Molecular detection and characterization of tick-borne pathogens of dogs

By

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Thesis summary

This thesis focuses on the molecular characterization of tick-borne parasites of dogs in South Africa. Emphasis is placed on *Babesia*, *Erhlichia*, incidental and novel parasite infections that may cause morbidity or mortality in infected dogs. An outbreak of canine babesiosis in the Netherlands is also reported in this thesis. Molecular techniques were employed to isolate, amplify and characterize genomic DNA of these parasites to species level. During preliminary screening of blood samples collected from various sites in the country, that included the Onderstepoort Veterinary Academic Hospital, SPCAs and private clinics throughout seven provinces in South Africa, it was discovered that domestic dogs harboured a wide variety of tick-borne pathogens.

The most frequently encountered parasites in South Africa were *Babesia rossi*, a novel *Theileria* species of dogs, *B. vogeli* and *Ehrlichia canis* respectively. The parasites occurred as single or mixed infections. Incidental infections that included *B. gibsoni* and *Trypanosoma congolense* were also detected using PCR. Although it was anticipated that zoonotic Ehrlichial infections of dogs would be detected, none were found. *Babesia vogeli* was reported for the first time in South Africa although, without any clinical significance. An outbreak of autochthonous canine babesiosis in the Netherlands was confirmed to have been caused by *Babesia canis*. *Dermacentor reticulatus* was implicated in the transmission of the parasite to naïve dogs. Clinical significance of *B. rossi* and the novel *Theileria* sp. of dogs was evaluated. *Babesia rossi* was found to be of significant clinical importance. Genotyping of *B. rossi* isolates revealed that parasite

genotypes could be correlated to disease phenotype. Additionally, specific genotypes could also be associated with fatalities. Although the characterization of the *Theileria* sp. in dogs was a first report in South Africa, the clinical significance of this infection in dogs appeared to be poorly resolved. The dangers of having non-endemic species becoming established in South Africa was highlighted with the incidental finding of a *B. gibsoni* infection in an imported dog.

The results of this thesis have shown therefore that populations of dogs that live in tick-endemic areas are exposed to single or multiple tick-borne pathogens. These pathogens continue to cause morbidity and mortality in susceptible dogs. Correct diagnosis (supported by molecular diagnostic tools) followed by appropriate treatment offers a better understanding and management of these tick-borne pathogens. Preventative measures should be fully evaluated and applied to prevent these tick-borne pathogens from adversely affecting the canine population in South Africa and elsewhere.

Chapter 1: General Introduction

1.1. Background

This thesis deals with tick-borne pathogens of dogs that are causative agents of severe diseases in dogs, particularly in South Africa. The focus is not limited to studies conducted on dogs in South Africa, however, but also includes work done in the Netherlands under a memorandum of understanding between the University of Pretoria and Utrecht University. These tick-borne pathogens are characterized using molecular techniques and their importance is analysed by assessing possible association with the clinical disease in domestic dogs.

1.2. Tick-borne pathogens

Being blood feeders, ticks are well adapted to transmitting disease agents such as viruses, bacteria and protozoa. Ticks attach securely to their host, facilitating effective transmission of infectious agents and also the spread of both ticks and the microorganisms to different geographical habitats via traveling pets (Shaw, Day, Birtles and Breitschwerdt, 2001). Ticks and the diseases they transmit have a zoogeographical range restricted by host movement, and to some extent, by climate factors. However, the increased mobility of pets has resulted in rapid extension of zoogeographical ranges for many species (Shaw et al., 2001).

Worldwide, ticks transmit a wide variety of infectious agents, making them important in both medical and veterinary fields. The most important tick-borne pathogens of dogs

causing severe clinical illness are *Babesia* and *Ehrlichia*. In the USA, Rocky Mountain Spotted fever (RMSF) and hepatozoonosis have been reported to be as common in dogs as are babesiosis and ehrlichiosis (Shaw et al., 2001). Other tick-borne pathogens of dogs include *Borrelia burgdorferi* and *Rickettsia conorii.* Infection with these pathogens often produces subclinical infections, however, making it difficult to evaluate their association with clinical disease in dogs (Levy and Magnarelli, 1992). Dogs are also susceptible to infection with *Coxiella burnetii* (Q fever) and tick-borne viral encephalitis (Weissenbock and Holzmann, 1996), but reports of clinical disease are uncommon. Other canine tick-transmitted infections include bartonellosis, tularaemia (*Francisella tularensis*) and rarely, louping illness (*Flaviviridae*) (Shaw et al., 2001).

The most important tick-transmitted infectious diseases that cause severe clinical illness in South African dogs are babesiosis (Collett, 2000) and ehrlichiosis (Rautenbach, Boomker and Villiers 1991; Van Heerden, 1982;). *Hepatozoon canis* has been reported to occur in South Africa (McCully, Basson, Bigalke, De Vos and Young, 1975), although it does not appear to be as pathogenic as *H. americanum*, the species reported to be endemic only in the northern part of South America, Central and Northern America (Baneth, Mathew, Shkap, Macintire, Barta and Ewing, 2003). There are no published reports on borreliosis and/or bartonellosis related infections of dogs in South Africa.

1.2.1. Zoonotic tick-borne infections

Several of the tick transmitted infections can cause serious disease in humans. These infections include: borreliosis, ehrlichiosis, babesiosis, Rocky Mountain Spotted Fever

(RMSF), *R. conorii* infection and tick-borne encephalitis (Kjemtrup and Conrad, 2000a; Shaw and Irwin, 2001; Centeno-Lima, Rosario, Parreira, Maia, Freudenthal, Nijhof, and Jongejan, 2003). It is reported that the potential zoonotic threat posed by dogs, is however, strongly influenced by the natural cycle of the specific pathogen with which the dog is infected (Shaw et al., 2001). Three general epidemiological scenarios that make transmission from dog to humans possible have been described (Shaw et al., 2001). Firstly, if transmission of an infectious agent involves ticks with a broad host range (such as *Ixodes ricinus*), dogs can act directly as sentinels for infection to humans (Shaw et al., 2001). Secondly, by acting as natural hosts for ticks such as *Rhipicephalus sanguineus* and *Ixodes canisuga*, dogs significantly increase contact between these species and humans, thereby increasing the risk of transmission (Mumcuoglu, Frish, Sarov, Manor, Gross, Gat and Galun, 1993). A study conducted in a rural community of Arizona (USA), has implicated *R. sanguineus* as a vector in an outbreak of RMSF in 10% of children and 70% of dogs in the outbreak community and 16% of children and 57% of dogs in a neighbouring community, highlighting the importance of ticks in the transmission of zoonotic pathogens from dogs to humans (Demma, Traeger, Blau, Gordon, Johnson, Dickson, Ethelbah, Piontkowski, Levy, Nicholson, Duncan, Heath, Cheek, Swerdlow and McQuiston, 2006). Thirdly, there is limited risk of transmission by exposure to infected-tick contents following damage to ticks during grooming of infested animals, a scenario that has been reported for *R. conorii* (Senneville, Ajana, Lecocq, Chidiac and Mouton, 1991). Thus it appears that the close association of dogs and man makes it possible for dogs to be source of zoonotic infections to man.

