive TaqMan MGB™ qPCR assay targeting a more conserved region of the *ema-1* gene for the detection of *T. equi* infections in South African horses.

5.3 Materials and Methods

5.3.1 Theileria equi field samples and isolates

Serum and EDTA-treated blood samples were collected from 107 horses at the South African National Yearling Sale in 2006 (Bhoora et al., 2009a; Chapter 2), and from 41 horses of unknown piroplasm status, resident on three stud farms in the Northern Cape Province, South Africa (Bhoora et al., 2009b; Chapter 3 Section 3.3.3). Field samples from horse (LFEQ23, LFEQ47, LFEQ189) and zebra (RBEQ101 and RBEQ96), for which the 18S rRNA gene had previously been characterized (Bhoora et al., 2009a; Chapter 2), were also used in this study. The *T. equi* WL isolate (Bhoora et al., 2009b; Chapter 3) was cultured as described previously (Zweygarth et al., 1995).

5.3.2 Serological assays

Sera from the 107 National Yearling Sale horse samples were tested for *T. equi* antibody, using (i) a standard IFAT protocol, as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Anonymous, 2008), with the exception that *in vitro*-cultured antigen produced locally in South Africa was used, and (ii) a commercially available cELISA kit as described by the manufacturer (VMRD Inc., Pullman, WA, USA). The IFAT was performed at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) and the cELISA was performed at the Department of Veterinary Tropical Diseases.

5.3.3 *In vitro culture diagnosis*

A modified HL-1 medium (BioWhittaker, Walkersville, MD, USA) (Holman et al., 1994), was used for the *in vitro* culture of *T. equi* from blood samples as described previously (Zweygarth et al., 1995). The medium was supplemented with 20% horse serum, 2 mM L-glutamine, 0.2 mM hypoxanthine, 1 mM L-cysteine hydrochloride, 0.02 mM 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulphonic acid disodium salt (bathocuproine sulphonate, BCS; Serva Feinbiochemica, Heidelberg, Germany), 100 IU/ml penicillin and 100 µg/ml streptomycin. The medium was buffered with 15 mM HEPES and 2.2 g l^{-1} $NaHCO_3$.

A piroplasm-free mare, which was confirmed seronegative for *T. equi* and *B. caballi* using IFAT, was used to collect horse red blood cells (HRBC) by venipuncture into sterile Vac-u-test® tubes containing EDTA as anticoagulant. They were washed four times by centrifugation (650 x g, 10 min, room temperature) and resuspended in a modified Vega y Martinez phosphate-buffered saline solution (Vega et al., 1985) omitting adenine, guanosine and the antibiotics (mVYM). After each wash, the white blood cell layer overlaying the HRBC was removed. After the fourth and final wash, the HRBC were resuspended in mVYM solution and stored at 4 °C until used.

For culture diagnosis, erythrocytes from each test sample were prepared as described above and 100 µl erythrocyte suspension was resuspended in 900 µl complete culture medium and distributed into 24-well culture plates. The plates were incubated at 37° C in either a humidified 5% CO_2-in-air atmosphere or a humidified gas mixture of 5% CO_2, 2% O_2 and 93% N_2. Medium was changed daily by replacement of 700 µl of medium overlaying the erythrocytes in each well. After 5 days, the plate was transferred to an incubator with 5% CO_2 in air.

The *in vitro* culture diagnosis of *T. equi* was performed at the ARC-OVI.

5.3.4 *DNA extraction*

Genomic DNA was extracted from 200 µl of EDTA-treated blood or 200 µl of *in vitro* culture material using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

5.3.5 Theileria equi-specific qPCR assays

The Ueti *ema-1* qPCR assay (primers and probes shown in Table 5.1) was used to detect *T. equi* infections in DNA extracted from 148 equine blood samples (n=107, National Yearling Sale 2006 and n=41, field samples, Northern Cape Province, South Africa). qPCR assays were performed in MicroAmp optical 96-well reaction plates using the StepOnePlus™ Real-time PCR instrument (v. 2.0, Applied Biosystems). Reactions were performed in a total volume of 20 µl comprising 1 x TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 0.9 µM of each forward and reverse primer, 0.25 µM TaqMan probe and 5 µl of genomic DNA. The qPCR cycling conditions were as follows: activation of the FastStart DNA polymerase at 95 ºC for 20 s, then 40 cycles of 1 s at 95 ºC and 20 s at 60 ºC.

Primers and probes (Table 5.1), previously designed for a *T. equi*-specific qPCR assay based on the amplification of a region of the 18S rRNA gene (Kim 18S qPCR assay) (Kim et al., 2008), were used to detect *T. equi* infections in DNA extracted from all 148 equine blood samples. qPCR was performed as described above.

Table 5.1 Oligonucleotide primer and probe sequences used in the *T. equi*-specific qPCR assays.

Primer/Probe	Sequence 5'- 3'	Reference
ema1_f	GAGTCCATTGACCACGTCACC	(Ueti et al., 2003)
ema1_r	GTGCCTGACGACAGTCTTTGG	(Ueti et al., 2003)
ema1_probe	6-FAM-TCGACAAGCAGTCCGAGGAGCACA-TAM	(Ueti et al., 2003)
Be18SF	GCGGTGTTTCGGTGATTCATA	(Kim et al., 2008)
Be18SR	TGATAGGTCAGAAACTTGAATGATACATC	(Kim et al., 2008)
Be18SP	6-VIC-AAATTAGCGAATCGCATGGCTT	(Kim et al., 2008)
RT_EMAF	CCGGCAAGAAGCACAY*CTT	This study
RT_EMAR	TGCCATCGACGAYCTTGAG	This study
RT_EMAprobe	6-FAM-TCCAGGCAAGCGC-MGB	This study

*Ambiguity code : Y=C/T

5.3.6 Amplification and sequencing

Published *T. equi ema-1* gene sequences [*B. equi* isolate 212, AB015212; *B. equi* isolate H-25, AB015208, *B. equi* USDA strain, AB043618 (Xuan et al., 2001); *B. equi* Russia, AB015211; *B. equi* isolate E12, AF261824; *B. equi* Brazil, U97167 (Knowles et al., 1997); *B. equi* Florida, L13784 (Kappmeyer et al., 1993); *B. equi* Morocco, U97168 (Knowles et al., 1997)], were used to design gene-specific primers, TE-EMA1F (5'-TCC ATT GCC ATT TCG AGC ATC CT-3') and TE-EMA1R (5'-TTG ATT CTG CCA TCG CCC TTG-3') for the amplification of a 602 bp fragment of the *T. equi ema-1* gene from South African field samples and isolates. The Genomiphi DNA amplification kit (Amersham Biosciences) was used to exponentially amplify genomic DNA from samples with low parasitaemias, prior to performing the PCR. 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 2.5 µl amplified genomic DNA. The cycling conditions were: an initial denaturation of 5 min at 95 ºC, followed by 35 cycles of 30 s at 95 ºC, 1 min at 60 ºC and 1 min at 72 ºC, and a final extension of 7 min at 72 ºC.

The V4 hypervariable region of the 18S rRNA gene was amplified using a nested PCR as described previously (Bhoora et al., 2009a; Chapter 2). Briefly, primary PCRs were performed using primers NBabesia1F (5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3') and 18SRev-TB (5'-GAA TAA TTC ACC GGA TCA CTC G-3'), and 2.5 µl of genomic DNA that had been amplified using the Genomiphi DNA amplification kit. Primers BT18S2F (5'-GGG TTC GAT TCC GGA GAG GG-3') and BT18S2R (5'-CCC GTG TTG AGT CAA ATT AAG CCG-3') were used to amplify the V4 hypervariable region from primary PCR products (Bhoora et al., 2009a; Chapter 2).

PCR products were purified using the Qiagen PCR purification kit (Qiagen, Hilden, Germany) and sequenced using BigDye chemistry (v.3.1, Applied Biosystems) on a 3130XL genetic analyzer (Applied Biosystems). Sequences were assembled and edited using the Staden software suite (Staden, 1996).

5.3.7 *Phylogenetic analyses*

Multiple sequence alignments were performed using MAFFT employing the FFT-NS-1 algorithm (Katoh et al., 2005) and alignments were adjusted manually using BioEdit, version 7.0.5.2 (Hall, 1999). Aligned *ema-1* nucleotide sequences were translated into amino acid sequences using the ExPasy translate tool (www.expasy.ch/tools/dna). Modeltest, v.3.7 (Posada and Crandall, 1998), was used to identify a TRN + I + G substitution model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites. PAUP* v4b10 (Swofford, 2003) was used to carry out the phylogenetic analyses of the 18S rRNA nucleotide sequences and *ema-1* nucleotide and amino acid sequences using distance, parsimony and maximum-likelihood methods. The EMA-1 phylogenetic tree was constructed (1000 replicates) using the merozoite surface amino acid sequence of *Plasmodium falciparum* (AAX55745) as an outgroup, while the 18S rRNA gene sequence of the *Hepatozoon canis* isolate Venezuela 1 (DQ439543) was used as an outgroup in the construction of the 18S rRNA phylogenetic tree. 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).

5.3.8 *Theileria equi ema-1-specific TaqMan MGB™ qPCR assay*

An alignment of *ema-1* gene sequences obtained from 19 South African samples and 8 published *T. equi ema-1* sequences available on Genbank (Accession numbers: AB015211, AF261824, U97167, U97168, L13784, AB043618, AB015208 and AB015212) was used to identify a more conserved region of the *ema-1* gene for the development of an *ema-1*-specific TaqMan minor-groove binder (MGB™) probe qPCR assay (Bhoora *ema-1*qPCR assay). The Primer express software v3.0 (Applied Biosystems) was used to design the qPCR primers and probes. A primer pair, RT-EMA1F and RT-EMA1R (Integrated DNA Technologies, IDT), in combination with a TaqMan MGB™ probe, RT-EMAprobe (Applied Biosystems), was designed to amplify and detect a 59 bp fragment of the *ema-1* gene (Table 5.1) using the StepOnePlus™ Real-time PCR instrument (Applied Biosystems). Reactions were performed as described above.

A ten-fold dilution series of the *in vitro*-cultured South African *T. equi* WL isolate, with a PE of approximately 13.8% (1.24 x 10^6 parasites/µl), was used to generate a standard curve from which the efficiency of the *T. equi ema-1* qPCR assay was determined. The dilution series (10^0 to 10^{-6}) was prepared in duplicate using uninfected equine red blood cells. DNA was extracted from 200 µl of all diluted samples and qPCR amplifications of the standard dilution series were repeated in triplicate and on ten separate days. The data generated from each of the 30 runs were used to calculate a linear regression equation of C_q (Bustin et al., 2009) against log copy number, from which the efficiency of the assay was determined.

SigmaPlot® (ver. 11) was used to plot a sigmoidal regression equation of sensitivity against log dilution, from which the sensitivity of the assay was determined. The estimated sensitivity and 95% confidence intervals for the true sensitivity of the dilution series prepared, was calculated using the standard error of the estimated sensitivity of the dilution series (Sibeko et al., 2008). The analytical specificity of the assay was evaluated by using DNA extracted from other protozoal parasites expected to occur in equids, including *Trypanosoma brucei evansi*, *Trypanosoma vivax*, *Trypanosoma brucei equiperdum* and *B. caballi*. DNA extracted from a piroplasm-free horse, confirmed negative by IFAT, was included in the assay as a negative control.

5.3.9 Evaluation of the T. equi qPCR assays

Forty-one serum and EDTA-treated blood samples, of unknown piroplasm status, were collected from horses resident on three stud farms in the Northern Cape Province, South Africa, where tick-control measures were only implemented when high tick burdens were observed. Sera were tested for the presence of antibody against *T. equi* using the IFAT (Bhoora et al., 2009b; Chapter 3 Table 3.3). DNA extracted from EDTA-treated blood samples was tested for the presence of *T. equi* parasites using the Kim 18S, Ueti *ema-1* and Bhoora *ema-1* qPCR assays.

5.4 Results

Of the 107 samples collected from horses at the National Yearling Sale (2006), the IFAT and cELISA detected *T. equi* antibodies in 90 and 88 samples, respectively (Appendix B). The *in vitro* culture technique detected *T. equi* in 90 samples. The Kim 18S qPCR assay detected *T. equi* in 92 samples, while the Ueti *ema-1* qPCR assay detected *T. equi* in only 67 samples. All five tests were in agreement for 74 samples (69.2%, 61 positive, 13 negative), although some

samples were detected by the Ueti *ema-1* qPCR assay at much higher C_q values than those obtained with the Kim 18S qPCR assay. Twenty samples that were *T. equi* positive using IFAT, cELISA, *in vitro* culture and the Kim 18S qPCR assay, tested negative using the Ueti *ema-1* qPCR assay. Conflicting results were obtained for the remaining 13 samples, which were positive by at least one test (Table 5.2). In addition, the Ueti *ema-1* qPCR assay detected DNA extracted from a South African *in vitro*-cultured *T. equi* isolate (1.38% PE) at an unexpectedly high C_q value of 36.18. In comparison, this sample tested positive at a C_q value of 22.7 using the Kim 18S qPCR assay.

Table 5.2 Conflicting test results for *T. equi* from 13 samples obtained from horses at the National Yearling Sale 2006, South Africa.

Sample	IFAT	cELISA	*In vitro* culture	Kim 18S rRNA qPCR assay (Kim et al., 2008)	Ueti *ema-1* qPCR assay (Ueti et al., 2003)
EQ2	+	+	+	-	-
EQ30	-	+	+	29.42	29.22
EQ33	-	-	+	31.51	33.9
EQ35	-	+	+	28.30	31.19
EQ50	+	-	+	31.21	-
EQ54	+	-	+	32.04	-
EQ63	+	+	+	-	36.52
EQ70	+	+	-	32.11	-
EQ71	+	+	-	35.15	-
EQ81	+	+	-	32.55	34.83
EQ94	+	-	+	31.25	33.22
EQ103	+	+	+	-	-
EQ107	-	-	-	37.45	-

A ~600 bp fragment of the *ema-1* gene was amplified and sequenced from the *in vitro*-cultured South African *T. equi* WL isolate and from 18 field samples. Sequences were obtained from 10 of the 20 *T. equi*-positive samples that were not detected using the Ueti *ema-1* qPCR assay (EQ6, EQ25, EQ37, EQ38, EQ43, EQ44, EQ46, EQ51, EQ59 and EQ67), from three samples that were detected by the Ueti *ema-1* qPCR assay at C_q values between 36.93 and 39.34 (EQ17, EQ47 and EQ74), and from five South African horse and zebra samples, for which the 18S rRNA gene had previously been characterized (LFEQ23, LFEQ47, RBEQ101, LFEQ189 and RBEQ96) (Bhoora et al., 2009a; Chapter 2). BLAST analysis confirmed that all sequences obtained were merozoite surface protein genes, which showed between 87.5 and 100% identity to previously published

T. equi ema-1 gene sequences (Accession numbers: AB015211, AF261824, U97167, U97168, L13784, AB043618, AB015208 and AB015212).

Phylogenetic analyses of both the *ema-1* nucleotide sequences and the predicted EMA-1 amino acid sequences yielded trees with almost identical topologies and high bootstrap or nodal support values. The analyses showed that the EMA-1 amino acid sequences obtained in this study fell into three main groups, designated A, B and C (Figure 5.1). Group A contained two EMA-1 sequences from samples for which the 18S rRNA gene sequence had previously been determined [LFEQ23 and LFEQ47 (Bhoora et al. 2009a; Chapter 2)]; these grouped together with a number of EMA-1 amino acid sequences from *T. equi* isolates from Brazil, Morocco, Florida and Russia (Kappmeyer et al., 1993; Knowles et al., 1997; Xuan et al., 2001) (Accession numbers, respectively: AAC38826, AAC38827, AAA72370, BAA32978) (Figure 5.1). Group B contained the *T. equi* USDA strain (Accession number: BAA96134), and four South African EMA-1 sequences. Two of these were obtained from samples for which the 18S rRNA gene had previously been characterized [RBEQ101 and RBEQ96 (Bhoora et al., 2009a; Chapter 2)], while two were obtained from samples that were detected by the Ueti *ema-1* qPCR assay at very high C_q values (EQ17 and EQ47). The remaining 13 South African EMA-1 sequences grouped together with two EMA-1 sequences from isolates 212 and H-25 (Accession numbers: BAA32979 and BAA32975) in group C. Ten of these South African sequences were from samples that were not detected by the Ueti *ema-1* qPCR assay, while two were from samples that were detected by the Ueti *ema-1* qPCR assay at very high C_q values (EQ74 and the *in vitro*-cultured South African *T. equi* WL isolate). The remaining sequence in group C was obtained from a sample for which the 18S rRNA gene had previously been characterized [LFEQ189 (Bhoora et al., 2009a; Chapter 2)].

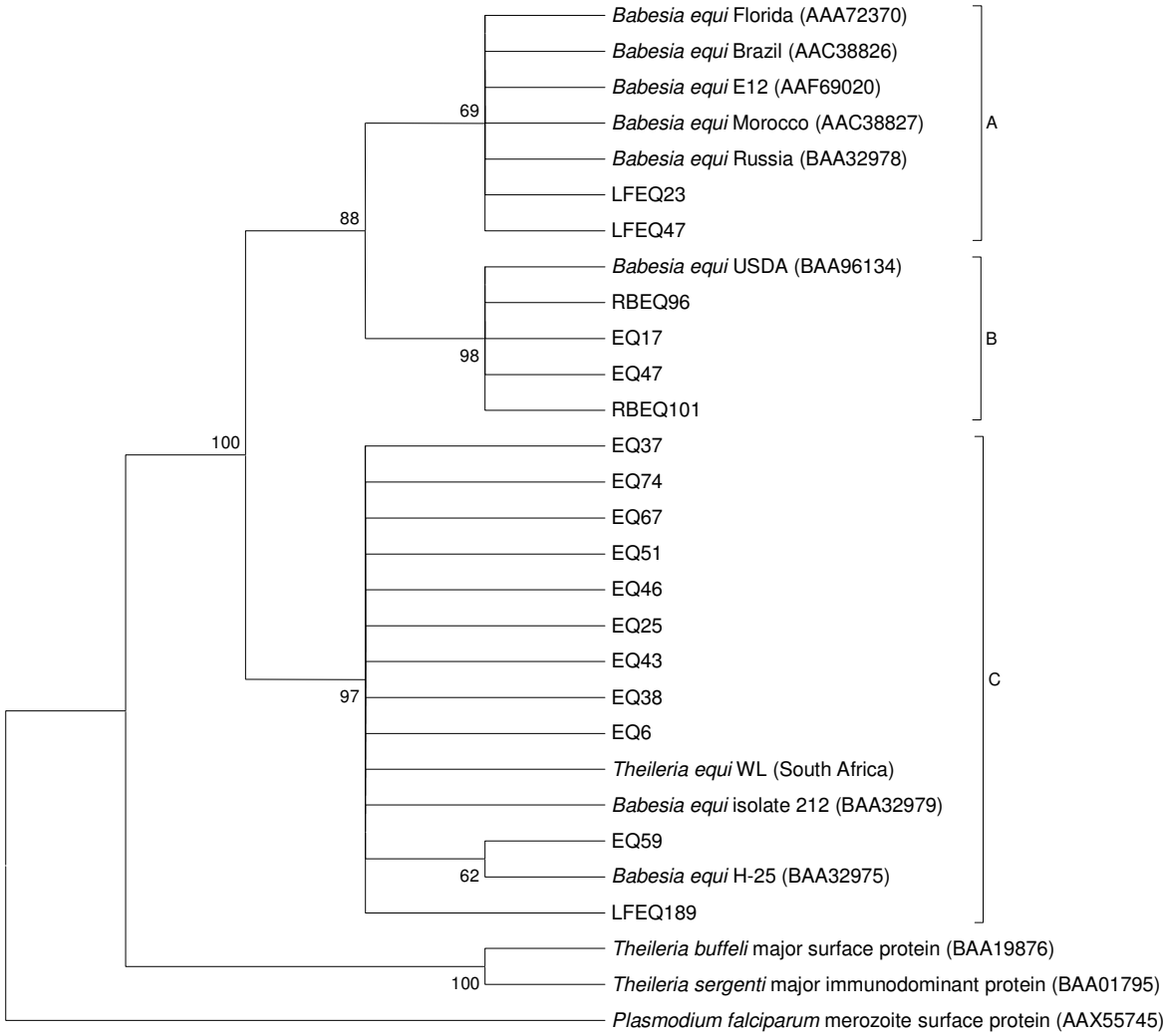


Figure 5.1 Rooted 50% majority-rule consensus cladogram, calculated using maximum-parsimony and bootstrap estimates from 1000 replicates of the EMA-1 amino acid sequences identified in this study and the *T. equi* EMA-1 sequences from the public sequence database (accession numbers are indicated in parenthesis). Bootstrap values are indicated on the nodes of the tree.