1.2.2. Co-infection

It is currently unknown whether the South African domestic dog population carries tick-borne infectious agents of human importance or whether multiplicity of infection (infection with more than one species of tick-borne organism) occurs. Infection with tick-borne disease agents, including several *Ehrlichia* and *Rickettsia* species, has been described in humans and dogs in North Carolina, USA (Archibald and Sexton, 1995). In addition, case reports of co-infection with multiple tick-borne organisms in humans and dogs have been published (Duffy, Pittlekow, Kolbert, Rutledge and Persing, 1997; Breitschwerdt, Hegarty and Hancock, 1998). Infection with multiple tick-transmitted pathogens or with multiple genotypes of the same pathogenic species can occur in an individual animal following heavy exposure to ticks (Breitschwerdt, Hegarty and Hancock 1998; Kordick, Breitschwerdt, Hegarty, Southwick, Colitz, Hancock, Bradley, Rumbough, Mcpherson and MacCormack, 1999). The implications are that dogs may harbour and serve as reservoirs for a variety of tick-borne pathogens. Infection with one tick-borne pathogen can be complicated by infection with other tick-borne pathogens that are transmitted by different species of ticks that share the same bio-habitat (Shaw et al., 2001). For instance *Ehrlichia phagocytophila,* the causative agent of human granulocytic ehrlichiosis (HGE), has been reported in dogs (Kordick et al., 1999). A PCR-based study conducted on co-infection with multiple tick-borne pathogens in 27 Walker hounds in North Carolina, USA, found that 15 dogs were infected with *Ehrlichia canis*, 9 with *Ehrlichia chaffeensis*, 8 with *Ehrlichia ewingii*, 3 with *Ehrlichia equi*, 9 with *Ehrlichia platys*, 20 with a *Rickettsia* species, 16 with a *Bartonella* species and 7 with *B. canis* (*senso lato*) (Kordick et al., 1999). The presence of different tick vectors

in dog populations increases the risk of transmission and the spread of these pathogens in non-endemic areas.

1.2.3. Babesia vectors

Three tick species (*Dermacentor reticulatus*, *Rhipicephalus sanguineus* and *Haemaphysalis elliptica**) are known vectors of the large *Babesia* parasites in dogs. Ticks are infected by *Babesia* merozoites when feeding on an infected host. Transmission of a *Babesia* parasite is through the bite of an infected tick and can also be transovarial and transstadial during the life cycle of the parasite (Friedhoff, 1988). Although *D. reticulatus* is endemic to southern Europe (Estrada-Peňa, Bouattour, Camicas and Walker, 2004), reports have indicated that there are localized populations of the tick in several non-endemic north-western European countries including Switzerland, Germany, Belgium and the Netherlands (Zahler and Gothe, 1997; Losson, Mollet, Avez, Malaise and Mignon, 1999; Duh, Tozon, Petrovec, Strasek and Avsic-Zupanc, 2004; Nijhof, Bodaan, Postigo, Nieuwenhuijs, Opsteegh, Franssen, Jebbink and Jongejan, 2007). Two vectors (*R. sanguineus* and *H. elliptica*) of *Babesia* parasites in dogs, have overlapping distributions in South Africa, and can also be found in mixed infestations on the same host (Horak, 1995). The distribution of *H. elliptica* coincides with the occurrence of *B. rossi* infections in dogs in South Africa (Lewis, Penzhorn, Lopez-Rebollar and De Waal, 1996). Tick surveys have indicated that *H. elliptica, R. sanguineus* and *Rhipicephalus simus* are the most prevalent species on domestic dogs in the areas where canine babesiosis is endemic (Norval, 1984; Horak, 1995). Although mixed infections of *B. rossi and B. vogeli* are rare, both tick vectors have been found on

**Haemaphysalis elliptica* of Southern Africa, previously synonymised with *Haemaphysalis leachi*, has been confirmed to be a distinct species (Apanaskevich, Horak and Camica, 2007). This is the nomenclature that will be followed here, when Southern African ticks are referred to.

the same host. It is not clear how the presence of both *B. rossi* and *B. vogeli* may influence the varied clinical manifestations of canine babesiosis in South Africa.

The tick species implicated in the transmission of the small piroplasms or the *B. gibsoni* (*senso lato*) group are *R. sanguineus*, *Haemaphysalis longicornis* and *Haemaphysalis bispinosa* (Shortt, 1973; Kuttler, 1988; Otsuka, Yamasaki, Yamato and Maede, 2002). *Haemaphysalis longicornis* and *H. bispinosa* are endemic to Asia, North America, Australia and Europe whereas *R. sanguineus* has a worldwide distribution. The only tick implicated in the transmission of *Theileria annae* in Spain is *Ixodes hexagonus* (Camacho, Pallas, Gestal, Guitian, Olmeda, Telford and Spielman, 2003). Reports of *T. annae* infections have only been limited to Spain. With the exception of *R. sanguineus,* none of the ticks mentioned above is prevalent in South Africa. To the best of my knowledge, *Babesia gibsoni* does not occur naturally in South Africa even though *R. sanguineus* is endemic in the country. It has been suggested in Japan that in the absence of the tick vector, there is a possibility that *B. gibsoni* might be transmitted through blood contamination during dog fights (Matsuu, Kawabe, Koshida, Ikadai, Okano and Higuchi, 2004). There are also sporadic reports on possible transplacental transmission of *B. gibsoni* (Abu, Hara, Nito and Sibauchi, 1973; Harvey, Taboda and Lewis, 1988). As far as I can ascertain, this mode of transmission has been evaluated on a limited scale (Fukumoto, Suzuki, Igarashi and Xuan, 2005).

1.3. Canine babesiosis

> "My first contact with canine babesiosis was in India in 1911 when my Irish terrier developed the disease in Lucknow. I had no idea then of the world wide distribution of *Piroplasmidea* and, more relevant to the present enquiry, of the family *Babesiidae*."
>
> -(Shortt, 1973)

Canine babesiosis is caused by two groups of intraerythocytic protozoan parasites: The large *Babesia* species known as the *B. canis* (*senso lato*) group (Uilenberg, Franssen, Perie and Spanjer, 1989) and the small *Babesia* species that includes the *B. gibsoni* (*senso lato*) group, *B. conradae* and *Theileria annae* (Zahler, Rinder, Schein and Gothe, 2000; Kjemtrup, Kocan, Whitworth, Meinkoth, Birkenheuer, Cummings, Boudreaux, Stockham, Irizarry-Rovira and Conrad, 2000b; Kjemtrup, Wainwright, Miller, Penzhorn and Carreno, 2006b). The large babesias of dogs have a wide distribution which includes South Africa (Uilenberg et al., 1989) while the small babesias of dogs occur in South-East Asia, North-East Africa, Spain, Australia and the USA (Zahler et al., 2000; Kjemtrup, et al., 2000b; Muhlnickel, Jefferies, Morgan-Ryan and Irwin, 2002; Kjemtrup et al., 2006b). These groups are morphologically distinguishable; i.e. the *Babesia canis* (*senso lato*) group is larger in size than the smaller *Babesia gibsoni* (*senso lato*) group (Kuttler, 1988). Despite their morphological similarity, *B. canis* (*senso lato*) isolates collected around the world show biological, genetic and antigenic differences (Uilenberg et al., 1989). Thus a trinomial system of nomenclature was proposed to differentiate

three subspecies: *B. rossi* transmitted by *Haemaphysalis elliptica, B. vogeli* transmitted by *Rhipicephalus sanguineus* and *B. canis* transmitted by *Dermacentor reticulatus* (Uilenberg et al., 1989). Further evidence provided by several other authors support the recognition of *B. rossi*, *B. vogeli* and *B. canis* as separate species (Schetters, Moubri, Precigout, Kleuskens, Scholtes and Gorenflot, 1997; Zahler, Schein, Rinder and Gothe, 1998). This nomenclature was followed in the current study. *Babesia rossi* is highly pathogenic (Uilenberg et al., 1989) and infected dogs require treatment to prevent fatalities (Schetters et al., 1997). *Babesia vogeli* causes mild to sub-clinical infections whereas *B. canis* causes mild to severe infections (Uilenberg et al., 1989). A novel large *Babesia* sp. has been described in a dog from north America, although its tick vector is still unknown (Birkenheuer, Neel, Ruslander, Levy and Breitschwerdt, 2004). The parasite identified was isolated from the bone marrow as well as the blood of a dog with haematological abnormalities consistent with canine babesiosis (Birkenheuer et al., 2004).

Babesia gibsoni (*senso lato*) occurs in Asia, North America, North and Eastern Africa and has also been reported in Europe (Casapulla, Baldi, Avallone, Sannino, Pazzanese and Mizzoni, 1998). The parasite measures 1-3µm in length and is rounded or oval (Kjemtrup et al., 2000b). *Babesia gibsoni* (*senso lato*) can be differentiated from *B. canis* (*senso lato*) based on morphology. However, morphological features alone are not sufficient to distinguish *B. gibsoni* from other small piroplasms of dogs. Isolates from dogs in California appear closely related to isolates from wildlife and humans from the western USA (Kjemtrup, Thomford, Robinson and Conrad, 2000c). Research has

revealed that there are at least three genetically distinct small piroplasms of dogs (Kjemtrup et al., 2000b). The first genetically distinct isolate, which was identified from Spain, is closely related to *Babesia microti* (Zahler et al., 2000) and has subsequently being named *Theileria annae*. The second distinct isolate has been described from dogs in the Midwestern United States (Kocan, Kjemtrup, Meinkoth, Whitworth, Murphy, Decker and Lorenz, 2001). The third isolate with distinct molecular, antigenic and morphologic characteristics of the Californian small canine piroplasms, in comparison to other small canine piroplasms, supported characterization of the isolate as a new species, which was named *Babesia conradae* (Kjemtrup et al., 2006b). The pathogenecity of the small piroplasms in dogs is uniformly high and it usually involves progressive anaemia (Kjemtrup and Conrad, 2006a). *Theileria annae,* which is endemic in the canine population of northwest Spain, causes a severe haemolytic anaemia and thrombocytopenia (Camacho et al., 2003). A comparison of *B. gibsoni*-infected dogs with *B. conradae*-infected dogs supports the similarity in clinical presentation (Meinkoth, Kocan, Loud and Lorenz, 2002). The comparisons also suggest that *B. conradae* infections, however, result in a higher parasitaemia and more pronounced anaemia. Moreover, *B. conradae* infections may be more pathogenic since a higher percentage of *B. conradae*-infected dogs (40%) died or were euthanized due to the disease, as compared to only 16% mortality in *B. gibsoni*-infected dogs. Recurrence of the parasite and disease even after treatment also appears more common with *B. conradae*-infected dogs as compared to *B. gibsoni* infected dogs (Meinkoth et al., 2002).

1.3.1. Canine babesiosis in South Africa

Canine babesiosis is an economically important disease of dogs in South Africa (Collett, 2000). Between 1988 and 1993, the disease was diagnosed in dogs presented at the Outpatients of the Onderstepoort Veterinary Academic Hospital (OVAH) at an average of 11.69% annually (Shakespeare, 1995). Two species, *B. rossi* and *B. vogeli*, are endemic to South Africa (Matjila, Penzhorn, Bekker, Nijhof and Jongejan, 2004). The clinical significance of the South African *B. vogeli* isolate has not been evaluated, whereas *B. rossi*, the most prevalent species in South Africa, causes a severe, often fatal disease (Böhm, Leisewitz, Thompson and Schoeman, 2006; Jacobson, 2006). Canine babesiosis caused by *B. rossi* is clinically classified as being either uncomplicated or complicated. The disease is said to be uncomplicated if the clinical changes could be attributed directly to a mild or moderate anaemia with no clinical evidence of organ dysfunction or failure (Jacobson and Clark, 1994). Dogs with mild anaemia are treated with an antibabesial drug and discharged. Dogs with moderate anaemia may receive a blood transfusion and are then discharged. The survival rate in this type of disease is almost 100% (Jacobson and Lobetti, 1996). Complicated cases of the disease are those where clinical presentation is complicated by evidence of organ dysfunction or failure (solid organ complications) or where anaemia itself is life threatening. Examples of complicated disease include acute renal failure, cerebral involvement, coagulopathy, icterus and hepatopathy, immune haemolytic anaemia, peracute manifestation, pulmonary oedema, haemoconcentration, pancreatitis, rhabdomyolysis and shock (Jacobson and Clark, 1994; Jacobson and Lobetti, 1996). These dogs all require admission to an intensive-care facility where advanced medical treatment is essential.