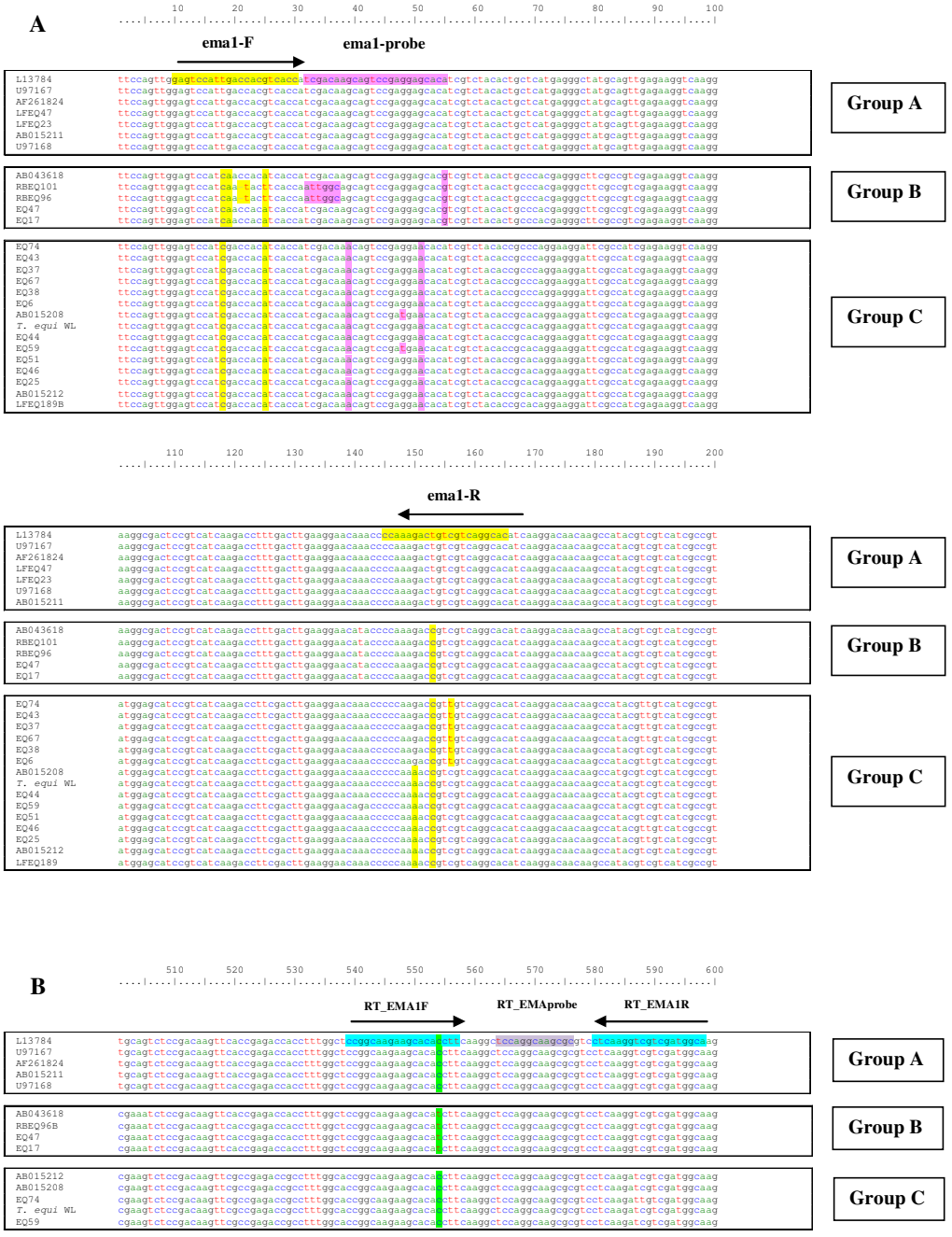


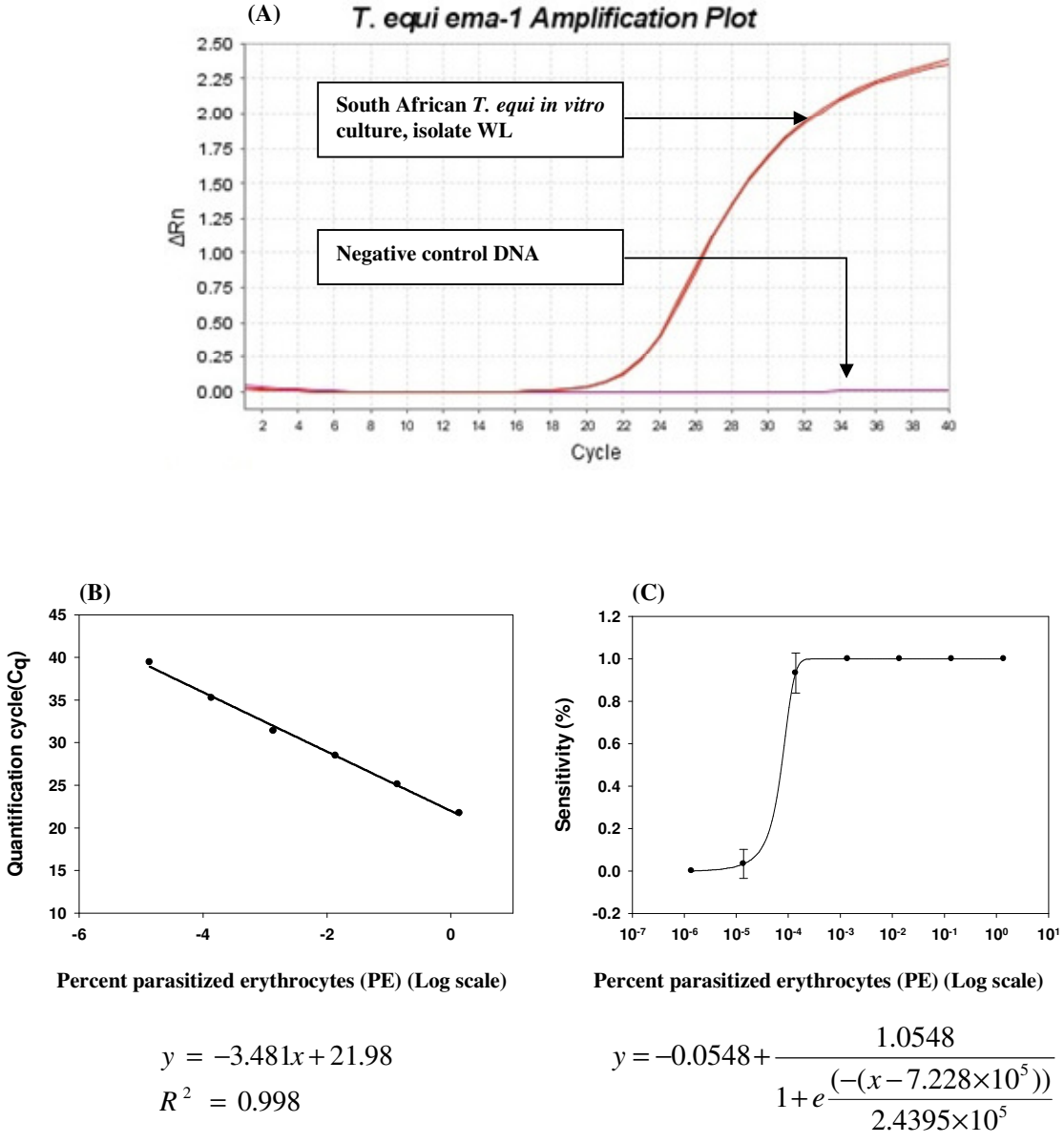
Figure 5.2 **(A)** Nucleotide alignment of *T. equi ema-1* gene sequences obtained in this study, to published *T. equi ema-1* gene sequences (*B. equi* isolate 212, AB015212; *B. equi* isolate H-25, AB015208, *B. equi* USDA strain, AB043618; *B. equi* Russia, AB015211; *B. equi* isolate E12, AF261824; *B. equi* Brazil, U97167; *B. equi* Florida, L13784; *B. equi* Morocco, U97168). The sequences of the real-time forward (ema1-F) and reverse (ema1-R) primers designed by Ueti et al. (2003) are highlighted in yellow. The probe sequence (ema1-probe) is highlighted in pink. Nucleotide differences in the primer and probe regions are highlighted in the same colors used to indicate the oligonucleotides. **(B)** An alignment of the *T. equi ema-1* gene sequences indicating the region where the Bhoora *ema-1* qPCR primers and probes were designed. The Bhoora *ema-1* qPCR primers (RT_EMA1F and RT_EMA1R) are highlighted in blue and the probe sequence (RT_EMAprobe), is highlighted in purple. Nucleotides highlighted in green indicate the position of primer degeneracy. Groups A, B and C represent the three *T. equi* 18S rRNA groups identified in this study.

Sequence alignments demonstrated the occurrence of nucleotide variation in the regions of the *ema-1* gene that the Ueti qPCR primers and probes had targeted (Figure 5.2A). Sequences in group A, which contained the Florida *ema-1* sequence (Accession number: L13784) used by Ueti et al. (2003) to design the qPCR test, showed no nucleotide heterogeneity in the regions where the primers and probe had been designed. Three sequences occurring in group B, including the *ema-1* gene sequence from the *B. equi* USDA strain (accession number AB043618), contained three nucleotide differences in the forward primer sequence, a single nucleotide difference in the reverse primer sequence and one difference at the 3' end of the qPCR probe sequence. Group B also contained two *ema-1* sequences amplified from zebra samples (RBEQ101 and RBEQ96), which showed five nucleotide differences in the forward primer region, seven differences in the probe region and one difference in the reverse primer sequence. Similarly, for *ema-1* sequences in group C, two nucleotide differences occurred in the forward and reverse primer regions, while up to three differences could be observed in the qPCR probe region.

The Bhoora *ema-1* qPCR primers and probe were designed in a conserved region of the *T. equi ema-1* gene (Figure 5.2B). The forward primer (RT_EMA1F) was designed to contain a single nucleotide degeneracy, which allows for the detection all sequences in each of the three *ema-1* groups identified. The Bhoora *ema-1* qPCR assay had an efficiency of 93.8% in amplifying a 59 bp conserved region of the *ema-1* gene from the *in vitro*-cultured *T. equi* WL isolate (Figure 5.3A and B). *Theileria equi* DNA was detected in all 30 replicates of the dilutions of the *in vitro*-cultured *T. equi* WL isolate ranging from 1.38 to 1.38 x 10^{-3}% PE. At lower PE values, sensitivity decreased (Figure 5.3C). The detection limit of the assay, defined as the concentration at which 95% of the positive samples were detected, was 1.4 x 10^{-4}% PE at a C_q of 35.4. The assay was specific for *T. equi*, in that no amplification signals were observed from DNA extracted from any of the other protozoal parasites expected to occur in equids including *Babesia caballi* as well as a number of *Trypanosoma* species (results not shown).

The Bhoora *ema-1* qPCR assay detected *T. equi* DNA in 90 (84%) of the 107 previously tested samples at C_q values ranging between 24.39 and 34.70. The IFAT, cELISA, *in vitro* culture, Kim 18S qPCR and Bhoora *ema-1* qPCR assays were in full agreement for 94 samples (87.9%, 81 positive, 13 negative). The Bhoora *ema-1* qPCR assay detected *T. equi* DNA in all 20 samples that tested negative using the Ueti *ema-1* qPCR assay, but which were *T. equi*-positive using the four other assays (IFAT, cELISA, *in vitro* culture and *T. equi* 18S rRNA qPCR assay); the C_q values obtained were comparable to those obtained using the Kim 18S qPCR assay (Table

5.3). In addition, the Bhoora *ema-1* qPCR assay detected *T. equi* DNA in the South African *in vitro*-cultured *T. equi* WL isolate at a C_q value of 20.0, in contrast to the C_q value of 36.18 obtained from using the Ueti *ema-1* qPCR assay (Figure 5.4).



$$y = -3.481x + 21.98$$
$$R^2 = 0.998$$

$$y = -0.0548 + \frac{1.0548}{1 + e^{\frac{(-(x - 7.228\times10^5))}{2.4395\times10^5}}}$$

Figure 5.3 **(A)** Detection of positive control DNA (*T. equi* WL *in vitro*-cultured isolate) using the Bhoora *ema-1* qPCR assay, indicated by an increase in the fluorescence signal. No increase in fluorescence was observed in the negative control sample (DNA extracted from blood from a certified piroplasm free horse). The threshold (ΔRn) value was set at 0.03. **(B)** Standard curve for the quantification of the *T. equi ema-1* gene. C_q values are plotted against the log% parasitaemia of the initial 10-fold dilution series of the parasite DNA equivalent to 1.242 x 10^5 to 1.242 parasites/µl of infected blood. **(C)** The sensitivity and 95% confidence intervals for the *ema-1* TaqMan MGB™ qPCR assay. The sensitivity was determined using a 10-fold dilution series from 10^{-1} to 10^{-7} prepared from an *in vitro* culture of *T. equi* WL with approximately 1.38% PE.

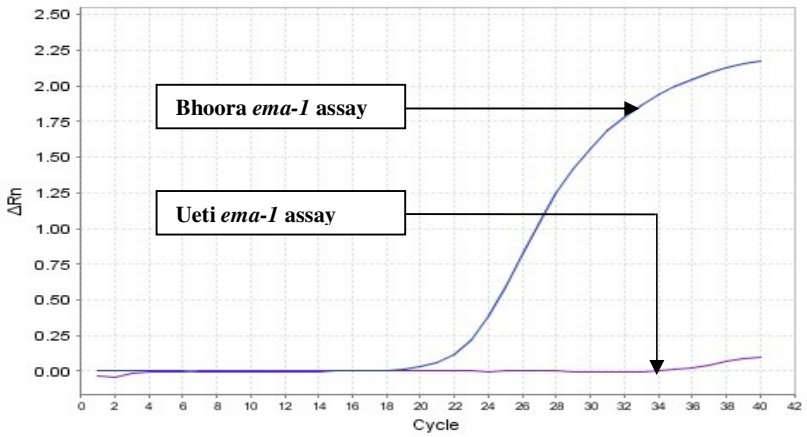


Figure 5.4 Comparison of the efficiencies of the Ueti and Bhoora *ema-1* qPCR assays in the amplification of the *T. equi ema-1* gene from DNA extracted from the South African *T. equi* WL *in vitro*-cultured isolate with approximately 1.38% PE. The threshold (ΔRn) value was set at 0.03.

Forty-one field samples were subsequently used to evaluate the *T. equi* qPCR assays (Bhoora *ema-1*, Ueti *ema-1* and Kim 18S) (Table 5.4). The three tests were in full agreement for 36 samples (87.8%, 32 positive, 4 negative). The Bhoora *ema-1* qPCR assay detected *T. equi* in all samples that were IFAT-positive, whereas the other two qPCR assays failed to detect *T. equi* in two IFAT-positive samples (Kim11 and Kim20). In addition, the Bhoora *ema-1* qPCR assay detected *T. equi* in three samples that were IFAT-negative (Kim18, Kim31 and Kim40); the other two qPCR assays detected *T. equi* DNA in only one of these samples (Kim18).

Although *T. equi* could be detected in most of the 41 field samples using both *ema-1* qPCR assays, the C_q values obtained using the Bhoora *ema-1* qPCR assay were consistently lower than those obtained using the Ueti *ema-1* qPCR assay (Table 5.4). Furthermore, in seven samples (Kim2, Kim6, Kim14, Kim22, Kim28, Kim29 and Kim38), the Bhoora *ema-1* qPCR assay detected *T. equi* DNA at C_q values that were considerably lower than those obtained using the Ueti *ema-1* qPCR assay.

Table 5.3 Comparison of test results for 20 samples obtained from horses at the National Yearling Sale 2006, South Africa, that tested negative for *T. equi* using the Ueti *ema-1* qPCR assay (Ueti et al., 2003), and tested positive using five other assays (Kim 18S qPCR assay, Bhoora *ema-1* qPCR assay, IFAT, cELISA and *in vitro* culture).

Sample	Ueti *ema-1* qPCR (Ueti et al., 2003)	Kim 18S qPCR assay (Kim et al., 2008)	Bhoora *ema-1* qPCR (this study)	IFAT	cELISA	*In vitro* culture
EQ6	-	28.85	26.88	+	+	+
EQ8	-	28.55	28.76	+	+	+
EQ10	-	29.40	29.56	+	+	+
EQ20	-	32.40	31.23	+	+	+
EQ25	-	28.58	25.92	+	+	+
EQ36	-	31.14	28.94	+	+	+
EQ37	-	31.54	29.94	+	+	+
EQ38	-	33.34	33.30	+	+	+
EQ43	-	31.96	27.76	+	+	+
EQ44	-	29.05	26.72	+	+	+
EQ46	-	28.99	26.32	+	+	+
EQ51	-	29.48	26.73	+	+	+
EQ53	-	31.05	28.92	+	+	+
EQ59	-	29.98	27.40	+	+	+
EQ60	-	32.85	31.01	+	+	+
EQ61	-	34.93	33.76	+	+	+
EQ64	-	30.93	28.62	+	+	+
EQ67	-	32.79	31.78	+	+	+
EQ79	-	32.52	30.35	+	+	+
EQ87	-	32.91	30.77	+	+	+

Phylogenetic analyses of the *ema-1* gene fragment amplified from 17 of the 41 field samples confirmed that the *ema-1* sequences fell within the same three groups described above (results not shown). Eleven sequences (Kim4, Kim10, Kim12, Kim16, Kim18, Kim23, Kim24, Kim25, Kim27, Kim32 and Kim41), which were all detected by the Ueti *ema-1* qPCR assay, clustered within group A. Four sequences (Kim6, Kim14, Kim20 and Kim38) clustered within group B, while the remaining two sequences (Kim28 and Kim30) occurred in group C. With the exception of Kim20 which was negative, the samples occurring in group B and C were detected using the Ueti *ema-1* qPCR assay, but at C_q values that were much higher than those obtained using the Bhoora *ema-1* qPCR assay.

Table 5.4 Comparison of test results from 41 field samples obtained from horses resident in the Northern Cape Province, South Africa.

Sample	IFAT	Ueti *ema-1* qPCR assay (Ueti et al., 2003)	*Bhoora ema-1* qPCR assay (this study)	Kim 18S qPCR assay (Kim et al. 2008)
Kim1	+	25.75	22.40	25.07
Kim2	+	30.43	22.30	25.14
Kim3	+	36.13	31.45	34.49
Kim4	+	24.28	21.16	24.18
Kim5	+	29.88	26.08	30.00
Kim6	+	31.40	23.94	25.53
Kim7	+	28.52	24.17	25.07
Kim8	+	33.93	30.85	34.13
Kim9	+	24.21	20.75	23.86
Kim10	+	24.78	21.81	24.19
Kim11	+	-	30.60	-
Kim12	+	22.16	18.87	22.37
Kim13	+	28.02	22.69	25.10
Kim14	+	30.38	21.53	24.09
Kim15	+	25.97	20.87	24.72
Kim16	+	25.86	22.84	26.04
Kim17	+	27.47	23.32	26.18
Kim18	-	28.85	27.02	30.13
Kim19	+	28.52	22.99	25.40
Kim20	+	-	25.61	-
Kim21	+	25.62	20.75	23.24
Kim22	+	32.61	22.21	25.03
Kim23	+	29.40	26.90	30.00
Kim24	+	22.15	19.41	22.88
Kim25	+	23.89	21.68	24.50
Kim26	+	33.09	30.27	32.16
Kim27	+	21.76	19.39	22.48
Kim28	+	34.23	19.75	22.86
Kim29	+	34.98	19.34	31.06
Kim30	+	31.58	28.21	23.17
Kim31	-	-	34.51	-
Kim32	+	29.25	26.14	29.09
Kim33	-	-	-	-
Kim34	-	-	-	-
Kim35	-	-	-	-
Kim36	-	-	-	-
Kim37	+	29.00	26.84	30.10
Kim38	+	37.33	27.67	30.44
Kim39	+	27.50	24.66	28.64
Kim40	-	-	35.40	-
Kim41	+	34.12	31.16	34.12

To determine whether the three *ema-1* groups identified in this study corresponded to the three 18S rRNA clusters identified previously (Bhoora et al., 2009a; Chapter 2), the V4 hypervariable region of the 18S rRNA gene was amplified and sequenced from 13 randomly selected field samples, representative of each EMA-1 phylogenetic group. The 18S rRNA sequence was also obtained from the *in vitro*-cultured South African *T. equi* WL isolate. Five 18S rRNA gene sequences determined previously [Accession numbers: EU888906, EU642510, EU888903, EU642507 and an unpublished sequence from RBEQ96 (Bhoora et al., 2009a; Chapter 2)] were included in the analysis from samples for which the *ema-1* gene was sequenced in this study (LFEQ23, LFEQ47, LFEQ189, RBEQ101 and RBEQ96, respectively). The 18S rRNA sequences from eleven of the samples clustered in group A, two in group B and six in group C (Figure 5.5). Of the eleven samples that clustered in 18S rRNA group A, only three samples (*T. equi* LFEQ23, Kim27 and Kim32) grouped in EMA-1 group A, two samples (Kim38 and EQ47) occurred in EMA-1 group B while the remaining six samples (*T. equi* WL, EQ38, EQ44, EQ25, EQ43 and Kim30) were found in EMA-1 group C (Figure 5.6). Both samples that were present in 18S rRNA group B (RBEQ101 and RBEQ96) also clustered in EMA-1 group B (Figure 5.6). Of the six samples that grouped in 18S rRNA group C, three (LFEQ47, Kim4 and Kim11) were found in EMA-1 group A, one (Kim20) in EMA-1 group B, and two (*T. equi* LFEQ189 and EQ6) in EMA-1 group C (Figure 5.6).

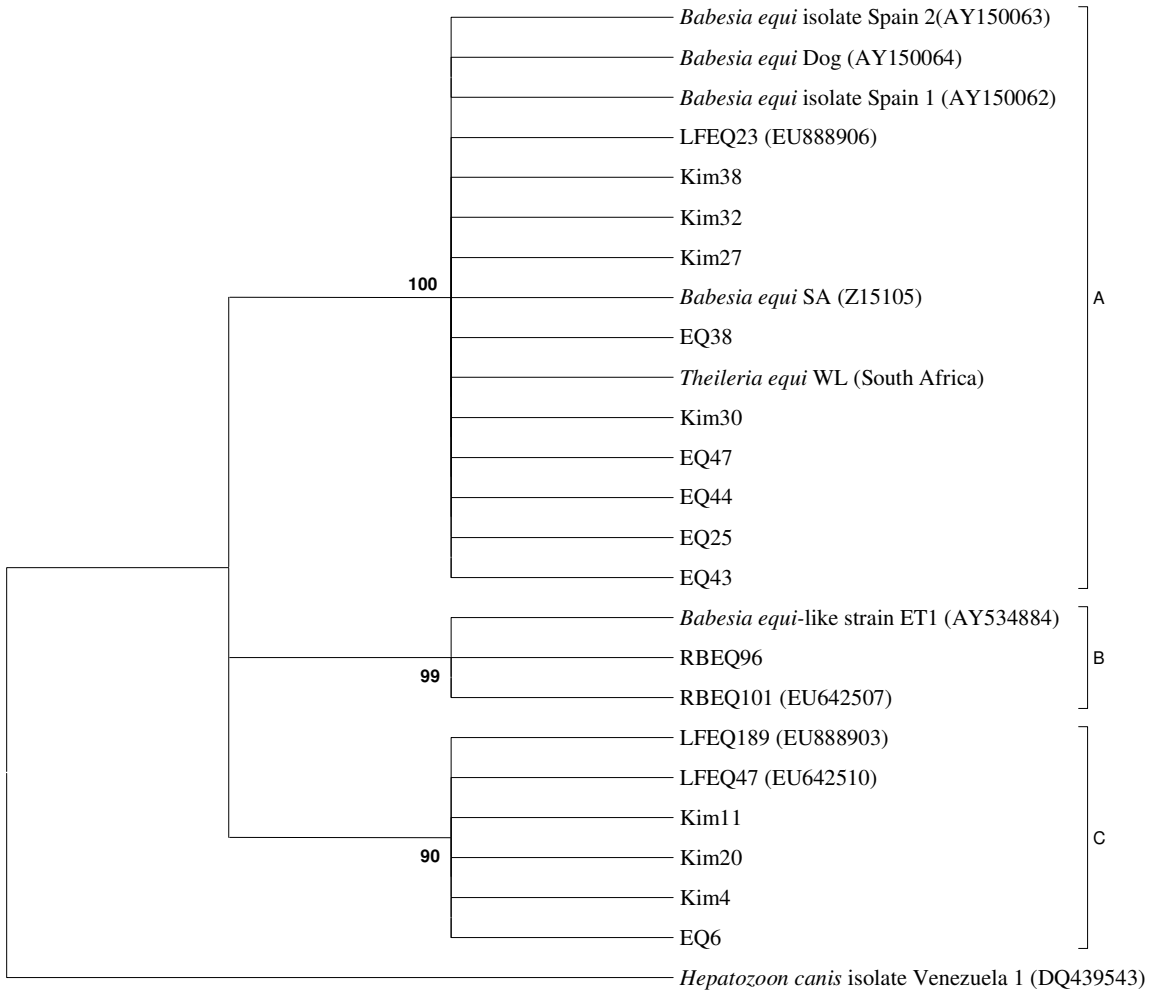


Figure 5.5 Rooted 50% majority-rule consensus cladogram, calculated using maximum-parsimony and bootstrap estimates from 1000 replicates, of the *T. equi* 18S rRNA gene sequences identified in this study and the *T. equi* 18S rRNA sequences from the public sequence databases (accession numbers are indicated in parenthesis). The *Hepatozoon canis* 18S rRNA gene sequence (accession number: DQ439543) was used as an outgroup. Bootstrap support values are indicated on the nodes of the trees.

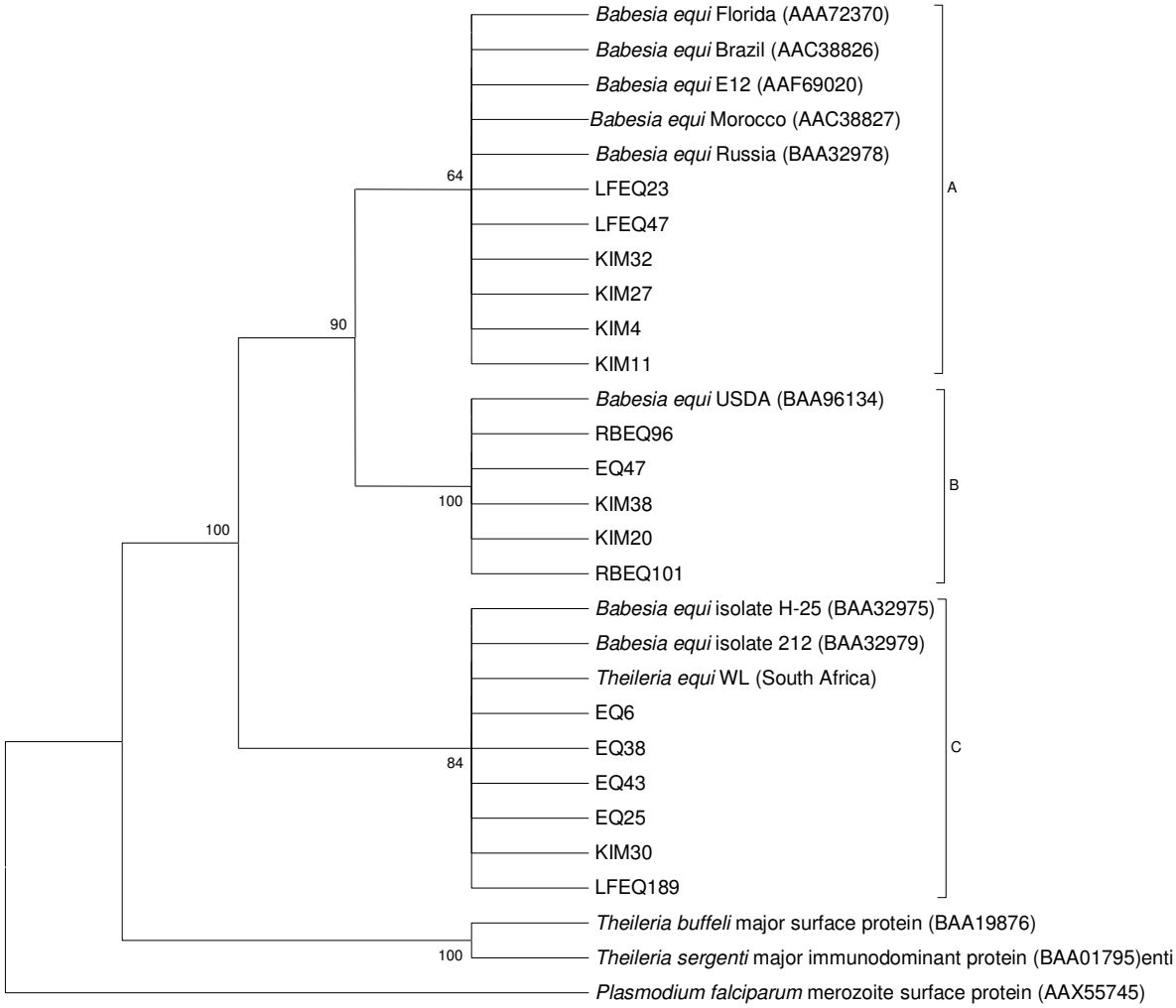


Figure 5.6 Rooted 50% majority-rule consensus cladogram, calculated using maximum-parsimony and bootstrap estimates from 1000 replicates of representative EMA-1 amino acid sequences identified in this study and the *T. equi* EMA-1 amino acid sequences from the public sequence database (accession numbers are indicated in parenthesis). Bootstrap values are indicated on the nodes of the tree.

5.5 Discussion

The gene encoding an immunodominant surface protein of *T. equi*, equi merozoite antigen-1 (EMA-1) has previously been used as a target in the development of a TaqMan qPCR assay to determine the expression levels of merozoite surface proteins at critical stages within the tick vectors (Ueti et al., 2003). It was subsequently used to determine the ability of tick vectors to acquire and transmit *T. equi* following feeding on a chronically infected horse with low piroplasm parasitaemia (Ueti et al., 2003; Ueti et al., 2005). Recently, a modified multiplex qPCR assay combining the Ueti *ema-1* qPCR assay and a *B. caballi rap-1* TaqMan qPCR assay

was used in the detection of equine piroplasmosis in Brazil (Ueti et al., 2003; Heim et al., 2007). In all instances, the Ueti *ema-1* qPCR assay was shown to reliably amplify the *ema-1* gene and therefore detect *T. equi* infections. In this study, however, the Ueti *ema-1* qPCR assay failed to detect parasite DNA in 20 South African samples that tested positive for *T. equi* using the IFAT, cELISA, *in vitro* culture and the Kim 18S qPCR assay. Although the Ueti *ema-1* qPCR assay has previously been reported to detect *T. equi* in chronically infected horses with parasitaemias ranging from a low of 1.99 to a high of 1000 parasites/µl (Ueti et al., 2005), this assay could only detect *T. equi* in the *in vitro*-cultured South African *T. equi* WL isolate (1.38% PE) at a high C_q value. The lack of specificity of the Ueti *ema-1* qPCR primers and/or probe sequences may explain the discrepancies observed between the high Ueti *ema-1* qPCR C_q values and the lower C_q values obtained using the Kim 18S qPCR assay, in samples that were positive by both assays. These results therefore suggested that the Ueti *ema-1* qPCR assay should be reassessed and the *ema-1* gene sequences of South African *T. equi* samples determined, to ensure that genetic variation within the qPCR primer and probe sequences was not overlooked.

There are several possible explanations for the conflicting results observed for the 13 samples that tested positive by at least one of the five assays used (Table 5.2). Sero-positive but qPCR-negative results could be explained by the fact that antibodies may be present in the absence of parasites (Holman et al., 1993). Such results might also be explained by the presence of PCR inhibitors or could indicate sequence heterogeneity in the qPCR primer and/or probe regions that prevent the detection of parasite DNA. Sero-negative but qPCR-positive results could indicate a new infection prior to the development of antibodies (Donnelly et al., 1982; Bhoora et al., 2009b, Chapter 3). One of the limitations of IFAT is that it is often difficult to differentiate between weak positive and negative reactions (Weiland, 1986). The cELISA has however been shown to have higher specificities for *T. equi* when compared to IFAT (Knowles et al., 1991b; Shkap et al., 1998; Xuan et al., 2002), which would explain the IFAT-negative but cELISA-positive results. In contrast, cELISA-negative but IFAT-positive results could indicate either the genetic inability of these horses to produce antibodies to the epitope defined by the monoclonal antibody, the absence of the epitope on the *T. equi* isolates that infected these horses or the reduced sensitivity of the cELISA assay (Knowles et al., 1991b).

Sequence analysis of the *T. equi ema-1* gene from South African field samples and isolates confirmed the existence of distinct *ema-1* sequences, which belonged to three main phylogenetic groups. To ensure the stability of the *ema-1* gene sequence used as a target in the qPCR assay, Ueti et al. (2005) reported on the cloning and sequencing of the full-length (816 bp) *ema-1* gene from the Florida strain of *T. equi* (Accession number: L13784) from 11 time points over 39 months. In their study, five clones containing single nucleotide substitutions in the qPCR forward primer and probe binding regions were identified and compared to clones without any changes. Despite the presence of these single nucleotide changes, the efficiency of the qPCR assay remained unaffected (Ueti et al., 2005). Our data suggest a marked decrease in the efficiency of amplification and detection of South African *T. equi* parasites with five or more sequence differences in the primer and probe regions. An alignment of the *ema-1* fragment sequenced from 38 South African *T. equi* samples indicated that the qPCR primers and probe sequence regions were conserved among all samples occurring in group A. However, nucleotide differences in sequences occurring in groups B and C probably explain either the failure, or the decreased sensitivity of the Ueti *ema-1* qPCR assay in detecting *T. equi* in some samples.

Based on the observed variation in the South African *ema-1* gene sequences, a TaqMan MGB™ qPCR (Bhoora *ema-1* qPCR assay), targeting a more conserved region of the gene was developed for the quantitative detection of *T. equi* from South African equine blood samples. The Bhoora *ema-1* qPCR assay was highly sensitive, able to detect as low as 1.4 x 10^{-4}% PE. The Ueti *ema-1* qPCR assay (Ueti et al., 2003; Ueti et al., 2005) could detect *T. equi* in chronically infected horses with parasitaemias ranging from a low of 1.99 parasites/µl to a high of 1000 parasites/µl. Kim et al. (2008) determined the detection limit of their 18S rRNA-based qPCR assay (Kim 18S qPCR assay) to be 1 x 10^{-5}% PE; in our hands, the detection limit (95% sensitivity) of this qPCR assay was 1.9 x 10^{-4}% PE (Bhoora et al., 2009b; Chapter 3). The sensitivity of the Bhoora *ema-1* qPCR assay is therefore comparable to that reported for the Kim 18S qPCR assay. Furthermore, no amplification could be observed from DNA of other protozoal parasites expected to occur in equids, thus indicating the high specificity of the assay. When tested for its ability to detect *T. equi* parasite DNA from the 107 field samples collected from horses at the National Yearling sale (2006), the Bhoora *ema-1* qPCR assay was shown to be more sensitive than the Ueti *ema-1* qPCR assay.

Other molecular assays that target the *ema-1* gene of *T. equi* have also been developed (Battsetseg et al., 2001; Nicolaiewsky et al., 2001; Alhassan et al., 2005; Salim et al., 2008). Using nested PCR, Nicolaiewsky et al. (2001) reported the amplification of a 102 bp *ema-1* fragment from equine blood samples with a parasitaemia as low as 6 x 10^{-6}%. In comparison, the Bhoora *ema-1* qPCR assay could detect *T. equi* at 1.4 x 10^{-4}% PE. However, real-time PCR technology provides several advantages over the use of conventional and nested PCR assays: there is no need for post-PCR manipulation which reduces the risk of contamination, and it is possible to quantify the amount of target DNA in the reaction. Although the internal primers (EMAI-F and EMAI-R), designed by Nicolaiewsky et al. (2001) to amplify the 102 bp fragment, would have been able to amplify all sequences occurring in group A, two and three nucleotide differences were observed in the EMAI-F primer region of sequences occurring in groups B and C, respectively. No nucleotide variations were observed in the EMAI-R primer region for sequences occurring in these two groups. These differences, although minor, may result in a reduced efficiency of amplification.

The *T. equi* qPCR assays were evaluated by testing their ability to detect parasites in 41 South African field samples (Table 5.4). Significant correlation (90%) was observed between the IFAT and the Bhoora *ema-1* qPCR assay results. The Ueti *ema-1* qPCR assay detected *T. equi* in fewer samples (80%) than the Bhoora *ema-1* qPCR assay; the results could be attributed to the observed variation in the *ema-1* gene, as discussed earlier. Both the Kim 18S and the Ueti *ema-1* qPCR assays failed to detect *T. equi* parasite DNA in four samples (Kim11, Kim20, Kim31 and Kim40) that could be detected using the Bhoora *ema-1* qPCR assay. Of these, we were only able to amplify and sequence the *ema-1* gene from Kim20. Phylogenetic analysis indicated that this sample grouped in clade B (Figure 5.6) and sequences occurring in this clade contained between one and seven nucleotide differences in the regions where the Ueti *ema-1* qPCR primers and probe had been designed. The *ema-1* sequence from Kim20 had three nucleotide differences in the forward primer region and one difference each in the reverse primer and probe regions (results not shown). The qPCR primers and probes developed by Kim et al. (2008) were designed in a region close to the 5'-end of the 18S rRNA gene. Again, we were only able to amplify and sequence the 5' fragment of the 18S gene from Kim20. Inspection of the 18S rRNA sequence of Kim20 indicated the occurrence of a single nucleotide difference in the qPCR forward primer region, while no differences were observed in the reverse primer and probe regions (results not shown). Single nucleotide variations in the qPCR forward primer region, previously reported for twelve South African *T. equi* variants, did not affect the ability of the

Kim 18S qPCR assay to detect each of these variants (Bhoora et al, 2009b; Chapter 3) and single nucleotide changes did not affect the efficiency of the Ueti *ema-1* qPCR assay (Ueti et al., 2005). Since we could not amplify and sequence the 5' end of the 18S rRNA gene from the other three samples, we can only speculate that single nucleotide changes in combination with low piroplasm parasitaemias (as indicated by high C_q values obtained using the Bhoora *ema-1* qPCR assay) may compromise the efficiencies of the qPCR assays, thus yielding negative results.

Recently, a multiplex qPCR assay targeting genes encoding antigenic proteins of *T. equi* and *B. caballi*, has been developed for the detection of equine piroplasmosis (Heim et al., 2007). The multiplex qPCR assay (MRT-PCR), described by Heim et al. (2007), was developed using the qPCR primers and probes initially described by Ueti et al. (2003). Based on the data presented in this study, it is evident that the MRT-PCR assay will not be able to detect all *T. equi ema-1* genotypes identified in South Africa. Redesigning of the MRT-PCR assay using the newly developed TaqMan MGB™ assay described in this study may overcome this problem.

Three distinct clades were identified by phylogenetic analysis of both *T. equi* 18S rRNA gene sequences and EMA-1 amino acid sequences. A comparison between the *T. equi* 18S rRNA and *T. equi* EMA-1 phylogenetic trees showed that samples which grouped in one *T. equi* 18S genotype did not group together in a particular EMA-1 phylogenetic clade, but rather occurred randomly between the three EMA-1 clades identified.

Ribosomal RNA (rRNA) genes are thought to be under tight structural and functional constraint, resulting in lower substitution rates; this and the absence of lateral gene transfer across lineages, are characteristics which make rRNA genes appropriate targets to assist in species identification (Chae et al., 1998; Katzer et al., 1998; Allsopp and Allsopp, 2006). Our previous studies have shown that extensive sequence heterogeneity exists in the 18S rRNA gene of *T. equi* parasites of both horses and zebra in South Africa (Bhoora et al., 2009a; Chapter 2; Chapter 4). It is probably not possible to use the 18S rRNA gene alone to decide whether these variants represent new species or subspecies (Chae et al., 1999; Allsopp and Allsopp, 2006). On the other hand, parasite outer membrane protein gene sequences, which are likely to be under intense selection pressure and therefore evolve at a more rapid rate than core function genes, will probably not give reliable phylogenetic information at the species level (Allsopp and Allsopp, 2006). In a study conducted to establish the phylogenetic relationship between different *Theileria* species, a comparison between homologous major merozoite/piroplasm surface antigen genes (mMPSA)

and the ribosomal rRNA genes from the different *Theileria* species investigated, showed that even though the phylogenetic clades were consistent with respect to the major groupings, inconsistencies between the analysis with the two types of gene sequence were found within the groups (Katzer et al., 1998). In addition, Katzer et al. (1998) identified four *Bems* gene sequences (*Bems*1-1, *Bems*1-2, *Bems*1-3 and *Bems*1-4) derived from genomic DNA of the *T. equi* (Florida) stock, which showed between 93.6-65.5% nucleotide identity to each other. The *Bems*1-3 sequence was found to be closely related to the *ema-2* sequence identified from the Florida isolate of *T. equi* (Knowles et al., 1997; Katzer et al., 1998). The recent identification and characterization of the *T. equi* EMA-3 suggests that these homologues either represent members of a related gene family within the genome of *T. equi* or distinct variants of the same gene (Katzer et al., 1998; Kumar et al., 2004; Ikadai et al., 2006). Such differences are not surprising, as surface exposed mMPSA genes are expected to be under selection pressure and have therefore evolved molecular mechanisms that generate diversity as a means of evading the immune system and succeeding in parasitism (Carcy et al., 2006). The use of these divergent mMPSA gene sequences may therefore be preferable for the comparison of closely related species (Shiels et al., 1995; Katzer et al., 1998).

The occurrence of such sequence variation may be related to antigenic variability and pathogenicity (Katzer et al., 1998). This has been demonstrated in a recent study on the association of *B. rossi* genotypes and the clinical manifestation of canine babesiosis (Matjila et al., 2009). Phylogenetic analyses of *Babesia rossi* erythrocyte membrane antigen 1 (Br*EMA1*) gene sequences enabled the identification of 13 Br*EMA1* genotypes, four of which could be linked to mild, moderate or severe disease phenotypes (Matjila et al., 2009). Unfortunately, it is not known whether the different *T. equi* 18S rRNA or *ema-1* genotypes can be associated with clinical differences in equine piroplasmosis cases.

In conclusion, this study reports on the development and evaluation of a sensitive TaqMan MGB™ qPCR assay, targeting the *ema-1* gene for the detection of *T. equi* infection in horses. Taking into consideration the discovery of extensive sequence heterogeneity both within the *ema-1* gene and the *T. equi* 18S rRNA gene, the use of this highly sensitive and specific *ema-1* qPCR assay, in conjunction with the *T. equi* 18S qPCR assay (Kim et al., 2008; Bhoora et al., 2009b; Chapter 3), may assist in the improved detection and diagnosis of equine piroplasmosis.

5.6 Reference List

Alhassan, A., Pumidonming, W., Okamura, M., Hirata, H., Battsetseg, B., Fujisaki, K., Yokoyama, N., Igarashi, I., 2005. Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood. Veterinary Parasitology 129, 43-49.