The mortality rate in this group is around 15%, irrespective of the nature of the treatment administered (Shakespeare, 1995).

1.3.2. Pathogenesis of babesiosis and malaria

The similarities between *B. rossi*-induced canine babesiosis and the pathogenesis and clinical picture of bovine babesiosis and human *falciparum* malaria have been recognized for years (Wright, Goodger and Clark, 1988; Schetters and Eling, 1999; Allred and Al-Khedery, 2004). All three protozoan parasites are highly pathogenic and red blood cells parasitized with these pathogens sometimes adhere to capillary endothelium, a phenomenon known as sequestration (Pardini, 2000; Miller, Baruch, Marsh and Doumbo, 2002; Allred and Al-Khedery, 2004). Adhesion to the vascular endothelium appears to be a parasite survival mechanism (to avoid splenic removal) (Allred and Al-Khedery, 2004; Sherwood, 1990) and maintenance of parasite virulence since isolates that do not adhere only cause mild to non-patent infections (Langreth and Peterson, 1985). The most striking similarities are those between the relatively mild clinical disease caused by *B. bigemina* and *Plasmodium vivax* and the much more severe and fatal clinical syndromes caused by *B. bovis* and *P. falciparum* (Cooke, Mohandas, Cowman and Coppel, 2005). The unique way in which *B. rossi, B. bovis* and *P. falciparum* modify their infected red blood cells to make them adhesive for a number of other cell types, however, is widely believed to be a major factor that contributes to enhanced pathogenecity of these parasites (Pardini, 2000; Miller et al., 2002; Allred and Al-Khedery, 2004).

1.3.3. Sequestration

The mechanisms involved in the cytoadherence of *B. rossi*-infected red blood cells in the microvasculature to capillary and venous endothelium are unknown. The *Plasmodium falciparum* and *B. bovis* cytoadherence models may provide insight in mechanisms involved in cytoadherence (Pongponratn, Riganti, Punpoowong and Aikawa, 1991; Aikawa, Pongponratn, Tegoshi, Nakamura, Nagatake, Cochrane and Ozaki, 1992; Schetters, Kleuskens, Scholtes and Gorenflot, 1998; Newbold, 1999; Nevils, Figueroa, Turk, Canto, Le, Ellersieck and Carson, 2000; Duffy and Fried, 2003). There is evidence that most adhesive interactions of malaria-infected cells are mediated by the same parasite ligand expressed on the surface of the infected red cell, *P. falciparum* erythrocyte membrane protein 1 (*PfEMP*1) (Baruch, Rogerson and Cooke, 2002; Kriek, Tilley, Horrocks, Pinches, Elford, Ferguson, Lingelbach and Newbold, 2003), a bifunctional protein that is responsible for cytoadherence to several ligands as well as for antigenic variation (Baruch, Ma, Singh, Bi, Pasloske and Howard, 1997; Baruch et al., 2002; Gardner and Hall, 2002). The *PfEMP*1 is encoded by various members of the *var* multi-gene family. Seventy-five *var* genes can be identified within the *P. falciparum* genome of the 3D7 isolate and a number of these appear to be transcribed within a particular infected red blood cell (Gardner and Hall, 2002; Cooke et al., 2005). *Var* genes vary in sequence and the repertoire within a single parasite may vary substantially from those in another parasite (Cooke et al., 2005).

Although cytoadherence ligands on *B. bovis*-infected red blood cell have yet to be definitely identified, preliminary evidence strongly implicates *VESA*1 involvement

(O'Connor, Long, and Allred, 1999; O'Connor and Allred, 2000). The protein, termed *B. bovis* variant erythrocyte surface antigen 1 (*VESA*1), is variable in antigenicity and apparent molecular weight between different parasite isolates and appears to cluster over stellate protrusions on the surface of parasite-infected red blood cell (O'Connor, Long, and Allred, 1999; O'Connor and Allred, 2000). Similar to *PfEMP*1, the protein is the product of a multi-gene family *ves* (Allred, Carlton, Satcher, Long, Brown, Patterson, O'Connor and Stroup, 2000). Although preliminary evidence strongly suggests a linkage of *VESA*1 and cytoadherence in *B. bovis* (O'Connor et al., 1999; O'Connor and Allred, 2000), and adhesive function for *VESA*1 has not been proven. *Babesia rossi* antigenic diversity is not well documented except at chromosomal level where polymorphism between two *B. rossi* laboratory strains was shown (Depoix, Carcy, Jumas-Bilak, Pages, Precigout, Schetters, Ravel and Gorenflot, 2002). Parasite-derived antigens are expressed on the surface of *B. canis*-infected red blood cells (Schetters et al., 1997), although their function on the surface of an infected red blood cell is currently unknown. Also, not much is known about antigens that are expressed on the surface of a *B. rossi*-infected erythrocyte.

1.4. Ehrlichiosis and Anaplasmosis

Canine ehrlichiosis is caused by tick-transmitted intracellular bacteria of the genus *Ehrlichia*, which in dogs have been identified parasitizing monocytes, granulocytes and platelets (Shaw et al., 2001). There are three genogroups identified targeting the 16S rRNA phylogenetic analysis (Drancourt and Raoult, 1994). Genogroup III includes *E. canis*, which has its geographical distribution linked to the distribution of *R. sanguineus*. Genogroup III also includes *E. chaffeensis* and *E. ewingii*, isolated from dogs with or

without ehrlichiosis symptoms (Breitschwerdt et al., 1998; Kordick et al., 1999). These species are reported to have more restricted geographical distributions but a potentially wider range of tick vectors than *E. canis* (Shaw et al., 2001). Genogroup II Ehrlichiae of pathogenic significance includes *Ehrlichia phagocytophilum* and *Ehrlichia platys* (Drancourt and Raoult, 1994). However, these species have been reclassified into the genus *Anaplasma* (Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa, 2001). Therefore *Anaplasma phagocytophilum* encompasses the three previously classified Ehrlichiae bacteria: *Ehrlichia phagocytophilum*, the aetiologic agent of tick-borne fever of sheep and cattle in Europe; *Ehrlichia equi*, which causes granulocytic ehrlichiosis in Europe and the United States; and the human granulocytic ehrlichiosis (HGE) agent (Dumler et al., 2001).