Alhassan, A., Thekisoe, O.M.M., Yokoyama, N., Inoue, N., Motloang, M.Y., Mbati, P.A., Yin, H., Katayama, Y., Anzai, T., Sugimoto, C., Igarashi, I., 2007. Development of loop-mediated isothermal amplification (LAMP) method for diagnosis of equine piroplasmosis. Veterinary Parasitology 143, 155-160.

Allsopp, B.A., Baylis, H.A., Allsopp, M.T.E.P., Cavalier-Smith, T., Bishop, R.P., Carrington, D.M., Sohanpal, B., Spooner, P., 1993. Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences. Parasitology 107, 157-165.

Allsopp, M.T.E.P., Allsopp, B.A., 2006. Molecular sequence evidence for the reclassification of some *Babesia* species. Annals of the New York Academy of Sciences 1081, 509-517.

Anonymous, 2008. Equine piroplasmosis. In: OIE Manual of diagnostic tests and vaccines for terrestrial animals. Office International des Epizooties, Paris, France, pp. 884-893.

Bashiruddin, J.B., Camma, C., Rebelo, E., 1999. Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. Veterinary Parasitology 84, 75-83.

Battsetseg, B., Xuan, X., Ikadai, H., Bautista, J.L., Byambaa, B., Boldbaatar, D., Battur, B., Battsetseg, G., Batsukh, Z., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K., 2001. Detection of *Babesia caballi* and *Babesia equi* in *Dermacentor nuttalli* adult ticks. International Journal for Parasitology 31, 384-386.

Bhoora, R., Franssen, L., Oosthuizen, M.C., Guthrie, A.J., Zweygarth, E., Penzhorn, B.L., Jongejan, F., Collins, N.E., 2009a. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Veterinary Parasitology 159, 112-120.

Bhoora, R., Quan, M., Franssen, L., Butler, C.M., van der Kolk, J.H., Guthrie, A.J., Zweygarth, E., Jongejan, F., Collins, N.E., 2009b. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. Veterinary Parasitology doi:10.1016/j.vetpar.2009.11.011.

Bhoora, R., Quan, M., Zweygarth, E., Guthrie, A.J., Prinsloo, S.A., Collins, N.E., 2010. Sequence heterogeneity in the gene encoding the rhoptry-associated protein-1 (RAP-1) of *Babesia caballi* isolates from South Africa. Veterinary Parasitology doi:10.1016/j.vetpar.2010.01.009.

Buling, A., Criado-Fornelio, A., Asenzo, G., Benitez, D., Barba-Carretero, J.C., Florin-Christensen, M., 2007. A quantitative PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina*. Veterinary Parasitology 147, 16-25.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 55, 611-622.

Caccio, S., Camma, C., Onuma, M., Severini, C., 2000. The beta-tubulin gene of *Babesia* and *Theileria* parasites is an informative marker for species discrimination. International Journal for Parasitology 30, 1181-1185.

Carcy, B., Precigout, E., Schetters, T., Gorenflot, A., 2006. Genetic basis for GPI-anchor merozoite surface antigen polymorphism of *Babesia* and resulting in antigenic diversity. Veterinary Parasitology 138, 33-49.

Chae, J.S., Allsopp, B.A., Waghela, S.D., Park, J.H., Kakuda, T., Sugimoto, C., Allsopp, M.T.E.P., 1999. A study of the systematics of *Theileria* spp. based upon small subunit ribosomal RNA gene sequences. Parasitology Research 85, 877-883.

Chae, J.S., Lee, J.M., Kwon, O.D., Holman, P.J., Waghela, S.D., Wagner, G.G., 1998. Nucleotide sequence heterogeneity in the small subunit ribosomal RNA gene variable (V4) region among and within geographic isolates of *Theileria* from cattle, elk and white-tailed deer. Veterinary Parasitology 75, 41-52.

d'Oliveira, C., Tijhaar, E.J., Shiels, B.R., van der Weide, M., Jongejan, F., 1996. Expression of genes encoding two major *Theileria annulata* merozoite surface antigens in *Escherichia coli* and a *Salmonella typhimurium aroA* vaccine strain. Gene 172, 33-39.

Donnelly, J., Phipps, L.P., Watkins, K.L., 1982. Evidence of maternal antibodies to *Babesia equi* and *B. caballi* in foals of seropositive mares. Equine Veterinary Journal 14, 126-128.

Geysen, D., Delespaux, V., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Veterinary Parasitology 110, 171-180.

Gubbels, J.M., de Vos, A.P., van der Weide, M., Viseras, J., Schouls, L.M., de Vries,E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. Journal of Clinical Microbiology 37, 1782-1789.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95-98.

Heim, A., Passos, L.M.F., Ribeiro, M.F.B., Costa-Junior, L.M., Bastos, C.V., Cabral, D.D., Hirzmann, J., Pfister, K., 2007. Detection and molecular characterization of *Babesia caballi* and *Theileria equi* isolates from endemic areas of Brazil. Parasitology Research 102, 63-68.

Holman, P.J., Chieves, L., Frerichs, W.M., Olson, D., Wagner, G.G., 1994. *Babesia equi* erythrocytic stage continuously cultured in an enriched medium. Journal of Parasitology 80, 232-236.

Holman, P.J., Frerichs, W.M., Chieves, L., Wagner, G.G., 1993. Culture confirmation of the carrier status of *Babesia caballi*-infected horses. Journal of Clinical Microbiology 31, 698-701.

Ikadai, H., Ishida, H., Sasaki, M., Taniguchi, K., Miyata, N., Koda, M., Igarashi, I., Oyamada, T., 2006. Molecular cloning and partial characterization of *Babesia equi* EMA-3. Molecular and Biochemical Parasitology 150, 371-373.

Jefferies, R., Ryan, U.M., Irwin, P.J., 2007. PCR-RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies. Veterinary Parasitology 144, 20-27.

Kappmeyer, L.S., Perryman, L.E., Knowles, D.P., 1993. A *Babesia equi* gene encodes a surface protein with homology to *Theileria* species. Molecular and Biochemical Parasitology 62, 121-124.

Katende, J.M., Goddeeris, B.M., Morzaria, S.P., Nkonge, C.G., Musoke, A.J., 1990. Identification of a *Theileria mutans*-specific antigen for use in an antibody and antigen detection ELISA. Parasite Immunology 12, 419-433.

Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Research 33, 511-518.

Katzer, F., McKellar, S., Kirvar, E., Shiels, B., 1998. Phylogenetic analysis of *Theileria* and *Babesia equi* in relation to the establishment of parasite populations within novel host species and the development of diagnostic tests. Molecular and Biochemical Parasitology 95, 33-44.

Kawazu, S.I., Sugimoto, C., Kamio, T., Fujisaki, K., 1992. Analysis of the genes encoding immunodominant piroplasm surface proteins of *Theileria sergenti* and *Theileria buffeli* by nucleotide sequencing and polymerase chain reaction. Molecular and Biochemical Parasitology 56, 169-175.

Kim, C.M., Blanco, L.B.C., Alhassan, A., Iseki, H., Yokoyama, N., Xuan, X., Igarashi, I., 2008. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. Veterinary Parasitology 151, 158-163.

Knowles, D.P., Kappmeyer, L.S., Perryman, L.E., 1997. Genetic and biochemical analysis of erythrocyte-stage surface antigens belonging to a family of highly conserved proteins of *Babesia equi* and *Theileria species*. Molecular and Biochemical Parasitology 90, 69-79.

Knowles, D.P., Kappmeyer, L.S., Stiller, D., Hennager, S.G., Perryman, L.E., 1992. Antibody to a recombinant merozoite protein epitope identifies horses infected with *Babesia equi*. Journal of Clinical Microbiology 30, 3122-3126.

Knowles, D.P., Perryman, L.E., Goff, W.L., Miller, C.D., Harrington, R.D., Gorham, J.R., 1991a. A monoclonal antibody defines a geographically conserved surface protein epitope of *Babesia equi* merozoites. Infection and Immunity 59, 2412-2417.

Knowles, D.P., Perryman, L.E., Kappmeyer, L.S., Hennager, S.G., 1991b. Detection of equine antibody to *Babesia equi* merozoite proteins by a monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 29, 2056-2058.

Kumar, S., Yokoyama, N., Kim, J.Y., Huang, X., Inoue, N., Xuan, X., Igarashi, I., Sugimoto, C., 2004. Expression of *Babesia equi* EMA-1 and EMA-2 during merozoite developmental stages in erythrocyte and their interaction with erythrocytic membrane skeleton. Molecular and Biochemical Parasitology 133, 221-227.

Matjila, P.T., Carcy, B., Leisewitz, A.L., Schetters, T., Jongejan, F., Gorenflot, A., Penzhorn, B.L., 2009. Preliminary evaluation of the Br*EMA1* gene as a tool for associating *Babesia rossi* genotypes and clinical manifestation of canine babesiosis. Journal of Clinical Microbiology 47, 3586-3592.

Matsuba, T., Sugimoto, C., Hattori, M., Sako, Y., Fujisaki, K., Onuma, M., 1995. Expression of a 32 kilodalton *Theileria sergenti* piroplasm surface protein by recombinant baculoviruses. International Journal for Parasitology 25, 939-943.

Nagore, D., Garcia-Sanmartin, J., Garcia-Perez, A.L., Juste, R.A., Hurtado, A., 2004. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: epidemiological survey and phylogenetic analysis. Veterinary Parasitology 123, 41-54.

Nicolaiewsky, T.B., Richter, M.F., Lunge, V.R., Cunha, C.W., Delagostin, O., Ikuta, N., Fonseca, A.S., da Silva, S.S., Ozaki, L.S., 2001. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. Veterinary Parasitology 101, 9-21.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

Salim, B.O.M., Hassan, S.M., Bakheit, M.A., Alhassan, A., Igarashi, I., Karanis, P., Abdelrahman, M.B., 2008. Diagnosis of *Babesia caballi* and *Theileria equi* infections in horses in Sudan using ELISA and PCR. Parasitology Research 103, 1145-1150.

Sevinc, F., Maden, M., Kumas, C., Sevinc, M., Ekici, O.D., 2008. A comparative study on the prevalence of *Theileria equi* and *Babesia caballi* infections in horse sub-populations in Turkey. Veterinary Parasitology 156, 173-177.

Shiels, B.R., d'Oliveira, C., McKellar, S., Ben-Miled, L., Kawazu, S.I., Hide, G., 1995. Selection of diversity at putative glycosylation sites in the immunodominant merozoite/piroplasm surface antigen of *Theileria* parasites. Molecular and Biochemical Parasitology 72, 149-162.

Shkap, V., Cohen, I., Leibovitz, B., Savitsky, Pipano, E., Avni, G., Shofer, S., Giger, U., Kappmeyer, L., Knowles, D.P., 1998. Seroprevalence of *Babesia equi* among horses in Israel using competitive inhibition ELISA and IFA assays. Veterinary Parasitology 76, 251-259.

Sibeko, K.P., Oosthuizen, M.C., Collins, N.E., Geysen, D., Rambritch, N.E., Latif, A.A., Groeneveld, H.T., Potgieter, F.T., Coetzer, J.A.W., 2008. Development and evaluation of a real-time polymerase chain reaction test for the detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle. Veterinary Parasitology 155, 37-48.

Staden, R., 1996. The Staden sequence analysis package. Molecular Biotechnology 5, 233-241.

Swofford, D.L., 2003. PAUP*. Phylogenetic Analysis using Parsimony (*and other methods). Version 4b10. Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution 24, 1596-1599.

Ueti, M.W., Palmer, G.H., Kappmeyer, L.S., Scoles, G.A., Knowles, D.P., 2003. Expression of equi merozoite antigen 2 during development of *Babesia equi* in the midgut and salivary gland of the vector tick *Boophilus microplus*. Journal of Clinical Microbiology 41, 5803-5809.

Ueti, M.W., Palmer, G.H., Kappmeyer, L.S., Statdfield, M., Scoles, G.A., Knowles, D.P., 2005. Ability of the vector tick *Boophilus microplus* to acquire and transmit *Babesia equi* following feeding on chronically infected horses with low-level parasitemia. Journal of Clinical Microbiology 43, 3755-3759.

Vega, C., Buening, G., Green, T., Carson, C., 1985. *In vitro* cultivation of *Babesia bigemina*. American Journal of Veterinary Research 46, 416-420.

Weiland, G., 1986. Species-specific serodiagnosis of equine piroplasma infections by means of complement fixation test (CFT), immunofluorescence (IIF), and enzyme-linked immunosorbent assay (ELISA). Veterinary Parasitology 20, 43-48.

Xuan, X., Chahan, B., Huang, X., Yokoyama, N., Makala, L.H., Igarashi, I., Fujisaki, K., Maruyama, S., Sakai, T., Mikami, T., 2002. Diagnosis of equine piroplasmosis in Xinjiang province of China by the enzyme-linked immunosorbent assays using recombinant antigens. Veterinary Parasitology 108, 179-182.

Xuan, X., Larsen, A., Ikadai, H., Tanaka, T., Igarashi, I., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., 2001. Expression of *Babesia equi* merozoite antigen 1 in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 39, 705-709.

Zweygarth, E., Just, M.C., de Waal, D.T., 1995. Continuous *in vitro* cultivation of erythrocytic stages of *Babesia equi*. Parasitology Research 81, 355-358.

CHAPTER 6

Sequence heterogeneity in the gene encoding the rhoptry associated protein-1 (RAP-1) of *Babesia caballi* isolates from South Africa

6.1 Abstract

A competitive-inhibition enzyme-linked immunosorbent assay (cELISA) developed for the detection of antibody specific for *Babesia caballi* was used to test sera collected from 1237 South African horses. None of these samples tested positive using the cELISA, although 63 samples tested positive for *B. caballi* antibody using the indirect fluorescent antibody test (IFAT). We therefore characterized the *rap-1* gene that codes for the antigen (rhoptry associated protein, RAP-1) used in the cELISA, from South African *B. caballi* isolates. Three sets of primers were designed to amplify the complete gene and flanking regions (~1800 bp), but only one set of primers yielded PCR products, and we were only able to amplify a region at the 5' end of the gene (615 bp) from ten South African *B. caballi in vitro*-cultured isolates. Sequence data from seven of these were obtained. The sequences showed between 79 and 81% identity to *B. caballi rap-1* gene sequences that have been reported in the literature (accession numbers: AF092736 and AB017700). The GenomeWalker Universal kit (Clonetech) was used to amplify the regions flanking the 615 bp *B. caballi rap-1* fragment from two South African isolates. Amplified products were cloned into the pGEM-T Easy vector and sequenced. The complete *rap-1* gene sequence, comprising a single open reading frame of 1479 bp that encodes a protein consisting of 493 amino acids, was obtained from the two South African isolates. These sequence data were used to redesign the amplification primers and *rap-1* homologues were obtained from a further eight isolates. BLASTP analysis indicated an amino acid identity of between 57.9% and 65.1% to the two RAP-1 protein sequences, AF092736 and AB017700, with most differences occurring at the carboxy-terminus. The amino acid sequence differences probably explain why it was not possible to detect *B. caballi* antibody in IFAT-positive sera from South Africa using the cELISA. Redesigning the current cELISA using a conserved epitope of the RAP-1 antigen, or a more conserved protein as the target antigen, may overcome this problem.

6.2 Introduction

Babesia caballi is a tick-borne haemoprotozoan parasite, and is one of the causes of equine piroplasmosis. The clinical manifestations of the disease are often variable, making it easy to confuse with *Theileria equi* infections. *Babesia caballi* infections, characterized by fever and anaemia, are considered less severe than *T. equi* infections, which are more commonly associated with haemoglobinuria and death (de Waal, 1992; Camacho et al., 2005). Many

infections caused by either parasite are subclinical and, in addition, animals that have recovered from the infections often remain carriers of the parasites for long periods of time.

The international movement of horses has led to the spread of equine piroplasmosis from its endemic tropical and subtropical regions to more temperate non-endemic regions. Many countries have introduced stringent import restrictions to prevent the introduction of these parasites into disease-free areas (Friedhoff et al., 1990). Serological methods of determining the carrier status of horses and other equid species are currently the prescribed methods for certifying animals free of these parasites. These include the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) (Donnelly et al., 1980; Weiland, 1986; Brüning et al., 1997). Although it is possible to distinguish between *T. equi* and *B. caballi* infections using IFAT, differentiation between weak positive and negative reactions can be difficult. Cross-reactions that occur between *B. caballi* and *T. equi* in the indirect ELISA prevent this test from being recognized as a differential diagnostic assay (Weiland, 1986). Recently, however, *T. equi* and *B. caballi* recombinant merozoite proteins and monoclonal antibodies to immunogenic epitopes on these proteins have been used in competitive inhibition ELISAs (cELISAs), with promising results. These cELISAs have been shown to have higher specificities for *T. equi* and *B. caballi*, when compared to those of the IFAT and indirect ELISA (Knowles et al., 1991; Knowles et al., 1992; Shkap et al., 1998; Kappmeyer et al., 1999; Katz et al., 2000; Xuan et al., 2002).

Secreted proteins from the apical organelles of apicomplexan parasites are thought to play pivotal roles in parasite attachment to, invasion of and expansion and maintenance within the host cell (Sam-Yellowe, 1996). Among these proteins is the rhoptry-associated protein-1 (RAP-1), which was initially described in *Babesia bovis* and *Babesia bigemina*, but has 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 family of proteins contains several immunogenic epitopes and antibodies directed against these proteins have been shown to inhibit merozoite invasion (Ikadai et al., 1999; Machado et al., 1999; Yokoyama et al., 2006). This phenomenon 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 a cELISA for the detection of *B. caballi* antibody in infected horses (Kappmeyer et al., 1999). This assay has been successfully used for the detection of *B. caballi* antibody in the sera of infected horses in North and South America and several European countries (Kappmeyer et al., 1999; Sevinc et al., 2008). A preliminary study in our laboratory, however, showed that the commercially available cELISA was not able to detect antibody in South African horses infected with *B. caballi*. This result led to the hypothesis that differences in the RAP-1 antigen within South African *B. caballi* isolates could prevent the detection of *B. caballi* antibody. This study was therefore focused on the characterization of *rap-1* gene homologues in South African *B. caballi* isolates in an attempt determine the cause of the failure of the commercial cELISA in South Africa.

6.3 Materials and Methods

6.3.1 Field samples and in vitro-cultured isolates

A total of 1237 whole blood samples were collected from horses at the National Sale of two-year-old thoroughbred horses in 2005 (n=273) and the National Yearling Sales in 2005 (n=455) and 2006 (n=509). Ten *in vitro*-cultured *B. caballi* isolates, designated Bcab5, Bcab9, Bcab13, Bcab19, Bcab105, Bcab167, Bcab418, Bcab443, Bcab502 and BcabE7 (Zweygarth et al., 2002), were also used in this study.

6.3.2 cELISA and IFAT

Sera obtained from the blood samples were examined for the presence of antibodies against *B. caballi* and *T. equi* using the commercially available cELISA kit, as described by the manufacturer (VMRD Inc., Pullman, WA, USA). The cELISA was performed at the Department of Veterinary Tropical Diseases.

The IFAT was conducted at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI). A standard IFAT protocol, as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Anonymous, 2008) was used, with the exception that cultured antigen, produced locally in South Africa, was used.

6.3.3 DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from 200 µl of each of the *in vitro*-cultured isolates using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Primers were designed to amplify complete *rap-1* homologues and flanking sequences (~1800 bp) from the South African isolates based on two *B. caballi rap-1* DNA sequences available in the public sequence databases [accession numbers: AF092736 (Kappmeyer et al., 1999) and AB017700 (Ikadai et al., 1999)]. Initially, primers BC-RAP1F and BC-RAP1R (Table 6.1, Figure 6.1) were designed to amplify the full-length gene and flanking sequences. Subsequently, primer pairs BC-RAP1F and BC-RAP3R, BC-RAP2F and BC-RAP2R, and BC-RAP3F and BC-RAP1R (Table 6.1) were designed to amplify overlapping fragments, respectively, at the 5' end, in the middle and at the 3' end of the ~1800 bp *rap-1* fragment (Figure 6.1). The FastPCR software program (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm) was used to design all primers used in this study, and to check for the formation of secondary structures and primer dimers. All PCR primers were obtained from Integrated DNA Technologies (IDT). Reactions were performed in a final volume of 25 µl, containing 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 5 min at 95 ºC, followed by 40 cycles of 30 s at 95 ºC, 1 min at 55 ºC and 1 min at 72 ºC, and a final extension of 7 min at 72 ºC.

PCR products were purified using the Qiagen PCR purification kit (Qiagen, Hilden, Germany). Samples were sequenced using BigDye chemistry (v.3.1, Applied Biosystems) on a 3130XL genetic analyser (Applied Biosystems). Sequences were assembled and edited using gap4 of the Staden software suite (Staden, 1996). Multiple sequence alignments were performed using the MAFFT alignment program (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 homologous nucleotide sequences were performed using BLASTN and for homologous protein sequences, BLASTP was used (Altschul et al., 1990).

Table 6.1 Nucleotide sequences of PCR primers used in this study.

Primer	Size (bp)	Sequence (5'- 3')	Tm	%GC
BC-RAP1F	23	CGTACAATGAGGTGTTCTGCGAG	57.8	52.1
BC-RAP1R	22	TATTCTCCGAGCAGTGCGATGG	59.0	54.5
BC-RAP2F	21	AGAGTGACGCTAATCCGGCCA	60.7	57.1
BC-RAP2R	23	GAATAAATCCTCGGCCCCCGTTG	60.2	56.5
BC-RAP3F	21	AGTGTCAACGGGGGCCGAGGA	65.5	66.6
BC-RAP3R	23	CGTCTTGTAGTAAAGCGTGGCCA	59.4	52.1
BC-GSP1F	30	CTGGATGCGTTTCAGGAGTGGCAAGAACCA	65.3	53.3
BC-GSP1R	28	CGCTACACGGCTGTTGTGGCGAATGGCC	68.5	64.2
BC-GSP2F	30	GGAGAAGAACGTGACTAGCGACCCCAACGT	65.8	56.6
BC-GSP2R	30	CCTTGCTACAAGCAAGAGGGCGCCGAAAAC	66.2	56.6
Bc9_RAPF	24	AGCAGTGCTGTATATGTCTGTGTC	56.7	45.8
Bc9_RAPR	23	GCTGATGCGATGTGTGTCGTAGG	60.0	56.5
Bc9_RAP2F	24	ACTAGCGACCCCAACGCTACTGAC	62.4	58.3
Bc9_RAP2R	22	TTGGAGCATGAAGTCCTTCAGC	57.5	50.0

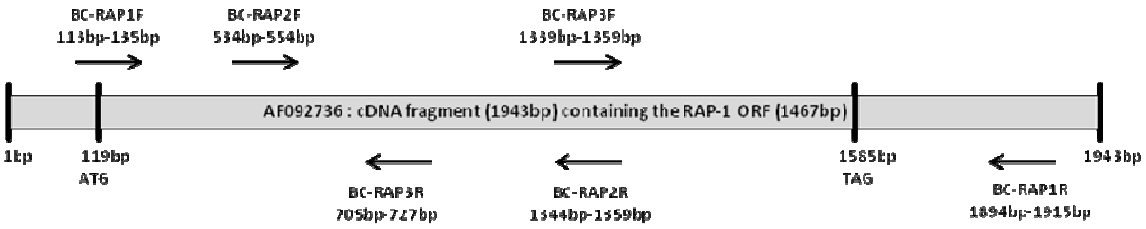


Figure 6.1 Schematic representation of positions of primers for amplification of *rap-1* gene homologues and flanking regions from South African *B. caballi* isolates.

6.3.4 Genome Walking

Since only one fragment close to the 5' end of the *rap-1* gene could be obtained by PCR amplification, the GenomeWalker™ Universal Kit (CloneTech) was used to amplify and sequence the remaining 5' and 3' flanking regions of two South African *B. caballi in vitro*-cultured isolates (Bcab9 and Bcab13). Genomic DNA from *B. caballi* isolates Bcab9 and Bcab13 were digested with four blunt-end restriction enzymes, *Dra*I, *Eco*RV, *Pvu*II and *Stu*I. GenomeWalker adaptors (Table 6.2) were ligated to 4 µl of each digested genomic DNA library.

Table 6.2 Nucleotide sequences of the GenomeWalker Adaptor and adaptor primers.

Oligonucleotide	Nucleotide Sequence
Adaptor	5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3'
	3' H_2N-CCCGACCA-PO_4-5'
Primer ADP1	5'-GTAATACGACTCACTATAGGGC-3'
Primer ADP2	5'-ACTATAGGGCACGCGTGGT-3'

Four gene-specific primers, BC-GSP1F, BC-GSP1R, BC-GSP2F and BC-GSP2R (Table 6.1), were designed for the amplification of the 5' and the 3' unknown flanking regions of the *rap-1* gene. Primary PCR reactions were performed using adaptor-specific primer ADP1 (Table 6.2) and gene-specific primer BC-GSP1R for the amplification of the 5' end of the *rap-1* gene, while ADP1 and BC-GSP1F were used to amplify the 3' end. Secondary PCR reactions were carried out using the adaptor-specific primer ADP2 (Table 6.2) and gene-specific primer BC-GSP2R to amplify the 5' end, and ADP2 and BC-GSP2F to amplify the 3' end of the *rap-1* gene. Both primary and secondary PCR reactions were carried out according to the manufacturer's instructions.

The resulting secondary PCR products were purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned using the pGEM®-T Easy Vector system II (Promega), according to the manufacturer's instructions. Transformants were screened using ImMedia™ Amp Blue (Invitrogen), and recombinant colonies were selected and grown using ImMedia™ Amp Liquid (Invitrogen). Plasmid DNA was isolated by means of a High Pure Plasmid Purification kit (Roche) and sequenced using the cloning vector primers, T7 and SP6. Sequences were edited and analysed as described above.

6.3.5 Amplification and sequencing of the rap-1 gene homologue from South African B. caballi isolates

An approximately 1500 bp fragment containing the *rap-1* gene was amplified from another eight South African *B. caballi in vitro*-cultured isolates using the primers, Bc9_RAPF and Bc9_RAPR (Table 6.1), that were designed based on the *rap-1* sequence data obtained from the South African *B. caballi* isolates Bcab9 and Bcab13. Reactions were performed in a final volume of 25 µl, containing High Fidelity PCR Master mix (Roche Diagnostics, Mannheim, Germany), 0.2 µM of each primer and 30 ng of genomic DNA. The cycle parameters included an initial denaturation of 5 min at 94 ºC, followed by 40 cycles of 30 s at 94 ºC, 1 min at 58 ºC and 1 min

at 72 ºC, and a final extension of 7 min at 72 ºC. Amplicons were purified and sequenced as described above using the amplification primers, Bc9_RAPF and Bc9_RAPR, and two internal sequencing primers, Bc9_RAP2F and Bc9_RAP2R (Table 6.1). The sequences were aligned and edited as described above. Representative South African *B. caballi rap-1* gene sequences have been deposited in GenBank under accession numbers GQ871778, GQ871779 and GQ871780.

6.3.6 *Phylogenetic analysis*

Rap-1 nucleotide sequences were aligned using the MAFFT alignment program (Multiple Sequence Alignment) employing the FFT-NS-1 algorithm (Katoh et al., 2005), then translated into amino acid sequences using the ExPasy translate tool (www.expasy.ch/tools/dna). 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. PAUP* v4b10 (Swofford, 2003) was used to explore distance, parsimony and maximum-likelihood phylogenetic methods for both nucleotide and amino acid sequences. Trees were constructed using the *Babesia divergens rap-1* gene sequence (Accession number: Z49818) as an outgroup. Analysis by Bayesian inference was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) which is accessible via the Computational Biology Service Unit at Cornell University (http://cbsuapps.tc.cornell.edu/mrbayes.aspx). All consensus trees were edited using MEGA4 (Tamura et al., 2007).

6.3.7 *Analysis of the 18S rRNA gene from South African in vitro-cultured B. caballi isolates*

The complete 18S rRNA gene of the *in vitro*-cultured *B. caballi* isolates was amplified and sequenced as described previously (Bhoora et al., 2009; Chapter 2), and phylogenetic analyses using distance, parsimony and maximum-likelihood methods were carried out using the program PAUP* v4b10 (Swofford, 2003). Trees were constructed using the 18S rRNA gene sequence of *B. divergens* (accession number: Z48751) as an outgroup.

6.4 Results

Of the field samples (n=1237) tested for the presence of *B. caballi* and *T. equi* antibodies using the commercially available cELISA kits, none of the samples tested positive for *B. caballi* antibodies, but 265 samples were positive for *T. equi* antibodies. The samples were also tested using the IFAT, which detected *B. caballi* antibodies in 63 samples and *T. equi* antibodies in 274 samples (Table 6.3).

No amplification product could be obtained from any of the ten South African *B. caballi in vitro*-cultured isolates using the BC-RAP1F and BC-RAP1R primer pair. Of the primers designed to amplify overlapping fragments at the 5' end, in the middle and at the 3' end of the *rap-1* fragment (Figure 6.1), only one primer pair, BC-RAP1F and BC-RAP3R, yielded a ~615 bp PCR product from the ten South African *B. caballi* isolates, and sequence data were obtained from seven of these. BLASTN analysis revealed that the sequences showed between 79 and 81% identity to published *B. caballi rap-1* nucleotide sequences (Table 6.4). Given this sequence heterogeneity at the 5' end of the *rap-1* gene, we hypothesized that even greater sequence heterogeneity present in the middle and at the 3' end of the *rap-1* gene in South African isolates prevented the downstream primers from binding to the template DNA, resulting in no amplification. An alternative method was therefore required to obtain full-length *rap-1* gene sequences from the South African *B. caballi* isolates.

Table 6.3 Comparison of serology results for (A) *B. caballi* and (B) *T. equi*.

(A) *B. caballi*

	cELISA positive	**cELISA negative**	**Total**
IFAT positive	0	63	**63**
IFAT negative	0	1174	**1174**
Total	**0**	**1237**	**1237**

(B) *T. equi*

	cELISA positive	**cELISA negative**	**Total**
IFAT positive	265	9	**274**
IFAT negative	0	963	**963**
Total	**265**	**972**	**1237**

Table 6.4 PCR and sequencing results obtained for *rap-1* homologues from ten South African *B. caballi in vitro*-cultured isolates.

Isolate	*rap*-1 PCR*	Sequencing result**
Bcab5	+	568 bp; 80% identity
Bcab9	+	591 bp; 80% identity
Bcab13	+	590 bp; 80% identity
Bcab19	+	417 bp; 77% identity
Bcab105	+	514 bp; 78% identity
Bcab341	+	573 bp; 80% identity
Bcab418	+	No sequence data
Bcab443	Multiple bands	No sequence data
Bcab502	+	518 bp; 79% identity
BcabE7	Double bands	No sequence data

* Amplification products obtained using primers BC-RAP1F and BC-RAP3R

**Number of base pairs sequenced and % identity to published RAP-1 sequence AF092736

Two *B. caballi in vitro*-cultured isolates (Bcab9 and Bcab13) were selected for subsequent genome walking experiments. The primary PCR using adaptor primer 1 (ADP1) and gene-specific primers (BC-GSP1F or BC-GSP1R) produced either smearing in some lanes or multiple fragments that ranged in size from 500 bp to 3000 bp. The secondary PCR using adaptor primer 2 (ADP2) and gene-specific primers (BC-GSP2F or BC-GSP2R) produced distinct PCR products in each of the four libraries for the 3' unknown region (Figure 6.2). For amplification of the 5' unknown region, PCR products were observed in all libraries except the *Stu*I digested library (Figure 6.2).

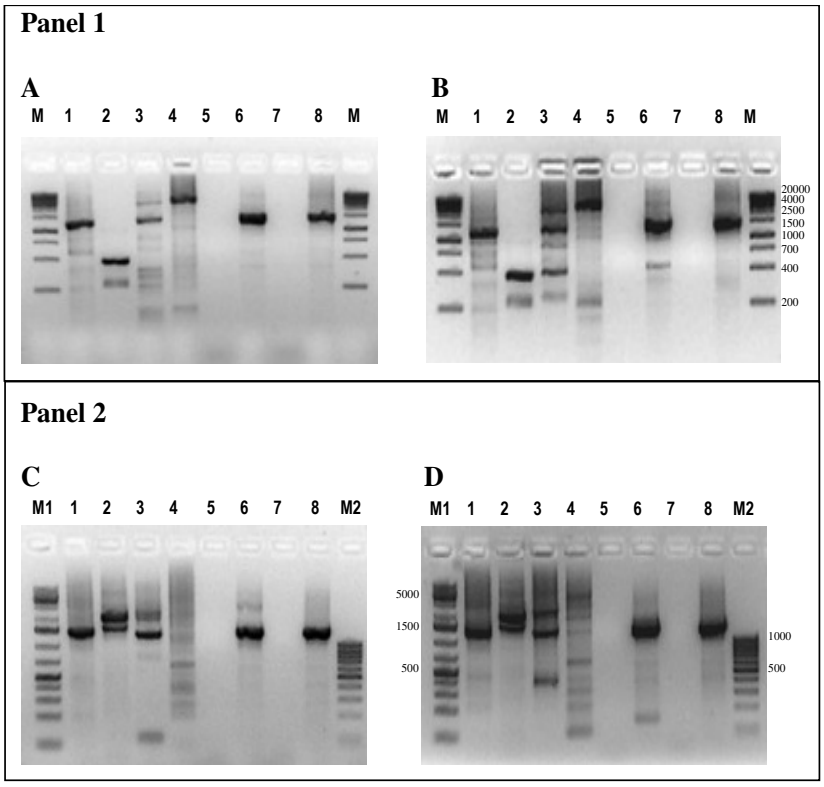


Figure 6.2 Genome walking PCR analysis of restriction enzyme digested genomic DNA using primers ADP2 and BC-GSP2F to amplify the 3'-end of the *rap-1* fragment (Panel 1) and primers ADP2 and BC-GSP2R to amplify the 5'-end (Panel 2). (A and C) *B. caballi in vitro*-cultured isolate Bcab9. (B and D) *B. caballi in vitro*-cultured isolate Bcab13. Lane 1: *Pvu*II library. Lane 2: *Dra*I library. Lane 3: *Eco*RV library. Lane 4: *Stu*I library. Lanes 5 and 7: negative controls. Lanes 6 and 8: Positive control (pre-constructed human genomic DNA library). Lane M: ZipRuler Express DNA ladder 2 (Fermentas). Lane M1: O'GeneRuler 1 kb DNA ladder plus DNA size markers (Fermentas). Lane M2: O'GeneRuler 100 bp DNA ladder (Fermentas).

PCR and sequence data generated from the *Pvu*II digested genome walking libraries were used in the construction of the complete *B. caballi* isolate 9 and *B. caballi* isolate 13 *rap-1* sequences. A single open reading frame of 1479 bp, encoding a peptide of 493 amino acids, was obtained from the two South African *B. caballi* isolates. BLAST analysis of the sequences confirmed that they were most closely related to the *B. caballi* RAP-1 family. The two South African *B. caballi rap-1* sequences were very similar to each other, with 99% nucleotide sequence identity, but they showed much lower identity (81.7 and 82.3%) to two published *B. caballi rap-1* sequences, AF092736 (Kappmeyer et al., 1999) and AB017700 (Ikadai et al., 1999) (Table 6.5).

Table 6.5 Nucleotide sequence identity of *rap*-1 sequences from the South African *B. caballi* isolates compared to published sequences AB017700 (Ikadai et al., 1999) and AF092736 (Kappmeyer et al., 1999) as determined by BLASTN analysis.

	AB017700 % identity	AF092736 % identity	Bcab9 % identity
AB017700	100.0	99.9	81.8
AF092736	99.9	100.0	82.3
Bcab5	81.9	82.4	99.4
Bcab9	81.8	82.3	100.0
Bcab13	81.7	82.1	99.3
Bcab19	82.0	82.0	99.3
Bcab105	81.9	82.4	99.4
Bcab167	81.9	82.0	87.5
Bcab418	82.4	82.4	89.8
Bcab443	81.9	82.0	87.6
Bcab502	81.8	82.3	99.4
BcabE7	82.0	82.0	87.5

Table 6.6 Amino acid sequence identity and similarity of predicted RAP-1 amino acid sequences from the South African isolates compared to published sequences AB017700 (Ikadai et al., 1999) and AF092736 (Kappmeyer et al., 1999) as determined by BLASTP analysis

	AB017700		AF092736		Bcab 9	
	% identity	% similarity	% identity	% similarity	% identity	% similarity
AB017700	100.0	100.0	99.8	99.8	63.7	77.5
AF092736	99.8	99.8	100.0	100.0	64.9	78.6
Bcab5	63.7	77.9	64.9	78.9	98.3	98.9
Bcab9	63.7	77.5	65.1	78.6	100.0	100.0
Bcab13	63.2	77.3	64.7	78.5	98.6	98.9
Bcab19	63.7	77.5	63.7	77.5	98.2	98.7
Bcab105	59.8	73.6	61.1	74.7	93.6	94.2
Bcab167	57.9	73.2	59.2	74.2	74.2	80.7
Bcab418	62.0	76.0	62.0	76.0	76.5	82.1
Bcab443	57.9	73.2	59.5	74.3	74.4	80.9
Bcab502	63.7	77.9	63.8	78.1	98.9	80.9
BcabE7	57.9	73.2	57.9	73.0	73.2	79.7

Primers Bc9_RAPF and Bc9_RAPR (Table 6.1), which were designed based on the *rap-1* sequences obtained from *B. caballi* isolates Bcab9 and Bcab13, were used to amplify and sequence almost complete *rap-1* open reading frames (~1500 bp) from an additional eight South African *B. caballi in vitro*-cultured isolates. *Rap-1* sequences similar to those obtained from

Bcab9 and Bcab13 were obtained from isolates Bcab5, Bcab19, Bcab105, and Bcab502, but a second distinct group of *B. caballi rap-1* nucleotide sequences was identified from Bcab167, Bcab418, Bcab443 and BcabE7, with nucleotide identities to Bcab9 ranging from 87.5 to 89.8% (Table 6.5). All ten of the South African *rap-1* sequences showed between 81.7 and 82.4% identity to the two published *B. caballi rap-1* sequences (Table 6.5). BLASTP analysis, on the other hand, indicated amino acid identities to the published *B. caballi* RAP-1 protein sequences which ranged from 57.9 to 65.1%, and amino acid similarities ranging between 73.0 and 78.9% (Table 6.6).

Nucleotide sequence alignments demonstrated that sequence variation between published sequences and their homologues from South African isolates occurred across the full-length of the *rap-1* gene (results not shown). An alignment of the amino acid sequences deduced from the *rap-1* nucleotide sequences of PCR products amplified from genomic DNA, showed that the RAP-1 amino acid sequences were fairly conserved in the amino-terminal region of the protein, but were more variable at the carboxy-terminus (Figure 6.3). Distinctive features of the RAP-1 family, including the presence of a signal peptide sequence and four conserved cysteine residues (Suarez et al., 1998), could be identified (Figure 6.3). The detection of putative conserved domains by BLASTP analysis further confirmed that the newly discovered South African *B. caballi* RAP-1 sequences belong to the RAP-1 superfamily.

Figure 6.3 Alignment of the RAP-1 predicted amino acid sequences from ten South African *B. caballi* isolates (Bcab5, Bcab13, Bcab9, Bcab19, Bcab105, Bcab167, Bcab418, Bcab443, Bcab502 and BcabE7) with the published RAP-1 amino acid sequences from the public sequence databases (accession numbers: AB01770 and AF092736). The potential signal peptide cleavage site is indicated by an arrow and conserved cystein residues are highlighted in yellow. The carboxy-terminal amino acid repeat regions are indicated in blocks. Identical sequences are indicated by dots (…) and missing sequences or gaps are indicated by dashes (---).

Phylogenetic analyses of both the *rap-1* nucleotide sequences and the predicted amino acid sequences yielded trees with almost identical topologies and high bootstrap or nodal support values. The analyses demonstrated that the *B. caballi* RAP-1 amino acid sequences fell into two main groups (Figure 6.4). Group A contained the RAP-1 amino acid sequences from *B. caballi* clone X6 (AF092736) (Kappmeyer et al., 1999) and the *B. caballi* pBC48/31 mRNA for a 48 KDa merozoite antigen (AB017700) (Ikadai et al., 1999), both of which were obtained from the USDA *B. caballi* strain. Group B, which could be further subdivided into groups B1 and B2, contained the new *B. caballi* RAP-1 amino acid sequences from South African isolates. Group B1 contained RAP-1 amino acid sequences from six *B. caballi* isolates (Bcab5, Bcab9, Bcab13, Bcab19, Bcab105 and Bcab502), while the remaining four RAP-1 amino acid sequences (Bcab167, Bcab418, Bcab443 and BcabE7) were found in group B2.

Phylogenetic analyses of complete 18S rRNA gene sequences from nine of the *in vitro*-cultured samples revealed that the sequences grouped within Clade B, which has previously been shown to include only 18S rRNA sequences from South African *B. caballi* samples (Bhoora et al., 2009; Chapter 2) (Figure 6.5). The 18S rRNA sequences from six of the *B. caballi* isolates (Bcab5, Bcab9, Bcab13, Bcab19, Bcab105 and Bcab502) were shown to group together in subgroup B1 with the original South African *B. caballi* sequence (accession number: Z15104), while the remaining three isolates (Bcab167, Bcab418 and Bcab443), grouped within the second subgroup, B2, that contained the recently published South African sequence CABRBEQ115 [accession number: EU642514 (Bhoora et al., 2009; Chapter 2)] (Figure 6.5).