Ehrlichia and *Anaplasma* species infecting humans and dogs have been documented in South Africa (Pretorius and Kelly, 1998; Pretorius, Venter, Ryst and Kelly, 1999), although the results of these reports were based only on serological diagnostic assays. It is currently unknown whether the South African domestic dog population carries tick-borne infectious agents of human importance, which can be detected using molecular techniques. To date we are aware of one report where a new *Anaplasma* species closely related to *Anaplasma phagocytophilum* was detected from canine blood (Inokuma, Oyamada, Kelly, Jacobson, Fournier, Itamoto, Okuda and Brouqui, 2005). *Anaplasma phagocytophilum* is the causative agent of canine (Egenvall, Bjoersdorff, Lilliehook, Engvall, Karlstam, Artursson, Hedhammar and Gunnarsson, 1998) and human granulocytic anaplasmosis (Wormser, Dattwyler, Shapiro, Halperin, Steere, Klempner,

Krause, Bakken, Strle, Stanek, Bockenstedt, Fish, Dumler and Nadelman, 2006). Another pathogen infecting dogs is *Ehrlichia chaffeensis* (Kordick et al., 1999), which can also cause human monocytic erhlichiosis (Dumler, Chen, Asanovich, Trigiani, Popov and Walker, 1995; Paddock, Sumner, Shore, Bartley, Elie, McQuade, Martin, Goldsmith and Childs, 1997).

1.4.1. Pathogenesis of Ehrlichiosis and / or Anaplasmosis

Disease manifestations caused by members of the *E. canis* genogroup (genogroup III) infecting dogs can be indistinguishable (Breitschwerdt et al., 1998) and there can be strain variation in pathogenecity (Hegarty, Levy, Gager and Breitschwerdt, 1997). Monocytic ehrlichiosis in dogs and humans is caused by *E. canis* and *E. chaffeensis,* respectively. Canine monocytic ehrlichiosis, the disease caused by *E. canis,* can be differentiated into three stages characterized by thrombocytopenia, leukopenia and anaemia (Van Heerden, 1982). The first, acute phase may be manifested by fever, dyspnoea, anorexia, and slight weight loss (Van Heerden, 1982). Haematological results often indicate thrombocytopenia, leukopenia, mild anaemia, and hypergammaglobulinaemia. The second phase is subclinical and follows the acute phase. During the subclinical phase, dogs can remain persistently infected for years without clinical signs but with mild thrombocytopenia (Codner and Farris-Smith, 1986). The chronic phase is the third stage, characterized by haemorrhages, epistaxis and oedema. Haematology results resemble those during the first phase of the disease. The course of the third phase is often complicated by co-infections by other microorganisms (Rikihisa, 1991; Rikihisa, Ewing, Fox, Siregar, Pasaribu and Malole, 1992; Iqbal,

Chaichanasiriwithaya and Rikihisa, 1994). Dogs infected with *E. canis* become life-long carriers, even after treatment (Wen, Rikihisa, Mott, Greene, Kim, Zhi, Couto, Unver and Bartsch, 1997).

Canine granulocytic ehrlichiosis (recently renamed canine granulocytic anaplasmosis by Dumler et al. [2001]) caused by *Anaplasma phagocytophilum,* is associated with two distinct clinical syndromes, which include chronic, moderate to severe anaemia and polyarthritis (Goldman, Breitschwerdt, Grindem, Hegarty, Walls and Dumler, 1998). Clinical signs are nonspecific and include fever, lethargy, anorexia, vomiting and diarrhoea (Goldman et al., 1998; Kohn, Galke, Beelitz and Pfister, 2008). Most often blood abnormalities are normocytic, normochromic non-regenerative, moderate thrombocytopenia with large platelets, lymphopenia and eosinopenia (Goldman et al., 1998, Kohn et al., 2008).

1.5. Hepatozoonosis

Canine hepatozoonosis is a disease caused by intraleukocytic *Hepatozoon* species (MacIntire, Vincent-Johnson, Dillon, Blagburn, Lindsay, Whitley and Banfield, 1997). Unlike most other tick-borne infections, *Hepatozoon* is transmitted by ingestion of an infected tick by dogs, rather than by the tick biting (Ewing and Panciera, 2003). There are currently two known species causing hepatozoonosis, *H. canis* and *H. americanum* (MacIntire et al., 1997; Ewing and Panciera, 2003). *Hepatozoon canis,* whose major vector is *R. sanguineus,* is endemic in Africa, southern Europe, the Middle East and Asia (MacIntire et al., 1997; Mathew, Ewing, Panciera and Woods, 1998; Panciera, Ewing,

Mathew, Cummings, Kocan, Breshears and Fox, 1998; Panciera, Ewing, Mathew, Lehenbauer, Cummings and Woods, 1999), whereas *H. americanum,* whose major vector is *Amblyomma maculatum,* is endemic in the southern USA (Vincent-Johnson, Macintire, Lindsay, Lenz, Baneth, Shkap and Blagburn, 1997). Hepatozoonosis caused by *H. canis* is often a subclinical infection whereas *H. americanum* causes a more severe disease (Baneth et al., 2003). Dogs infected with *H. americanum* are often febrile, stiff, lethargic, and depressed (Ewing and Panciera, 2003). Gait abnormalities and muscle wasting are usually obvious, as is copious mucopurulent ocular discharge. Atrophy of head muscles is especially noticeable (Ewing and Panciera, 2003). Dogs may eat readily when food is placed immediately in front of them, but they often refuse to move to food and water, presumably owing to intense pain, which derives in part from periosteal bone proliferation and inflamed muscles (Vincent-Johnson et al., 1997; MacIntire et al., 1997; Ewing, Mathew, Lehenbauer, Cummings and Woods, 1999).

1.6. Theileriosis

The only *Theileria* species known to cause disease in dogs is *Theileria annae*, which has been reported only in Spain (Garcia, 2006). *Ixodes hexagonus* is suspected to be the tick vector responsible for the transmission of *Theileria annae* (Camacho et al., 2003). The disease caused by *T. annae* is characterized by severe regenerative anaemia and thrombocytopenia. Azotaemia is found in many cases (Camacho, Guitian, Pallas, Gestal, Olmeda, Goethert, Telford and Spielman, 2004). Abnormally high serum concentrations of urea and creatinin, together with elevated concentrations of inorganic phosphorus, hypoalbuminaemia, hypercholesterolaemia, proteinuria, high protein /

creatinin and presence of hyaline and granular casts in the microscopic examination of urine sediment suggest a glomerular component of the disease (Garcia, 2006).