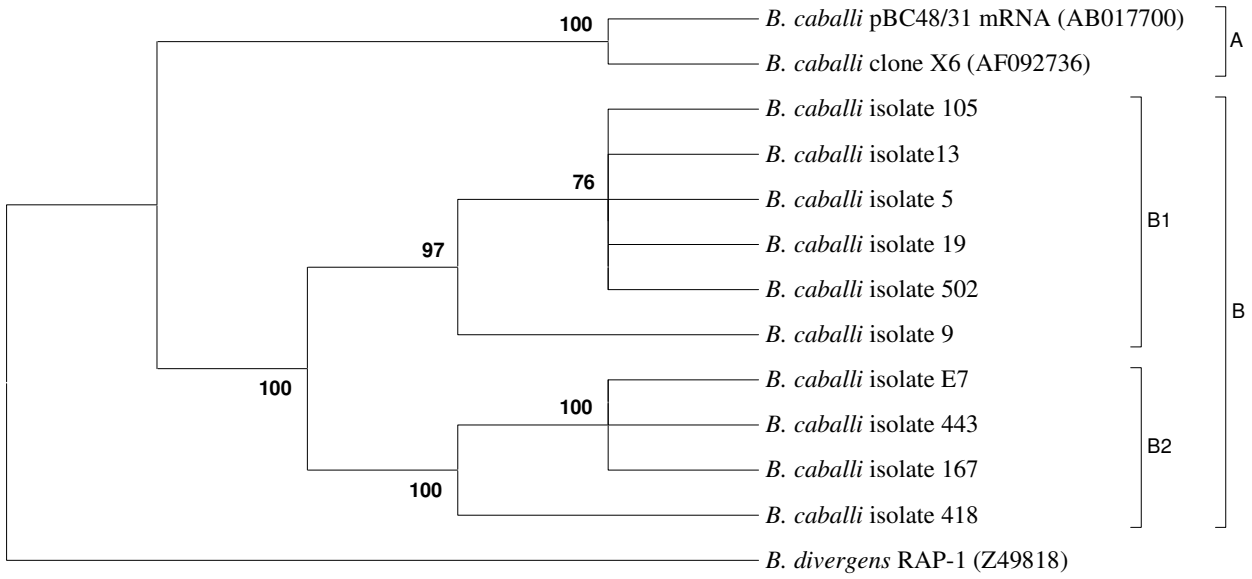


Figure 6.4 Rooted 50% majority-rule consensus cladogram, calculated using maximum-parsimony and bootstrap estimates from 1000 replicates, of the predicted *B. caballi* RAP-1 amino acid databases (accession numbers are indicated in parentheses). The *B. divergens* RAP-1 amino acid sequence (accession number: Z49818) was used as an outgroup. Bootstrap support values are indicated on the nodes of the trees.

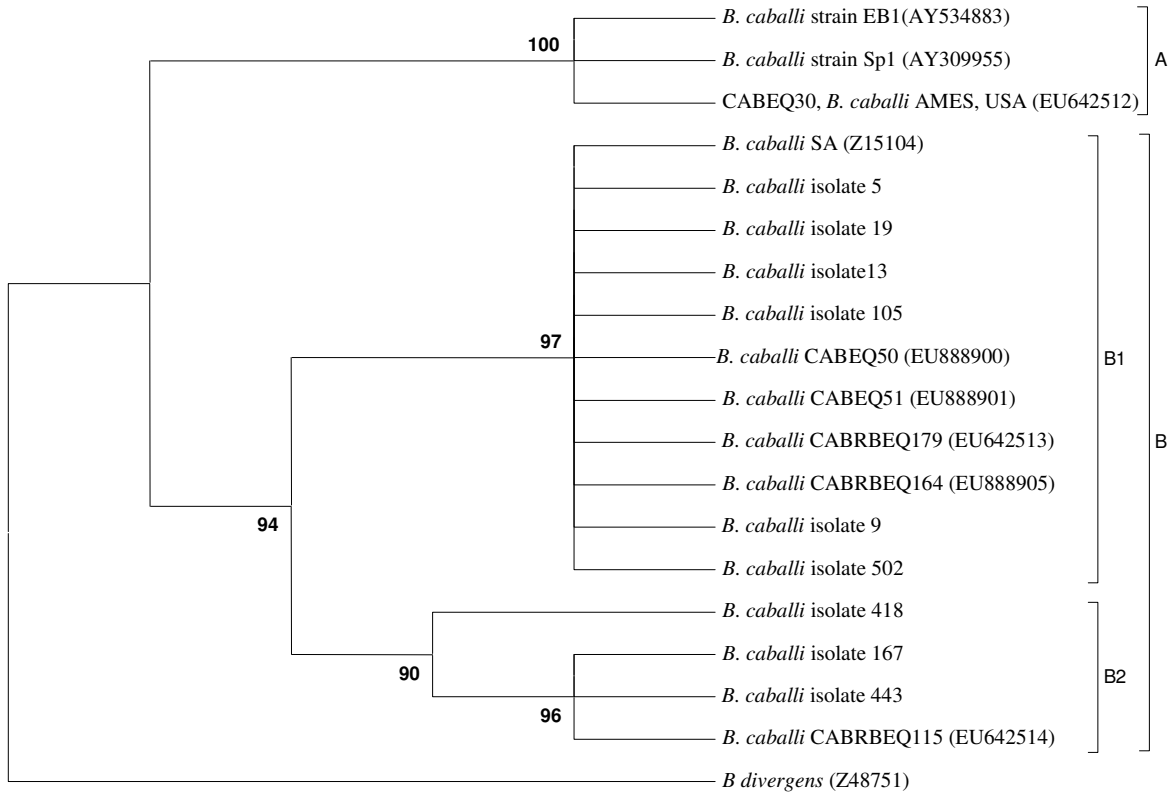


Figure 6.5 Rooted 50% majority-rule consensus cladogram, calculated using maximum-parsimony and bootstrap estimates from 1000 replicates, of the *B. caballi* 18S rRNA gene sequences identified in this study and the *B. caballi* 18S sequences from the public sequence databases (accession numbers are indicated in parenthesis). The *B. divergens* 18 rRNA gene sequence (accession number: Z48751) was used as an outgroup. Bootstrap support values are indicated on the nodes of the trees.

6.5 Discussion

Monoclonal antibody technology coupled with cELISA has been used to develop specific and sensitive serological assays for the detection of antibody to a number of infectious agents, including *B. caballi* (Knowles, 1996). A cELISA that was developed using a monoclonal antibody to RAP-1, proved to be effective in the detection of *B. caballi* antibody in the sera of infected horses from 21 different countries in the Americas and Europe (Shkap et al., 1998; Kappmeyer et al., 1999; Sevinc et al., 2008). However, in our hands the commercial cELISA kit was unable to detect *B. caballi* antibody in infected horses in South Africa, although *B. caballi* antibody was detected in 63 samples using the IFAT. Sera collected from horses during the early stages of infection and prior to the development of antibody, may result in false-negative cELISA results (Kappmeyer et al., 1999). However, this is unlikely to explain the failure of the

cELISA to detect *B. caballi* antibody in all 63 IFAT positive samples. We therefore characterized the gene encoding RAP-1 from ten South African *B. caballi* positive *in vitro*-cultured isolates, in an attempt to explain the failure of the assay.

Two of the three primer sets which were designed based on two published *B. caballi rap-1* nucleotide sequences, AF092736 (Kappmeyer et al., 1999) and AB017700 (Ikadai et al., 1999), obtained from the USDA *B. caballi* strain, failed to amplify the homologous *rap-1* gene and flanking regions (~1800bp) from the ten South African *B. caballi in vitro*-cultured samples. Sequence analysis of the one *rap-1* fragment obtained (~615 bp) revealed sequence heterogeneity in the regions where the PCR primers had been designed, suggesting that even greater sequence heterogeneity present in the middle and at the 3' end of the *rap-1* gene in the South African isolates may have prevented the downstream amplification primers from binding to the template DNA. The PCR-based genome walking method (Rishi et al., 2004) was therefore used to amplify and sequence the unknown flanking regions of the *rap-1* fragment. This technique is commonly used to isolate upstream regions of known DNA sequences (Rishi et al., 2004), and has been successfully applied in the amplification and sequencing of a number of genes from various tick-borne pathogens that include *Ehrlichia canis* (McBride et al., 1999), *Ehrlichia ruminantium* (van Heerden et al., 2004), *Anaplasma phagocytophilum* (Zhi et al., 2002) and *Theileria parva* (Sohanpal et al., 2000). Similarly, we were able to obtain *rap-1* gene sequences from *Pvu*II digested genomic DNA of *B. caballi* isolates Bcab9 and Bcab13. An alignment of the two new sequences allowed us to design primers to amplify and sequence the *rap-1* gene from an additional eight South African *B. caballi* isolates. A nucleotide sequence alignment of all *rap-1* sequences obtained in this study and the published sequences AF092736 and AB017700, confirmed the existence of nucleotide heterogeneity in the regions where the amplification and sequencing primers had originally been designed.

Phylogenetic analyses of predicted RAP-1 amino acid sequences showed that the South African *B. caballi* sequences grouped separately from the RAP-1 amino acid sequences obtained from the USDA *B. caballi* strain (Ikadai et al., 1999; Kappmeyer et al., 1999). In addition, the South African *B. caballi* RAP-1 amino acid sequences could be further divided into two subgroups. Subgroup B1 contained RAP-1 sequences from six isolates, which showed an average of 63.4% amino acid identity to *B. caballi* RAP-1 amino acid sequences AF092736 and AB017700, while the four sequences in subgroup B2 had an average of 59.3% amino acid identity to these sequences. The groupings of RAP-1 sequences from the South African *B. caballi* isolates were

consistent with the clades observed by phylogenetic analysis of the 18S rRNA gene. This could indicate the occurrence of two antigenically different *B. caballi* strains in South Africa, which are distinct from those occurring in other parts of the world. It would be interesting to determine whether there is an association between the different *B. caballi* genetic groups identified and the clinical manifestation of equine piroplasmosis.

The testing of antigens for use in ELISAs, based on the apical complex surface-exposed epitopes, is done on the assumption that these epitopes are widely conserved among isolates (Madruga et al., 1996). The 48-kDa immunodominant rhoptry protein isolated from a USDA strain of *B. caballi* has been shown to be present in both European and South American strains of *B. caballi* (Böse et al., 1994; Böse and Hentrich, 1994; Ikadai et al., 1999). Characterization of the 48-kDa rhoptry protein indicated the occurrence of highly conserved sequences also present in the Bv60/p58 family of rhoptry genes (*rap-1* gene family) isolated from *B. bovis* and *B. bigemina* (Ikadai et al., 1999). Significant homology has been found in the nucleotide and amino acid sequences of members of the *rap-1* gene family, but sequence identity is limited to short oligopeptides, with marked sequence variation occurring in other parts of the molecule (Palmer and McElwain, 1995). While the 58 kDa RAP-1 protein of *B. bigemina* and the 60 kDa RAP-1 protein of *B. bovis* have been shown to be antigenically conserved among all the geographic strains tested (McElwain et al., 1987; Palmer et al., 1991; Suarez et al., 1994), genomic sequence polymorphism occurs in *rap-1* genes among antigenically different strains of both *B. bigemina* and *B. bovis* (Suarez et al., 1994). Four different variants of the *B. bigemina* RAP-1 protein have been identified in a biological clone (Mishra et al., 1991; Mishra et al., 1992); the amino acid sequences of these variants were highly conserved in the central region, but the sequences at the amino- and carboxy-termini of the protein were found to be variable.

The monoclonal antibody (MAb 79/17.18.5) used in the *B. caballi*-specific cELISA has been shown to bind to a peptide epitope within the carboxy-terminal repeat region of the *B. caballi* RAP-1 amino acid sequence (Kappmeyer et al., 1999). Tandemly repeated amino acid residues occurring in the carboxy-terminal region of the RAP-1 protein were hypothesized to be surface-exposed merozoite epitopes that could be used to potentially block infectivity for host erythrocytes (Suarez et al., 1991). Close inspection of the carboxy-terminal region of the RAP-1 amino acid sequences obtained from the South African *B. caballi* isolates clearly indicates marked amino acid sequence differences in this repeat region and thus the probable absence of the monoclonal antibody binding site. This observation probably explains the failure of the

cELISA to detect antibody to *B. caballi* in sera of infected horses in South Africa. It is therefore likely that variation in RAP-1 sequences in South African *B. caballi* isolates is immunologically significant, as has been shown for *B. bigemina* (Hötzel et al., 1997). RAP-1 of *B. bigemina* has two regions of sequence dimorphism at the carboxy- and amino-terminal ends, respectively. Antibodies recognizing surface-exposed B-cell epitopes present in the amino-terminal variant type 1 (NT-1) do not cross react with NT-2, and CD4 T-cells recognizing epitopes in the carboxy-terminal variant type 1 (CT-1) do not recognise CT-2 (Hötzel et al., 1997).

In the development and testing of the cELISA assay for *B. caballi*, discrepancies between CFT and cELISA results were reported for five samples, each of which originated from a different country (Kappmeyer et al., 1999). Four of these five CFT positive but cELISA negative samples were confirmed to be true positives by IFAT. The observed discrepancy was thought to be due to the early sampling of sera from recently infected horses and it was suggested that, since the sera did not originate from one place, the difference in results was not due to a lack in conservation of the carboxy-terminal repeat region. In the light of our results, however, it may be possible that the latter was indeed the case.

In this study we have shown significant heterogeneity in *rap-1* gene sequences from South African *B. caballi* isolates, which is reflected in the predicted amino acid sequences, particularly in the carboxy-terminal regions. We were able to identify two distinct groups of novel RAP-1 amino acid sequences occurring among ten South African isolates. Examination of a larger number of *B. caballi* isolates from different geographical locations around South Africa, and other countries, may lead to the identification of additional, as yet unidentified RAP-1 sequences. Such variation complicates the development of sensitive and reliable serological and molecular diagnostic assays for the detection of all *B. caballi* infections. We therefore recommend that the cELISA for the detection of antibodies to *B. caballi* should be redesigned using a monoclonal antibody that binds to a more conserved epitope of the RAP-1 antigen, or alternatively by choosing a more conserved antigenic target protein.

6.6 Reference List

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403-410.

Anonymous, 2008. Equine piroplasmosis. In: OIE Manual of diagnostic tests and vaccines for terrestrial animals. Office International des Epizooties, Paris, France, pp. 884-893.

Bhoora, R., Franssen, L., Oosthuizen, M.C., Guthrie, A.J., Zweygarth, E., Penzhorn, B.L., Jongejan, F., Collins, N.E., 2009. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Veterinary Parasitology 159, 112-120.

Böse, R., Hentrich, B., 1994. Identification of antigens diagnostic for European isolates of *Babesia equi* by two-dimensional electrophoresis and Western blotting. Parasitology Research 80, 182-185.

Böse, R., Peymann, B., Barbosa, I.P., 1994. Identification of diagnostic antigens for South American *Babesia caballi* infections. International Journal for Parasitology 24, 255-258.

Brüning, A., Phipps, P., Posnett, E., Canning, E.U., 1997. Monoclonal antibodies against *Babesia caballi* and *Babesia equi* and their application in serodiagnosis. Veterinary Parasitology 68, 11-26.

Camacho, A.T., Guitian, F.J., Pallas, E., Gestal, J.J., Olmeda, A.S., Habela, M.A., Telford, S.R., III, Spielman, A., 2005. *Theileria* (*Babesia*) *equi* and *Babesia caballi* infections in horses in Galicia, Spain. Tropical Animal and Health Production 37, 293-303.

Dalrymple, B.P., Casu, R.E., Peters, J.M., Dimmock, C.M., Gale, K.R., Boese, R., Wright, I.G., 1993. Characterisation of a family of multi-copy genes encoding rhoptry protein homologues in *Babesia bovis*, *Babesia ovis* and *Babesia canis*. Molecular and Biochemical Parasitology 57, 181-192.

de Waal, D.T., 1992. Equine piroplasmosis: a review. British Veterinary Journal 148, 6-14.

Donnelly, J., Joyner, L.P., Graham-Jones, O., Ellis, C.P., 1980. A comparison of the complement fixation and immunofluorescent antibody tests in a survey of the prevalence of *Babesia equi* and *Babesia caballi* in horses in the Sultanate of Oman. Tropical Animal Health and Production 12, 50-60.

Friedhoff, K.T., Tenter, A.M., Muller, I., 1990. Haemoparasites of equines: impact on international trade of horses. Revue Scientifique et Technique 9, 1187-1194.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95-98.

Hötzel, I., Suarez, C.E., McElwain, T.F., Palmer, G.H., 1997. Genetic variation in the dimorphic regions of *rap-1* genes and rap-1 loci of *Babesia bigemina*. Molecular and Biochemical Parasitology 90, 479-489.

Ikadai, H., Xuan, X., Igarashi, I., Tanaka, S., Kanemaru, T., Nagasawa, H., Fujisaki, K., Suzuki, N., Mikami, T., 1999. Cloning and expression of a 48-kilodalton *Babesia caballi* merozoite rhoptry protein and potential use of the recombinant antigen in an enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 37, 3475-3480.

Kappmeyer, L.S., Perryman, L.E., Hines, S.A., Baszler, T.V., Katz, J.B., Hennager, S.G., Knowles, D.P., 1999. Detection of equine antibodies to *Babesia caballi* by recombinant *B. caballi* rhoptry-associated protein 1 in a competitive-inhibition enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 37, 2285-2290.

Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Research 33, 511-518.

Katz, J., Dewald, R., Nicholson, J., 2000. Procedurally similar competitive immunoassay systems for the serodiagnosis of *Babesia equi*, *Babesia caballi, Trypanosoma equiperdum*, and *Burkholderia mallei* infection in horses. Journal of Veterinary Diagnostic Investigation 12, 46-50.

Knowles, D.P., Kappmeyer, L.S., Stiller, D., Hennager, S.G., Perryman, L.E., 1992. Antibody to a recombinant merozoite protein epitope identifies horses infected with *Babesia equi*. Journal of Clinical Microbiology 30, 3122-3126.

Knowles, D.P., Perryman, L.E., Kappmeyer, L.S., Hennager, S.G., 1991. Detection of equine antibody to *Babesia equi* merozoite proteins by a monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 29, 2056-2058.

Knowles, J., 1996. Equine Babesiosis (piroplasmosis): A problem in the international movement of horses. British Veterinary Journal 152, 123-126.

Machado, R.Z., McElwain, T.F., Pancracio, H.P., Freschi, C.R., Palmer, G.H., 1999. *Babesia bigemina*: Immunization with purified rhoptries induces protection against acute parasitemia. Experimental Parasitology 93, 105-108.

Madruga, C.R., Suarez, C.E., McElwain, T.F., Palmer, G.H., 1996. Conservation of merozoite membrane and apical complex B cell epitopes among *Babesia bigemina* and *Babesia bovis* strains isolated in Brazil. Veterinary Parasitology 61, 21-30.

McBride, J.W., Yu, X., Walker, D.H., 1999. Molecular cloning of the gene for a conserved major immunoreactive 28-kilodalton protein of *Ehrlichia canis*: a potential serodiagnostic antigen. Clinical and Diagnostic Laboratory Immunology 6, 392-399.

McElwain, T.F., Perryman, L.E., Davis, W.C., McGuire, T.C., 1987. Antibodies define multiple proteins with epitopes exposed on the surface of live *Babesia bigemina* merozoites. Journal of Immunology 138, 2298-2304.

Mishra, V.S., McElwain, T.F., Dame, J.B., Stephens, E.B., 1992. Isolation, sequence, and differential expression of the p58 gene family of *Babesia bigemina*. Molecular and Biochemical Parasitology 53, 149-158.

Mishra, V.S., Stephens, E.B., Dame, J.B., Perryman, L.E., McGuire, T.C., McElwain, T.F., 1991. Immunogenicity and sequence analysis of recombinant p58: a neutralization-sensitive, antigenically conserved *Babesia bigemina* merozoite surface protein. Molecular and Biochemical Parasitology 53, 207-212.

Palmer, G.H., McElwain, T.F., 1995. Molecular basis for vaccine development against anaplasmosis and babesiosis. Veterinary Parasitology 57, 233-253.

Palmer, G.H., McElwain, T.F., Perryman, L.E., Davis, W.C., Reduker, D.R., Jasmer, D.P., Shkap, V., Pipano, E., Goff, W.L., McGuire, T.C., 1991. Strain variation of *Babesia bovis* merozoite surface-exposed epitopes. Infection and Immunity 59, 3340-3342.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Rishi, A.S., Nelson, N.D., Goyal, A., 2004. Genome walking of large fragments: an improved method. Journal of Biotechnology 111, 9-15.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

Sam-Yellowe, T.Y., 1996. Rhoptry organelles of the apicomplexa: Their role in host cell invasion and intracellular survival. Parasitology Today 12, 308-316.

Sevinc, F., Maden, M., Kumas, C., Sevinc, M., Ekici, O.D., 2008. A comparative study on the prevalence of *Theileria equi* and *Babesia caballi* infections in horse sub-populations in Turkey. Veterinary Parasitology 156, 173-177.

Shkap, V., Cohen, I., Leibovitz, B., Savitsky, Pipano, E., Avni, G., Shofer, S., Giger, U., Kappmeyer, L., Knowles, D.P., 1998. Seroprevalence of *Babesia equi* among horses in Israel using competitive inhibition ELISA and IFA assays. Veterinary Parasitology 76, 251-259.

Skuce, P.J., Mallon, T.R., Taylor, S.M., 1996. Molecular cloning of a putative rhoptry associated protein homologue from *Babesia divergens*. Molecular and Biochemical Parasitology 77, 99-102.

Sohanpal, B., Wasawo, D., Bishop, R., 2000. Cloning of telomere-associated DNA using single-specific-primer polymerase chain reaction provides evidence for a conserved sequence directly adjacent to *Theileria parva* telomeric repeats. Gene 255, 401-409.

Staden, R., 1996. The Staden sequence analysis package. Molecular Biotechnology 5, 233-241.

Suarez, C.E., McElwain, T.F., Echaide, I., Toriani de Echaide, S., Palmer, G.H., 1994. Interstrain conservation of babesial RAP-I surface-exposed B-cell epitopes despite *rap-1* genomic polymorphism. Infection and Immunity 62, 3576-3579.

Suarez, C.E., Palmer, G.H., Florin-Christensen, M., Hines, S.A., Hötzel, I., McElwain, T.F., 2003. Organization, transcription, and expression of rhoptry associated protein genes in the *Babesia bigemina* rap-1 locus. Molecular and Biochemical Parasitology 127, 101-112.

Suarez, C.E., Palmer, G.H., Hötzel, I., Hines, S.A., McElwain, T.F., 1998. Sequence and functional analysis of the intergenic regions separating *Babesial* rhoptry-associated protein-1 (*rap-1*) genes. Experimental Parasitology 90, 189-194.

Suarez, C.E., Palmer, G.H., Jasmer, D.P., Hines, S.A., Perryman, L.E., McElwain, T.F., 1991. Characterization of the gene encoding a 60-kilodalton *Babesia bovis* merozoite protein with conserved and surface exposed epitopes. Molecular and Biochemical Parasitology 46, 45-52.

Swofford, D.L., 2003. PAUP*. Phylogenetic Analysis using Parsimony (*and other methods). Version 4b10. Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0 Molecular Biology and Evolution 24, 1596-1599.

van Heerden, H., Steyn, H.C., Allsopp, M.T.E.P., Zweygarth, E., Josemans, A.I., Allsopp, B.A., 2004. Characterization of the pCS20 region of different *Ehrlichia ruminantium* isolates. Veterinary Microbiology 101, 279-291.

Weiland, G., 1986. Species-specific serodiagnosis of equine piroplasma infections by means of complement fixation test (CFT), immunofluorescence (IIF), and enzyme-linked immunosorbent assay (ELISA). Veterinary Parasitology 20, 43-48.

Xuan, X., Chahan, B., Huang, X., Yokoyama, N., Makala, L.H., Igarashi, I., Fujisaki, K., Maruyama, S., Sakai, T., Mikami, T., 2002. Diagnosis of equine piroplasmosis in Xinjiang province of China by the enzyme-linked immunosorbent assays using recombinant antigens. Veterinary Parasitology 108, 179-182.

Yokoyama, N., Okamura, M., Igarashi, I., 2006. Erythrocyte invasion by *Babesia* parasites: Current advances in the elucidation of the molecular interactions between the protozoan ligands and host receptors in the invasion stage. Veterinary Parasitology 138, 22-32.

Zhi, N., Ohashi, N., Tajima, T., Mott, J., Stitch, R.W., Grover, D., Telford III, S.R., Lin, Q., Rikihasa, Y., 2002. Transcript heterogeneity of the *p44* multigene family in a human granulocytic *Ehrlichiosis* agent transmitted by ticks. Infection and. Immunity 70, 1175-1184.

Zweygarth, E., Lopez-Rebollar, L.M., Nurton, J.P., Guthrie, A.J., 2002. Culture, isolation and propagation of *Babesia caballi* from naturally infected horses. Parasitology Research 88, 460-462.

CHAPTER 7

General Discussion

In most organisms, nuclear ribosomal RNA (rRNA) genes are thought to be under tight structural and functional constraint resulting in lower substitution rates and the absence of lateral gene transfer across lineages (Allsopp and Allsopp, 2006). In this study, unexpected sequence heterogeneity was found within the 18S rRNA genes of *Theileria equi* and *Babesia caballi* parasites, which led to the identification of three distinct *T. equi* and two *B. caballi* genetic groups in South African equids (Bhoora et al., 2009a; Chapter 2). 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). Sequence heterogeneity in the 18S rRNA gene has also been reported within some species of protozoa and genetically distinct *T. equi* and *B. caballi* genotypes infecting Spanish horses have also been identified previously (Criado-Fornelio et al., 2004; Nagore et al., 2004). Although piroplasm infections in zebra were first described at the turn of the century, the molecular epidemiology and possible influence that their existence may have had on horse piroplasms has largely been overlooked. In this study, the molecular epidemiology of *T. equi*-like piroplasms of zebra was also examined with the view to further elucidating the genetic variation of *T. equi* parasites in South Africa (Chapter 4).

Sequence analysis of both horse and zebra parasites confirmed the existence of as many as 25 distinct 18S rRNA sequences for *T. equi*, which belonged to three main groups (Bhoora et al., 2009a; Chapter 2; Chapter 4). Group A predominantly contained *T. equi* 18S rRNA sequences from horses, while group B, which was shown to contain a *T. equi*-like 18S rRNA sequence from a horse in Spain (Nagore et al., 2004), thus far only contains *T. equi* 18S sequences from zebra in South Africa. Novel *T. equi* 18S rRNA sequences from both zebra and horses in South Africa grouped together in the third group, group C. These results suggest that (a) there exists an assortment of *T. equi* 18S genotypes in the equid species investigated and (b) there may be more variation in the *T. equi* genotypes in South African equids than has been previously reported. *Babesia caballi* occurred at extremely low parasitaemias in zebra and we were thus unable to draw conclusions regarding the extent of sequence heterogeneity amongst these parasites infecting zebra (Chapter 4). Although less variation was observed amongst the *B. caballi* samples from horses, six different *B. caballi* 18S sequences which formed two distinct genetic groups (A and B), were identified (Bhoora et al., 2009a; Chapter 2). Group B could be further

subdivided into subgroups B1 and B2. Taken together, these observations explained the failure of many previously reported molecular assays that target the 18S rRNA gene (Bashiruddin et al., 1999; Rampersad et al., 2003; Nagore et al., 2004) to detect both *T. equi* and *B. caballi* in South Africa.

The molecular epidemiological study of piroplasms infecting zebra revealed the existence of a *T. equi*-like genotype in zebra, which has thus far not been identified in South African horse samples. It would be interesting to examine more samples collected from horses as well as from each of the three zebra species that live in sub-Saharan Africa to determine whether the *T. equi*-like parasite identified in zebra in this study is indeed exclusive to zebra in Africa. In addition, we have not examined any samples from donkeys or mules in South Africa. It would be interesting to determine the distribution of *T. equi* and *B. caballi* genotypes in these equids as well.

Accurate diagnosis of equine piroplasmosis is essential for effective control measures. It is therefore imperative that reliable and accurate molecular diagnostic assays be developed. A conserved region outside of the V4 hypervariable region of the 18S rRNA gene has been identified previously and targeted in the development of a quantitative TaqMan real-time PCR (qPCR) assay for the detection of *T. equi* (Kim et al., 2008). Despite the existence of extensive sequence variation in South African *T. equi* 18S rRNA gene sequences, an evaluation of this assay on South African isolates indicated that it is both sensitive and specific (Bhoora et al., 2009b; Chapter 3). The *T. equi*-specific qPCR assay was further shown to be able to detect DNA in samples representative of each of the three previously identified *T. equi* 18S rRNA genotypes (groups A, B and C) (Bhoora et al., 2009b; Chapter 3). The development of an 18S rRNA-based TaqMan MGB™ qPCR assay for the detection of *B. caballi* infections in equine blood samples is described in chapter 3; this test was both sensitive and specific. Previous assays proved to be limited in their ability to detect *B. caballi* infections in field blood samples due to extremely low parasitaemias that rarely exceed 1% (Hanafusa et al., 1998) or the complete absence of circulating parasites in the blood of carrier animals (Frerichs et al., 1969; Holman et al., 1993). In addition, the *B. caballi*-specific TaqMan MGB™ qPCR assay developed in this study could detect parasite DNA from samples representative of each of the previously identified *B. caballi* 18S rRNA genotypes (A, B1 and B2).

Due to the lack of sensitivity of many of the molecular diagnostic assays used previously, the prevalence of equine piroplasmosis in South African equids has probably been under-reported. This was clearly shown for zebra samples (Chapter 4), in which only 35% of samples tested were positive for *T. equi* and *B. caballi* parasites using the reverse line blot (RLB) hybridization assay, whereas 87% were positive using the qPCR assays. The application of the *B. caballi*-specific TaqMan MGB™ qPCR assay in conjunction with the *T. equi*-specific qPCR assay enabled the rapid and accurate quantification of both *B. caballi* and *T. equi* from sub-clinically infected or carrier animals (Bhoora et al., 2009b; Chapter 3) and also provided a more accurate report on the molecular epidemiology of piroplasms infecting zebra in South Africa (Chapter 4). The *B. caballi*-specific TaqMan MGB™ qPCR assay detected as low as 1.14 x 10^{-4} % PE, while the detection limit of the *T. equi*-specific qPCR assay was 1.9 x 10^{-4} % PE. Although an evaluation of these assays on South African isolates has indicated that they are both sensitive and specific, we cannot rule out the possible existence of as yet undetected *T. equi* and *B. caballi* 18S rRNA gene sequence variants, which may compromise the sensitivities of these assays. It might therefore be advantageous to incorporate additional *T. equi*-specific and *B. caballi*-specifc qPCR assays, which target other genes, in a multiplex qPCR assay for the detection of equine piroplasmosis. To this end, we examined the ability of a previously developed *T. equi ema-1*-specific qPCR assay (Ueti et al., 2003) to detect parasite DNA in South African field samples which were shown to be positive by other tests including cELISA, IFAT, *in vitro* culture and *T. equi*-specific 18S qPCR. However, this qPCR was unable to detect parasite DNA in many *T. equi*-positive field samples. Sequence analysis of the *T. equi ema-1* gene from South African field samples and isolates confirmed the existence of distinct *ema-1* sequences, which belonged to three main phylogenetic groups. An alignment of the South African *ema-1* gene sequences indicated that the qPCR primers and probe sequence regions were conserved among all isolates occurring in group A, but nucleotide differences in sequences occurring in groups B and C explained either the failure or the reduced sensitivity of the Ueti *ema-1* TaqMan qPCR assay in detecting *T. equi* in some samples. We therefore developed and evaluated a second TaqMan MGB™ qPCR assay targeting the *T. equi ema-1* gene. Again, this assay was shown to be both sensitive and specific in the detection of *T. equi* infections (Chapter 5).

Currently, serological assays prescribed for equine piroplasmosis, which include the use of the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA), are restricted by antibody detection limits and cross-reactivity and have been shown to have a low sensitivity for detecting latent infections (Donnelly et al., 1980; Tenter and Friedhoff, 1986;

Weiland, 1986; Böse et al., 1995; Anonymous, 2008). Recombinant *T. equi* and *B. caballi* merozoite surface proteins, implicated in the pathogenesis of haemoprotozoan diseases, have recently been used, together with monoclonal antibodies to immunogenic epitopes on these proteins, in the development of competitive inhibition ELISAs (cELISA) (Knowles et al., 1991; Sam-Yellowe, 1996). In chapters 5 and 6, we determined whether the commercially available cELISAs were able to detect antibody to *T. equi* and *B. caballi* in the sera of infected horses from South Africa. The recombinant equi merozoite antigen (EMA-1) used in the development of the cELISA, has been shown previously to be conserved among geographically distant *T. equi* isolates (Xuan et al., 2001) and orthologous gene sequences have also 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). In this study, the cELISA detected antibody to *T. equi* in the sera of infected horses from South Africa, although some discrepencies between the IFAT and cELISA alluded to the fact that there may be heterogeneity in the EMA-1 epitopes of some South African *T. equi* isolates (Chapter 5). In contrast, the cELISA developed using a monoclonal antibody to a recombinant apical complex rhoptry-associated protein (RAP-1), and which has been reported to successfully detect *B. caballi* antibody in the sera of infected horses from North and South America and various European countries (Kappmeyer et al., 1999; Sevinc et al., 2008), failed to detect *B. caballi* antibody in the sera of infected horses in South Africa (Bhoora et al., 2010; Chapter 6). In chapter 6 we looked at RAP-1 amino sequences from South African *B. caballi* isolates to ascertain the reasons for the failure of the cELISA.

Due to the extent of sequence variation in the *rap-1* gene from South African *B. caballi* isolates, a PCR-based genome walking method had to be used in order to amplify the complete open reading frame (1479 bp) encoding the RAP-1 protein (493 amino acids). Phylogenetic analyses confirmed the existence of two RAP-1 groups, with South African *B. caballi* RAP-1 amino acid sequences grouping separately from other published RAP-1 amino acid sequences (Bhoora et al., 2010; Chapter 6). Sequences from the two South African RAP-1 groups showed an average of 59.3 and 63.4% amino acid identities to published *B. caballi* RAP-1 sequences. Marked amino acid sequence differences in the carboxy-terminal repeat region of the *B. caballi* RAP-1 amino acid sequence in South African isolates, and thus the possible absence of the monoclonal antibody binding site, probably explain the failure of the cELISA to detect *B. caballi* antibody in infected horses in South Africa (Bhoora et al., 2010; Chapter 6). Since these results are based on analysis of a small sample population, examination of a larger number of *B. caballi* isolates from different geographical locations around South Africa and the rest of the world may lead to the

identification of additional RAP-1 amino acid sequences, the occurrence of which could further complicate the serological diagnosis of equine piroplasmosis. Based on our findings in this chapter, it is evident that the current cELISA developed for the detection of *B. caballi* antibody cannot be implemented as a prescribed serological assay for the diagnosis of equine piroplasmosis.

A comparison between the *T. equi* 18S rRNA and *ema-1* gene sequences obtained from the same samples showed inconsistencies between the phylogenetic groupings obtained, which precludes the possibility that the three *T. equi* 18S groupings identified represent different strains or lineages (Chapter 5). In contrast, the groupings of the *rap-1* sequences from South African *B. caballi* isolates were consistent with the clades observed by phylogenetic analysis of the 18S rRNA gene, indicating the potential occurrence of two antigenically different *B. caballi* strains in South Africa, which are distinct from those occurring in other parts of the world (Bhoora et al., 2010; Chapter 6). The results therefore suggest that *T. equi* and *B. caballi* parasites in South Africa have developed distinct evolutionary patterns. However, based on these observations, it also becomes evident that it is not possible to use the 18S rRNA gene to decide whether variants represent new species or subspecies. On the other hand, parasite outer membrane protein gene sequences, which are likely to be under intense selection pressure and therefore evolve at a more rapid rate than core function genes, will probably not give reliable phylogenetic information at the species level (Allsopp and Allsopp, 2006). Given the variation, particularly in *T. equi* in South Africa, sequence data obtained from other regions such as the ribosomal internal transcriber spacer (ITS) or the beta-tubulin gene may allow for better delineation of species and subspecies.

Owing to the extent of variation observed both within the *T. equi* 18S rRNA sequences as well as within the *B. caballi* RAP-1 and *T. equi* EMA-1 amino acid sequences, we cannot overlook the fact that there may be more variation in the 18S rRNA gene than we have already identified. Therefore, although the qPCR assays developed in this study were able to detect all currently known *T. equi* and *B. caballi* 18S rRNA sequence variants in South Africa, it is recommended that a multiplex qPCR assay, including a "catch-all" TaqMan probe similar to the *Theileria/Babesia* genus-specific probe used in the RLB, should be developed so as to ensure that if a piroplasm parasite with a novel 18S rRNA gene sequence is present, it will at least be detected by the genus-specific probe.

Another recommendation would be to evaluate the ability of the recently developed *B. caballi rap-1*-specific qPCR assay (Heim et al., 2007) in detecting parasite DNA in South African *B. caballi* isolates. Inspection of the *rap-1* nucleotide sequences from South African *B. caballi* isolates indicates the occurrence of variation in the qPCR primer and probe regions, however, which could potentially compromise the sensitivity of the *rap-1* qPCR assay. Since we have already established that the sensitivity of the *ema-1* qPCR assay developed by Ueti et al. (2003) is compromised by nucleotide sequence variation in the *ema-1* gene of South African *T. equi* parasites, the multiplex qPCR assay described by Heim et al. (2007), which employs the Ueti et al. (2003) *ema-1* qPCR assay, cannot be used to detect equine piroplasmosis in South Africa. It should be noted that the variation observed in the RAP-1 amino acid sequences in South African *B. caballi* isolates is based on data obtained from a relatively small number of samples, and, as mentioned above, investigation of a larger number of samples may reveal the existence of other RAP-1 amino acid sequences not identified in this study. Once the extent of sequence variation in the *rap-1* gene has been more thoroughly examined, a qPCR assay targeting a more conserved region of the *rap-1* gene could be developed and incorporated together with the *T. equi ema-1*-specific TaqMan MGB™ qPCR assay (Chapter 5), the *T. equi*-specific 18S rRNA qPCR assay (Kim et al., 2008) and the *B. caballi* TaqMan MGB™ qPCR assay (Bhoora et al., 2009b; Chapter 3) in a multiplex qPCR assay for the definitive detection of equine piroplasmosis.

Finally, we propose that a study be conducted to determine whether there is an association between the different *T. equi* and *B. caballi* genotypes identified in this study and disease phenotypes. A study conducted on the *B. rossi BrEMA-1* gene led to the identification of 13 *B. rossi* genotypes, four of which could be associated with complicated canine babesosis (Matjila et al., 2009). The identification of pathogenic *T. equi* strains or variants could be useful in the development of suitable control strategies for the parasite.

Equine piroplasmosis has a substantial impact on the international transport of horses, as positive animals are generally not permitted to enter piroplasm-free countries. Currently, regulations require the serological testing of horses by means of the IFAT or the ELISA to confirm seronegativity and to identify seropositive horses whose movement is restricted (Böse et al., 1995; Brüning, 1996). Due to the lack of sensitivity of these assays, however, the possible introduction of false-negative and therefore inapparent carrier animals into areas where competent tick vectors are prevalent can lead to an epizootic spread of the disease (Anonymous; 2008).

The findings in this thesis clearly highlight the challenges involved in developing rapid, sensitive and specific diagnostic assays for piroplasm parasites. Despite these challenges, we were able to develop three real-time PCR assays, which have significantly improved the sensitivity and specificity of detection of *T. equi* and *B. caballi* infections in South African horses over the currently prescribed serological assays.

Although the molecular epidemiology of equine piroplasmosis in South Africa has been studied previously (Posnett and Ambrosio, 1989; Posnett et al., 1991), results were based on the detection of parasites using DNA probe technology. This study therefore represents the first comprehensive molecular study, employing DNA sequence analysis and more advanced technologies, including real-time PCR, in the detection of equine piroplasmosis in South Africa. Our results will add to the existing knowledge of equine piroplasmosis worldwide and will be invaluable in the development of further molecular or serological diagnostic assays and in the development and implementation of successful control strategies. It is envisaged that, once validated, the possible incorporation of the real-time PCR assays developed in this study as prescribed tests by the World Organization for Animal Health (OIE), may assist in the accurate identification of carrier animals and therefore provide greater control over the spread of equine piroplasmosis globally.

7.1 Reference List

Allsopp, M.T.E.P., Allsopp, B.A., 2006. Molecular sequence evidence for the reclassification of some *Babesia* Species. Annals of the New York Academy of Sciences 1081, 509-517.

Allsopp, M.T.E.P., Lewis, B.D., Penzhorn, B.L., 2007. Molecular evidence for transplacental transmission of *Theileria equi* from carrier mares to their apparently healthy foals. Veterinary Parasitology 148, 130-136.

Anonymous, 2008. Equine piroplasmosis. In: OIE Manual of diagnostic tests and vaccines for terrestrial animals. Office International des Epizooties, Paris, France, pp. 884-893.

Bashiruddin, J.B., Camma, C., Rebelo, E., 1999. Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. Veterinary Parasitology 84, 75-83.

Bhoora, R., Franssen, L., Oosthuizen, M.C., Guthrie, A.J., Zweygarth, E., Penzhorn, B.L., Jongejan, F., Collins, N.E., 2009a. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Veterinary Parasitology 159, 112-120.

Bhoora, R., Quan, M., Franssen, L., Butler, C.M., van der Kolk, J.H., Guthrie, A.J., Zweygarth, E., Jongejan, F., Collins, N.E., 2009b. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. Veterinary Parasitology doi:10.1016/j.vetpar.2009.11.011.

Bhoora, R., Quan, M., Zweygarth, E., Guthrie, A.J., Prinsloo, S.A., Collins, N.E., 2010. Sequence heterogeneity in the gene encoding the rhoptry-associated protein-1 (RAP-1) of *Babesia caballi* isolates from South Africa. Veterinary Parasitology doi:10.1016/j.vetpar.2010.01.009.

Böse, R., Jorgensen, W.K., Dalgliesh, R.J., Friedhoff, K.T., de Vos, A.J., 1995. Current state and future trends in the diagnosis of *babesiosis*. Veterinary Parasitology 57, 61-74.

Brayton, K.A., Lau, A.O., Herndon, D.R., Hannick, L., Kappmeyer, L.S., Berens, S.J., Bidwell, S.L., Brown, W.C., Crabtree, J., Fadrosh, D., Feldblum, T., Forberger, H.A., Haas, B.J., Howell, J.M., Khouri, H., Koo, H., Mann, D.J., Norimine, J., Paulsen, I.T., Radune, D., Ren, Q., Smith, R.K. Jr, Suarez, C.E., White, O., Wortman, J.R., Knowles, D.P. Jr, McElwain, T.F., Nene, V.M., 2007. Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. PLoS Pathogens 3, 1401-1413.

Brüning, A., 1996. Equine piroplasmosis an update on diagnosis, treatment and prevention. British Veterinary Journal 152, 139-151.

Criado-Fornelio, A., Gonzalez-del-Rio, M.A., Buling-Sarana, A., Barba-Carretero, J.C., 2004. The "expanding universe" of piroplasms. Veterinary Parasitology 119, 337-345.

d'Oliveira, C., Tijhaar, E.J., Shiels, B.R., van der Weide, M., Jongejan, F., 1996. Expression of genes encoding two major *Theileria annulata* merozoite surface antigens in *Escherichia coli* and a *Salmonella typhimurium aroA* vaccine strain. Gene 172, 33-39.

Donnelly, J., Joyner, L.P., Graham-Jones, O., Ellis, C.P., 1980. A comparison of the complement fixation and immunofluorescent antibody tests in a survey of the prevalence of *Babesia equi* and *Babesia caballi* in horses in the Sultanate of Oman. Tropical Animal Health and Production. 12, 50-60.