1.7. Molecular detection and analysis

Detection and identification of tick-borne pathogens has largely relied on morphological and biological observations. Previously, parasitological (blood smear) examinations used in conjunctions with serology (immunoflourescent antibody test, IFAT) were methods of choice in diagnosing tick-borne infections. Parasitological examinations have limited specificity, however, and there is often antigenic cross-reactivity in the use of serology. Molecular diagnosis is increasingly being used as a reliable tool for the detection and characterization of blood-parasite infection in the host (Caccio, Antunovic, Moretti, Mangili, Marinculic, Baric, Slemenda and Pieniazek, 2002; Birkenheuer et al., 2004) and in the vector (Inokuma, Beppu, Okuda, Shimada and Sakata, 2003). Advances in molecular methodology, particularly automated DNA sequencing, have made it possible to ascertain the evolutionary relationships of species from genes (Stevens, Noyes, Schofield and Gibson, 2001). Within the piroplasmids (Babesiidae and Theileriidae) the 18S rRNA gene proves to be highly conserved in demonstrating genotypic diversity between the species. Phylogenetic analysis of the 18S rRNA gene proved to be useful in proving that multiple small canine piroplasm species exist (Kjemtrup and Conrad, 2006a). A more complete phylogenetic analysis of the 18S rRNA gene that included canine *Babesia* species from Asia, the Midwestern United States, California, Africa, Africa and Spain confirmed that there are three genotypical distinct small *Babesia* species of canines (Kjemtrup et al., 2000b). A recent and

complete analysis of the 18S rRNA gene, suggests that piroplasms may be divided into five clades: (1) *B. microti* group, containing *Babesia rodhaini*, *Babesia felis*, *Babesia leo*, *B. microti* and *B. microti*-type canine isolate; (2) western USA *Theileria*-like group, containing *B. conradae*; (3) *Theileria* group, containing all *Theileria* species from bovines; (4) a first group of *Babesia* species including *B. canis* and *B. gibsoni* from canines together with *Babesia divergens* and *Babesia odocoilei*; and (5) a second group composed mainly of *Babesia* species from ungulates: *Babesia caballi*, *Babesia bigemina*, *Babesia ovis*, *Babesia bovis* and *Babesia* sp. from cattle (Criado-Fornelio, Martinez-Marcos, Buling-Sarana and Barba-Carretero, 2003). Phylogenetic analysis of the 18S rRNA gene was also used to reinforce the designation of *Hepatozoon americanum* as a new species separate from *Hepatozoon canis* (Baneth, Mathew, Shkap, Macintire, Barta and Ewing, 2000). There is sufficient evidence to suggest that studies of the 18S rRNA gene have added important information in understanding the taxonomic position of many piroplasm species, particularly those from canines (Kjemtrup and Conrad, 2006a).

The 16S rRNA gene is useful in the analysis and differentiation between species of tick-borne pathogens of dogs belonging to the genera *Ehrlichia* and / or *Anaplasma*. Multiple analyses and alignments of the 16S rRNA gene sequences of *Ehrlichia* and *Anaplasma* species have revealed four distinct clusters, two of which are relevant to the current study since they are tick-transmitted: (1) the *Anaplasma* group, which includes the *Ehrlichia phagocytophila* group, *Ehrlichia platys* and *Ehrlichia bovis*; and (2) the *Ehrlichia canis* / *Cowdria* group, which includes *E. canis*, *E. chaffeeensis*, *E. ewingii*, *E.*

muris and *Cowdria ruminantium* (Dumler et al., 2001). The remaining two clusters of groups of parasites are transmitted via infected metacercariae or cercariae of flukes that infest snails, fish or aquatic insects and are therefore of no relevance to this study.

1.8. Objectives of the study

The objectives of this study were to identify tick-borne pathogens of domestic dogs using molecular techniques. The main focus of the study was to characterize parasites of the genus *Babesia* and to correlate parasite infection to disease manifestations. Furthermore, the relationship between *B. rossi* genotype and disease phenotype was investigated. The second focus of the study was to identify *Ehrlichia* and *Anaplasma* species, especially the zoonotic species, and to evaluate the degree of co-infection with multiple tick-borne pathogens. Additionally we also aimed to identify tick-borne pathogens that were incidental or were previously unknown pathogens of dogs.

1.9. Overview of the thesis

The characterization and identification of the tick-borne pathogens infecting dogs in South Africa is the subject of this thesis. There is an abundance of tick vectors of many species with overlapping geographic distribution which may be found on the same host. It is therefore difficult to establish the cause of disease associated with a single pathogen. This thesis covers the initial sampling of dogs, isolating and detection of parasites (both single and mixed infections) by molecular techniques and the clinical relevance of the characterized parasite species in the infected dogs.

In chapter 2 the occurrence of *Babesia vogeli* in domestic dogs in South Africa is reported. This is the first report of *B. vogeli* in South Africa and an indication that it occurs less frequently than *B. rossi*. Previously *B. rossi* was thought to be the only species responsible for canine babesiosis in South Africa. The detection of *B. vogeli* has implications with regard to the occurrence and varied clinical manifestations of canine babesiosis in South Africa.

In chapter 3 the occurrence of tick-borne pathogens infecting domestic dogs is studied. The screening of blood samples of domestic dogs collected from seven of the nine provinces of South Africa (four inland and 3 coastal provinces) indicates a wide distribution and a variety of parasite species (protozoal and ehrlichial) circulating in a significant number of canine populations in South Africa.

Chapter 4 deals with the importance of correlating *B. rossi* genotypes to clinical canine babesiosis. *Babesia rossi* genotypes have been previously identified by typing of a polymorphic repetitive region from the gene *Babesia rossi* Erythrocyte Membrane antigen (*BrEMA*1). Parasite genotype was correlated to disease phenotype. The association between *BrEMA*1 polymorphism and uncomplicated and complicated canine babesiosis is investigated for the first time in South Africa.

The memorandum of understanding between the Faculty of Veterinary Science, University of Pretoria, and the Faculty of Veterinary Medicine, Utrecht University, resulted in a collaborative study of an outbreak of canine babesiosis, caused by *Babesia*

canis, in dogs that had never been outside the Netherlands. Chapter 5 deals with autochthonous cases and the implications of having an endemic canine babesiosis situation in the Netherlands.