Frerichs, W.M., Holbrook, A.A., Johnson, A.J., 1969. Equine piroplasmosis: complement-fixation titers of horses infected with *Babesia caballi*. American Journal of Veterinary Research 30, 697-702.

Gunderson, J.H., Sogin, M.L., Wollet, G., Hollingdale, V.F., de la Cruz, V.F., Waters, A.P., McCutchen, T.F., 1987. Structurally distinct, stage-specific-ribosomes occur in *Plasmodium*. Science 238, 933-937.

Hanafusa, Y., Cho, K.O., Kanemaru, T., Wada, R., Sugimoto, C., Onuma, M., 1998. Pathogenesis of *Babesia caballi* infection in experimental horses. Journal of Veterinary Medical Science 60, 1127-1132.

Heim, A., Passos, L.M.F., Ribeiro, M.F.B., Costa-Junior, L.M., Bastos, C.V., Cabral, D.D., Hirzmann, J., Pfister, K., 2007. Detection and molecular characterization of *Babesia caballi* and *Theileria equi* isolates from endemic areas of Brazil. Parasitology Research 102, 63-68.

Holman, P.J., Frerichs, W.M., Chieves, L., Wagner, G.G., 1993. Culture confirmation of the carrier status of *Babesia caballi*-infected horses. Journal of Clinical Microbiology 31, 698-701.

Kappmeyer, L.S., Perryman, L.E., Hines, S.A., Baszler, T.V., Katz, J.B., Hennager, S.G., Knowles, D.P., 1999. Detection of equine antibodies to *Babesia caballi* by recombinant *B. caballi* rhoptry-associated protein 1 in a competitive-inhibition enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 37, 2285-2290.

Katende, J.M., Goddeeris, B.M., Morzaria, S.P., Nkonge, C.G., Musoke, A.J., 1990. Identification of a *Theileria mutans*-specific antigen for use in an antibody and antigen detection ELISA. Parasite Immunology 12, 419-433.

Kawazu, S.I., Sugimoto, C., Kamio, T., Fujisaki, K., 1992. Analysis of the genes encoding immunodominant piroplasm surface proteins of *Theileria sergenti* and *Theileria buffeli* by nucleotide sequencing and polymerase chain reaction. Molecular and Biochemical Parasitology 56, 169-175.

Kim, C.M., Blanco, L.B.C., Alhassan, A., Iseki, H., Yokoyama, N., Xuan, X., Igarashi, I., 2008. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. Veterinary Parasitology 151, 158-163.

Knowles, D.P., Perryman, L.E., Goff, W.L., Miller, C.D., Harrington, R.D., Gorham, J.R., 1991. A monoclonal antibody defines a geographically conserved surface protein epitope of *Babesia equi* merozoites. Infection & Immunity 59, 2412-2417.

Laughery, J.M., Lau, A.O.T., White, S.N., Howell, J.M., Suarez, C.E., 2009. *Babesia bovis*: Transcriptional analysis of rRNA gene unit expression. Experimental Parasitology 123, 45-50.

Matjila, P.T., Carcy, B., Leisewitz, A.L., Schetters, T., Jongejan, F., Gorenflot, A., Penzhorn, B.L., 2009. Preliminary evaluation of the Br*EMA1* gene as a tool for associating *Babesia rossi* genotypes and clinical manifestation of canine babesiosis. Journal of Clinical Microbiology 47, 3586-3592.

Matsuba, T., Sugimoto, C., Hattori, M., Sako, Y., Fujisaki, K., Onuma, M., 1995. Expression of a 32 kilodalton *Theileria sergenti* piroplasm surface protein by recombinant baculoviruses. International Journal for Parasitology 25, 939-943.

Nagore, D., Garcia-Sanmartin, J., Garcia-Perez, A.L., Juste, R.A., Hurtado, A., 2004. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: epidemiological survey and phylogenetic analysis. Veterinary Parasitology 123, 41-54.

Posnett, E.S., Ambrosio, R.E., 1989. Repetitive DNA probes for the detection of *Babesia equi*. Molecular and Biochemical Parasitology 34, 75-78.

Posnett, E.S., Fehrsen, J., de Waal, D.T., Ambrosio, R.E., 1991. Detection of *Babesia equi* in infected horses and carrier animals using a DNA probe. Veterinary Parasitology 39, 19-32.

Rampersad, J., Cesar, E., Campbell, M.D., Samlal, M., Ammons, D., 2003. A field evaluation of PCR for the routine detection of *Babesia equi* in horses. Veterinary Parasitology 114, 81-87.

Reddy, R.G., Chakrabarti, D., Yowell, C.A., Dame, J.B., 1991. Sequence microheterogeneity of the three small subunit ribosomal RNA genes of *Babesia bigemina*: expression in eukaryotic culture. Nucleic Acids Research 19, 3641-3645.

Sam-Yellowe, T.Y., 1996. Rhoptry organelles of the apicomplexa: Their role in host cell invasion and intracellular survival. Parasitology Today 12, 308-316.

Sevinc, F., Maden, M., Kumas, C., Sevinc, M., Ekici, O.D., 2008. A comparative study on the prevalence of *Theileria equi* and *Babesia caballi* infections in horse sub-populations in Turkey. Veterinary Parasitology 156, 173-177.

Shiels, B.R., d'Oliveira, C., McKellar, S., Ben-Miled, L., Kawazu, S.I., Hide, G., 1995. Selection of diversity at putative glycosylation sites in the immunodominant merozoite/piroplasm surface antigen of *Theileria* parasites. Molecular and Biochemical Parasitology 72, 149-162.

Tenter, A.M., Friedhoff, K.T., 1986. Serodiagnosis of experimental and natural *Babesia equi* and *B. caballi* infections. Veterinary Parasitology 20, 49-61.

Ueti, M.W., Palmer, G.H., Kappmeyer, L.S., Scoles, G.A., Knowles, D.P., 2003. Expression of equi merozoite antigen 2 during development of *Babesia equi* in the midgut and salivary gland of the vector tick *Boophilus microplus*. Journal of Clinical Microbiology 41, 5803-5809.

Weiland, G., 1986. Species-specific serodiagnosis of equine piroplasma infections by means of complement fixation test (CFT), immunofluorescence (IIF), and enzyme-linked immunosorbent assay (ELISA). Veterinary Parasitology 20, 43-48.

Xuan, X., Larsen, A., Ikadai, H., Tanaka, T., Igarashi, I., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., 2001. Expression of Babesia equi merozoite antigen 1 in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 39, 705-709.

SCIENTIFIC PUBLICATIONS

Bhoora, R., Franssen, L., Oosthuizen, M.C., Guthrie, A.J., Zweygarth, E., Penzhorn, B.L., Jongejan, F., Collins, N.E., 2009. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Veterinary Parasitology 159, 112-120.

Bhoora, R., Quan, M., Franssen, L., Butler, C.M., van der Kolk, J.H., Guthrie, A.J., Zweygarth, E., Jongejan, F., Collins, N.E., 2009. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. Veterinary Parasitology doi:10.1016/j.vetpar.2009.11.011.

Bhoora, R., Quan, M., Zweygarth, E., Guthrie, A.J., Prinsloo, S.A., Collins, N.E., 2010. Sequence heterogeneity in the gene encoding the rhoptry-associated protein-1 (RAP-1) of *Babesia caballi* isolates from South Africa. Veterinary Parasitology doi:10.1016/j.vetpar.2010.01.009.

APPENDICES

Appendix A Quantitative PCR results obtained for the ten-fold dilution series from the *in vitro*-cultured South African *B. caballi* 502 and *T. equi* WL isolates

***B. caballi* 502**

%PE		8.1	0.81	0.081	0.0081	0.00081	0.000081	0.0000081				
Log%PE		0.9084850	0.0915150	-1.0915150	-2.0915150	-3.0915150	-4.0915150	-5.0915150	Slope	Intercept	Efficiency	Amplification
	1	19.66	23.06	26.54	29.82	33.19	37.53	Negative	-3.34	22.90	0.99	1.99
	2	19.23	22.81	26.38	29.46	33.34	37.37	Negative	-3.52	22.60	0.92	1.92
REP1	3	19.50	22.76	26.06	30.67	33.39	37.68	Negative	-3.58	22.75	0.90	1.90
	4	19.38	22.82	25.95	29.43	32.74	Negative	Negative	-3.26	22.62	1.03	2.03
	5	19.25	22.74	26.08	29.35	32.76	35.97	Negative	-3.28	22.56	1.02	2.02
REP2	6	19.25	22.78	25.94	29.30	32.28	37.68	Negative	-3.48	22.43	0.94	1.94
	7	19.30	22.83	26.07	29.29	32.82	37.20	Negative	-3.34	22.54	0.99	1.99
	8	19.02	22.56	25.87	29.07	32.71	37.50	36.42	-3.13	22.54	1.09	2.09
REP3	9	18.97	22.63	25.93	29.17	33.33	35.09	Negative	-3.26	22.43	1.03	2.03
	10	19.32	22.96	26.18	29.36	33.23	35.55	37.21	-3.03	22.86	1.14	2.14
	11	19.11	22.77	26.09	29.14	32.67	35.69	Negative	-3.25	22.51	1.03	2.03
REP4	12	19.24	22.72	26.00	29.24	33.46	Negative	Negative	-3.43	22.52	0.96	1.96
	13	19.39	22.80	25.98	29.57	32.31	35.56	Negative	-3.17	22.64	1.07	2.07
	14	19.24	22.81	26.13	29.19	32.42	37.20	Negative	-3.42	22.49	0.96	1.96
REP5	15	19.22	22.72	25.93	29.47	33.30	35.32	Negative	-3.25	22.58	1.03	2.03
	16	19.30	22.75	26.01	29.42	32.91	36.61	36.91	-3.08	22.76	1.11	2.11
	17	19.22	22.76	25.94	29.37	33.45	36.31	Negative	-3.40	22.54	0.97	1.97
REP6	18	19.23	22.70	25.96	29.45	32.70	37.12	Negative	-3.45	22.46	0.95	1.95
	19	19.29	22.70	25.91	29.32	32.61	Negative	Negative	-3.26	22.52	1.03	2.03
	20	19.21	22.70	26.01	29.26	32.24	37.11	Negative	-3.41	22.43	0.97	1.97
REP7	21	19.20	22.70	26.09	29.21	32.26	37.29	Negative	-3.43	22.43	0.96	1.96
	22	19.26	22.66	26.01	29.62	32.88	37.36	37.60	-3.22	22.69	1.05	2.05
	23	19.17	22.62	26.03	29.44	32.60	37.64	Negative	-3.53	22.40	0.92	1.92
REP8	24	19.07	22.70	25.90	29.29	32.69	Negative	Negative	-3.31	22.43	1.00	2.00
	25	19.32	22.71	25.94	29.34	32.88	37.18	Negative	-3.46	22.49	0.95	1.95
	26	19.22	22.70	25.95	29.35	32.78	36.24	Negative	-3.34	22.50	0.99	1.99
REP9	27	19.26	22.64	25.93	29.08	32.70	35.40	Negative	-3.20	22.50	1.05	2.05
	28	19.12	22.61	25.80	29.42	32.61	34.93	Negative	-3.16	22.47	1.07	2.07
	29	19.03	22.60	25.84	29.33	33.23	35.37	Negative	-3.29	22.43	1.01	2.01
REP10	30	19.03	22.59	25.90	29.45	32.81	35.56	Negative	-3.28	22.43	1.02	2.02
MEAN		19.23	22.73	26.01	29.40	32.84	36.52	37.03	-3.37	22.52	0.98	1.98

***T. equi* WL**

%PE		13.8	1.38	0.138	0.0138	0.00138	0.000138	0.0000138				
Log %PE		1.1398791	0.1398791	-0.8601209	-1.8601209	-2.8601209	-3.8601209	-4.8601209	Slope	Intercept	Efficiency	Amplification
	1	19.03	22.79	26.46	29.53	32.96	37.68	Negative	-3.32	23.20	1.00	2.00
	2	19.01	22.80	26.38	29.60	32.57	35.55	Negative	-3.39	23.15	0.97	1.97
REP1	3	18.76	22.70	26.36	29.84	32.61	Negative	Negative	-3.48	23.05	0.94	1.94
	4	18.87	22.71	26.49	29.40	32.89	35.62	Negative	-3.35	23.11	0.99	1.99
	5	18.82	22.72	26.35	29.70	32.69	Negative	Negative	-3.47	23.07	0.94	1.94
REP2	6	18.58	22.79	26.23	29.73	33.24	39.99	Negative	-4.05	22.91	0.76	1.76
	7	18.88	22.52	26.59	29.65	32.78	38.54	Negative	-3.49	23.08	0.93	1.93
	8	19.00	22.87	26.51	29.65	32.91	34.65	Negative	-3.19	23.26	1.06	2.06
REP3	9	18.77	22.72	26.48	29.65	32.87	35.02	Negative	-3.28	23.12	1.02	2.02
	10	18.72	22.69	26.35	29.55	32.89	37.90	Negative	-3.71	22.97	0.86	1.86
	11	18.88	22.68	26.40	29.85	32.22	36.14	Negative	-3.38	23.09	0.98	1.98
REP4	12	18.70	22.83	26.46	29.90	Negative	Negative	39.03	-3.35	23.16	0.99	1.99
	13	18.95	22.58	26.31	29.48	33.21	35.28	Negative	-3.34	23.09	0.99	1.99
	14	18.72	22.75	26.40	29.44	32.87	35.96	Negative	-3.42	23.04	0.96	1.96
REP5	15	18.49	22.72	26.25	29.46	33.46	35.84	Negative	-3.49	22.95	0.93	1.93
	16	18.84	22.75	26.45	29.85	33.15	36.48	Negative	-3.51	23.14	0.93	1.93
	17	18.84	22.76	26.38	29.73	32.84	35.78	Negative	-3.38	23.12	0.98	1.98
REP6	18	18.79	22.68	26.29	29.68	33.00	36.06	Negative	-3.45	23.06	0.95	1.95
	19	18.89	22.60	26.58	29.73	33.32	37.32	Negative	-3.64	23.12	0.88	1.88
	20	18.87	22.80	26.44	29.74	33.19	35.74	Negative	-3.39	23.18	0.97	1.97
REP7	21	18.75	22.66	26.49	29.62	32.57	36.21	Negative	-3.44	23.04	0.95	1.95
	22	18.98	22.93	26.56	29.72	32.87	37.84	Negative	-3.64	23.20	0.88	1.88
	23	19.02	22.81	26.62	29.79	32.98	36.44	Negative	-3.45	23.24	0.95	1.95
REP8	24	18.82	22.74	26.33	29.87	33.06	35.21	38.49	-3.24	23.19	1.04	2.04
	25	19.06	22.88	26.75	30.03	33.01	35.33	Negative	-3.29	23.37	1.02	2.02
	26	18.96	22.87	26.57	29.88	32.89	35.94	Negative	-3.38	23.25	0.98	1.98
REP9	27	18.99	22.89	26.57	29.80	32.95	36.53	Negative	-3.46	23.25	0.95	1.95
	28	19.36	22.86	26.41	29.65	32.97	36.46	Negative	-3.40	23.32	0.97	1.97
REP10	29	18.93	23.55	26.44	31.22	32.57	Negative	Negative	-3.50	23.53	0.93	1.93
MEAN		**18.87**	**22.78**	**26.44**	**29.75**	**32.91**	**36.33**	**38.76**	**-3.33**	**23.21**	**1.00**	**2.00**

Appendix B Comparison of test results obtained for the National Yearling Sale 2006 samples.

Sample	cELISA	IFAT	*In vitro* culture	*T. equi* 18S rRNA TaqMan qPCR	*T. equi ema*-1 TaqMan qPCR (Ueti et al., 2003)	*T. equi ema*-1 Taqman MGB qPCR
EQ1	-	-	-	-	-	-
EQ2	+	+	+	-	-	-
EQ3	+	+	+	30.00	30.21	27.31
EQ4	+	+	+	33.61	33.27	31.18
EQ5	+	+	+	29.57	28.38	26.35
EQ6	+	+	+	28.85	-	26.88
EQ7	+	+	+	28.57	28.94	25.3
EQ8	+	+	+	28.55	-	28.76
EQ9	+	+	+	31.34	31.28	28.5
EQ10	+	+	+	29.4	-	29.56
EQ11	+	+	+	28.42	29.48	25.55
EQ12	+	+	+	29.68	36.42	26.7
EQ13	+	+	+	30.11	29.25	26.4
EQ14	-	-	-	-	-	-
EQ15	-	-	-	-	-	-
EQ16	-	-	-	-	-	-
EQ17	+	+	+	31.98	38.29	29.51
EQ18	-	-	-	-	-	-
EQ19	+	+	+	29.65	27.83	27.46
EQ20	+	+	+	32.4	-	31.23
EQ21	+	+	+	32.2	32.28	28.88
EQ22	-	-	-	-	-	-
EQ23	-	-	-	-	-	-
EQ24	+	+	+	30.85	30.31	27.96
EQ25	+	+	+	28.58	-	25.92
EQ26	+	+	+	28.75	28.45	26.02
EQ27	+	+	+	28.83	28.5	25.74
EQ28	+	+	+	27.85	27.3	24.39
EQ29	-	-	-	-	-	-
EQ30	+	-	+	29.42	29.22	26.73
EQ31	+	+	+	30.27	29.7	27.53
EQ32	+	+	+	29.08	37.97	28.96
EQ33	-	-	+	31.51	33.9	28.84
EQ35	+	-	+	28.3	31.19	26.02
EQ37	+	+	+	31.54	-	29.94
EQ38	+	+	+	33.34	-	33.3
EQ39	+	+	+	32.54	31.97	29.59
EQ40	+	+	+	28.67	29.7	25.71
EQ41	+	+	+	31.52	31.41	28.15
EQ42	+	+	+	27.98	27	24.85
EQ43	+	+	+	31.95	-	27.76
EQ44	+	+	+	29.05	-	26.72
EQ45	+	+	+	29.45	30.46	27.17
EQ46	+	+	+	28.99	-	26.32
EQ47	+	+	+	32.48	39.34	29.28
EQ48	+	+	+	31.03	36.03	28.79
EQ49	+	+	+	29.42	26.28	27.21
EQ50	-	+	+	31.21	-	29.28
EQ51	+	+	+	29.48	-	26.73
EQ52	+	+	+	30.98	30.08	27.97
EQ53	+	+	+	31.05	-	28.92
EQ54	-	+	+	32.04	-	29.85
EQ55	-	-	-	-	-	-

Sample	cELISA	IFAT	*In vitro* culture	*T. equi* 18S rRNA TaqMan qPCR	*T. equi ema*-1 TaqMan qPCR (Ueti et al., 2003)	*T. equi ema*-1 Taqman MGB qPCR
EQ56	+	+	+	28.29	28.4	25.56
EQ57	+	+	+	29.03	29.52	25.25
EQ58	-	-	-	-	-	-
EQ59	+	+	+	29.98	-	27.4
EQ60	+	+	+	32.85	-	31.01
EQ61	+	+	+	34.93	-	33.76
EQ62	+	+	+	27.16	27.43	24.59
EQ63	+	+	+	-	36.52	-
EQ64	+	+	+	30.93	-	28.62
EQ65	+	+	+	29.33	27.95	27.82
EQ66	+	+	+	32.05	28.94	29.48
EQ67	+	+	+	32.79	-	31.78
EQ68	+	+	+	31.09	30.3	28.9
EQ69	+	+	+	30.12	30.71	27.75
EQ70	+	+	-	32.11	-	34.77
EQ71	+	+	-	35.15	-	32.12
EQ72	+	+	+	33.03	33.57	30.68
EQ73	+	+	+	31.96	31.69	28.95
EQ74	+	+	+	32.53	36.93	30.39
EQ75	+	+	+	32.51	36.68	32.6
EQ76	+	+	+	30.43	29.58	27.51
EQ77	+	+	+	31.9	31.56	29.46
EQ78	+	+	+	29.8	29.27	26.84
EQ79	+	+	+	32.52	-	30.35
EQ80	+	+	+	29.51	29.39	26.85
EQ81	+	+	-	32.55	34.83	29.91
EQ82	+	+	+	29.21	36.16	26.17
EQ83	+	+	+	33.39	32.74	27.87
EQ84	+	+	+	29.77	28.85	26.58
EQ85	+	+	+	33.39	34.09	32.21
EQ86	+	+	+	33.18	34.47	30.21
EQ87	+	+	+	32.91	-	30.77
EQ88	+	+	+	29.95	29.37	27.01
EQ89	+	+	+	29.91	33.94	24.8
EQ90	+	+	+	30.56	35.32	27.78
EQ91	+	+	+	31.37	30.45	26.88
EQ92	-	-	-	-	-	-
EQ93	+	+	+	29.59	28.81	26.82
EQ94	-	+	+	31.25	33.22	28.66
EQ95	+	+	+	32.44	31.08	29.86
EQ96	+	+	+	33.28	31.8	29.68
EQ97	-	-	-	-	-	-
EQ98	+	+	+	28.63	28.18	25.9
EQ99	-	-	-	-	-	-
EQ100	+	+	+	28.97	28.72	26.57
EQ101	+	+	+	30.86	30.56	27.93
EQ102	+	+	+	28.73	28.34	25.85
EQ103	+	+	+	-	-	-
EQ104	+	+	+	28.97	27.79	25.17
EQ105	+	+	+	29.89	28.59	24.57
EQ106	+	+	+	31.37	31.66	27.53
EQ107	-	-	-	37.45	-	-