Chapter 6 deals with the detection of a *Theileria* sp. isolated from domestic dogs in South Africa. The only *Theileria* species associated with clinical disease in dogs is *Theileria annae,* which has only been detected in Spain. We report for the first time a *Theileria* sp. identified in South African dogs. Evidence suggested that the parasite may be linked to clinical signs of haemolysis and immune-mediated syndrome encountered in the infected dogs. This information added yet another microorganism to the list of haemoprotozoans infecting dogs.

Chapter 7 highlighted the importance of using molecular techniques in screening infected blood samples. *Babesia gibsoni*, a species not endemic to South Africa, was detected and characterized in a pit-bull pup recently imported into South Africa. The infected dog did not respond to conventional chemotherapy, usually administered to dogs infected with large piroplasms or the *B. canis* (*senso lato*) group. It was only after molecular diagnosis that it was realized that the dog was in fact infected with a different species of *Babesia* not frequently encountered in South Africa. The danger of having previously non-endemic species becoming endemic in South Africa and the importance of using the correct treatment for the correct parasite are emphasized.

Finally, chapter 8 contains the general discussion about the occurrence of tick-borne pathogens and their clinical relevance. It also discusses the implications of having various species of tick vectors, mixed parasite infections and the significance these have on treatment and successful recovery of dogs from parasitic infections. The most pertinent issues from the preceding chapters and future strategies for molecular diagnoses for tick-borne pathogens are discussed.

1.10. References

Abu, M., Hara, I., Nito, I., Sibauchi, T., 1973. *Babesia* infections in puppies: probably due to transplacental transmission. Journal of Veterinary Medicine 609, 203-206.

Aikawa, M., Pongponratn, E., Tegoshi, T., Nakamura, K.I., Nagatake, T., Cochrane, A., Ozaki, L.S., 1992. A study on the pathogenesis of human cerebral malaria and cerebral babesiosis. Memórias do Instituto Oswaldo Cruz 87, 297-301.

Allred, D.R., Al-Khedery, B., 2004. Antigenic variation and cytoadhesion in *Babesia bovis* and *Plasmodium falciparum*: different logics achieve the same goal. Molecular and Biochemical Parasitology 134, 27-35.

Allred, D.R., Carlton, J.M., Satcher, R.L., Long, J.A., Brown, W.C., Patterson, P.E., O'Connor, R.M., Stroup, S.E., 2000. The *ves* multigene family of *B. bovis* encodes components of rapid antigenic variation at the infected erythrocyte surface. Molecular Cell 5, 153-162.

Apanaskevich D.A., Horak, I.G., Camica J.-L., 2007. Redescription of *Haemaphysalis* (*Rhipistoma*) *elliptica* (Koch, 1844), an old taxon of the *Haemaphysalis* (*Rhipistoma*) *leachi* group from East and Southern Africa, and of *Haemaphysalis* (*Rhipistoma*) *leachi* (Audouin, 1826) (Ixodida, Ixodidae). Onderstepoort Journal of Veterinary Research 74, 181-208.

Archibald, L.K., Sexton, D.J., 1995. Long-term sequelae of Rocky Mountain spotted fever. Clinical Infectious Diseases 20, 1122-1125.

Baneth, G., Barta, J.R., Shkap, V., Martin, D.S., Macintire, D.K., Vincent-Johnson, N., 2000. Genetic and antigenic evidence supports the separation of *Hepatozoon canis*

and *Hepatozoon americanum* at the species level. Journal of Clinical Microbiology 38, 1298-301.

Baneth, G., Mathew, J.S., Shkap, V., Macintire, D.K., Barta, J.R., Ewing, S.A., 2003. Canine hepatozoonosis: two disease syndromes caused by separate *Hepatozoon* sp. Trends in Parasitology 27-31.

Baruch, D.I., Ma, X.C., Singh, H.B., Bi, X., Pasloske, B.L., Howard, R.J., 1997. Identification of a region of *PfEMP*1 that mediates adherence of *Plasmodium falciparum* infected erythrocytes to CD36: conserved function with variant sequence. Blood 90, 3766-3775.

Baruch, D.I., Rogerson, S.J., Cooke, B.M., 2002. Asexual blood stages of malaria antigens: cytoadherence. Chemical Immunology 80, 144-162.

Birkenheuer, A.J., Neel, J., Ruslander, D., Levy, M.G., Breitschwerdt, E.B., 2004. Detection and molecular characterization of a novel large *Babesia* species in a dog. Veterinary Parasitology 124, 151-160.

Böhm, M., Leisewitz, A.L., Thompson, P.N., Schoeman, J.P., 2006. Capillary and venous *Babesia canis rossi* parasitaemias and their association with outcome of infection and circulatory compromise. Veterinary Parasitology 141, 18-29.

Breitschwerdt, E.B., Hegarty, B.C., Hancock, S.I., 1998. Sequential evaluation of dogs naturally infected with *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia equi*, *Ehrlichia ewingii*, or *Bartonella vinsonii*. Journal of Clinical Microbiology 36, 2645-2651.

Caccio, S.M., Antunovic, B., Moretti, A., Mangili, V., Marinculic, A., Baric, R.R., Slemenda, S.B., Pieniazek, N.J., 2002. Molecular characterisation of *Babesia*

canis canis and *Babesia canis vogeli* from naturally infected European dogs. Veterinary Parasitology 106, 285-292.

Callow, L.L., Dalgliesh, R.J., 1982. Immunity and immunopathology in babesiosis. In: Cohen, S., Warren, K.S. (Eds), Immunology of Parasitic Infections. Blackwell Scientific, Oxford, pp. 475-526.

Camacho, A.T., Guitian, E.J., Pallas, E., Gestal, J.J., Olmeda, A.S., Goethert, H.K., Telford, S.R., III, Spielman, A., 2004. Azotemia and mortality among *Babesia microti*-like infected dogs. Journal of Veterinary Internal Medicine 18, 141-146.

Camacho, A.T., Pallas, E., Gestal, J.J., Guitian, F.J., Olmeda, A.S., Telford, S.R., Spielman, A., 2003. *Ixodes hexagonus* is the main candidate as vector of *Theileria annae* in northwest Spain. Veterinary Parasitology 112, 157-163.

Casapulla, R., Baldi, L., Avallone, V., Sannino, R., Pazzanese, L., Mizzoni, V., 1998. Canine piroplasmosis due to *Babesia gibsoni*: clinical and morphological aspects. Veterinary Record 142, 168-169.

Centeno-Lima, S., do Rosario, V., Parreira, R., Maia, A.J., Freudenthal, A.M., Nijhof, A.M., Jongejan, F., 2003. A fatal case of human babesiosis in Portugal: molecular and phylogenetic analysis. Tropical Medicine and International Health. 8, 760-764.

Codner, E.C., Farris-Smith, L.L., 1986. Characterization of the subclinical phase of ehrlichiosis in dogs. Journal of the American Veterinary Medical Association 189, 47-50.

Collett, M.G., 2000. Survey of canine babesiosis in South Africa. Journal of the South African Veterinary Association 71, 180-186.

Cooke, B.M., Mohandas, N., Cowman, A.F., Coppel, R.L., 2005. Cellular adhesive phenomena in apicomplexan parasites of red blood cells. Veterinary Parasitology 132, 273-295.

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 II. Phylogenetic analysis and evolutionary history. Veterinary Parasitology 114, 173-194.

Demma, L. J., Traeger, M., Blau, D., Gordon, R., Johnson, B., Dickson, J., Ethelbah, R., Piontkowski, S., Levy, C., Nicholson, W, L., Duncan, C., Heath, K., Cheek, J., Swerdlow, D. L., McQuiston, J. H., 2006. Serologic evidence for exposure to *Rickettsia rickettsii* in eastern Arizona and recent emergence of Rocky Mountain Spotted Fever in this region. Vector-Borne and Zoonotic Diseases 6, 423-429.

Depoix, D., Carcy, B., Jumas-Bilak, E., Pages, M., Precigout, E., Schetters, T.P., Ravel, C., and Gorenflot, A., 2002. Chromosome number, genome size and polymorphism of European and South African isolates of large *Babesia* parasites that infect dogs. Parasitology 125, 313-321.

Drancourt, M., Raoult, D., 1994. Taxonomic position of the Rickettsiae: Current knowledge. FEMS Microbiology Review 13, 13-24.

Duffy, J., Pittlekow, M.R., Kolbert, C.P., Rutledge, B.J., Persing, D.H., 1997. Co-infection with *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis. Lancet 349, 399-399.

Duffy, P.E., Fried, M., 2003. *Plasmodium falciparum* adhesion in the placenta. Current Opinions in Microbiology 6, 371-376.

Duh, D., Tozon, N., Petrovec, M., Strasek, K., Avsic-Zupanc, T., 2004. Canine babesiosis in Slovenia: molecular evidence of *Babesia canis canis* and *Babesia canis vogeli*. Veterinary Research 35, 363-368.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., Rurangirwa, F.R., 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. International Journal of Systematic and Evolutionary Microbiology 51, 2145-2165.

Dumler, J.S., Chen, S.M., Asanovich, K., Trigiani, E., Popov, V.L., Walker, D.H., 1995. Isolation and characterization of a new strain of *Ehrlichia chaffeensis* from a patient with nearly fatal monocytic ehrlichiosis. Journal of Clinical Microbiology 33, 1704-1711.

Egenvall, A., Bjoersdorff, A., Lilliehook, I., Engvall, E.O., Karlstam, E., Artursson, K., Hedhammar, A., Gunnarsson, A., 1998. Early manifestations of granulocytic ehrlichiosis in dogs inoculated experimentally with a Swedish *Ehrlichia* species isolate. Veterinary Record 143, 412-417.

Estrada-Peňa, A., Bouattour, A., Camicas, J.-L., Walker, A.R., 2004. Ticks of Domestic Animals in the Mediterranean Region, A guide to the Identification of Species. University of Zaragoza, Spain. Serial (Book, Monograph).

Ewing, S.A., Panciera, R.J., 2003. American canine hepatozoonosis. Clinical Microbiology Review 16, 688-97.

Friedhoff, K.T., 1988. Transmission of *Babesia*. In: Ristic, M. (Ed), Babesiosis of domestic animals and man. CRC Press, Baco Raton, Florida, pp. 23-52.

Fukumoto, S., Suzuki, H., Igarashi, I., Xuan, X., 2005. Fatal experimental transplacental *Babesia gibsoni* infections in dogs. International Journal of Parasitology 35, 1031-1035.

Garcia, A.T.C., 2006. Piroplasma-infection in dogs in northern Spain. Veterinary Parasitology 138, 97-102.

Gardner, M.J., Hall, N., 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419, 498-511.

Goldman, E.E., Breitschwerdt, E.B., Grindem, C.B., Hegarty, B.C., Walls, J.J., Dumler, J.S., 1998. Granulocytic ehrlichiosis in dogs from North Carolina and Virginia. Journal of Veterinary Internal Medicine 12, 61-70.

Harvey, J., Taboda, J., Lewis, J.C, 1988. Babesiosis in a litter of pups. Journal of the American Veterinary Medical Association 192, 1751-1753.

Hegarty, B.C., Levy, M.G., Gager, R.F., Breitschwerdt, E.B., 1997. Immunoblot analysis of the immunoglobulin G response to *Ehrlichia canis* in dogs: an international survey. Journal of Veterinary Diagnostic Investigation 9, 32-38.

Horak, I.G., 1995. Ixodid ticks collected at the Faculty of Veterinary Science, Onderstepoort, from dogs diagnosed with *Babesia canis* infection. Journal of the South African Veterinary Association 66, 170-171.

Inokuma H., Beppu T., Okuda M., Shimada Y., Sakata Y., 2003. Epidemiological survey of *Anaplasma platys* and *Ehrlichia canis* using ticks collected from dogs in Japan. Veterinary Parasitology 115, 343-348.

Inokuma, H., Oyamada, M., Kelly, P.J., Jacobson, L.A., Fournier, P.E., Itamoto, K., Okuda, M., Brouqui, P., 2005. Molecular detection of a new *Anaplasma* species closely related to *Anaplasma phagocytophilum* in canine blood from South Africa. Journal of Clinical Microbiology 43, 2934-2937.

Iqbal, Z., Chaichanasiriwithaya, W., Rikihisa, Y., 1994. Comparison of PCR with other tests for early diagnosis of canine ehrlichiosis. Journal of Clinical Microbiology 32, 1658-1662.

Jacobson, L.S., 2006. The South African form of severe and complicated canine babesiosis: Clinical advances 1994-2004. Veterinary Parasitology 138, 126-139.

Jacobson, L.S., Clark, I.A., 1994. The pathophysiology of canine babesiosis: new approaches to an old puzzle. Journal of the South African Veterinary Association 65, 134-145.

Jacobson, L.S., Lobetti, R.G., 1996. Rhabdomyolysis as a complication of canine babesiosis. Journal of Small Animal Practice 37, 286-291.

Kjemtrup, A.M., Conrad, P.A., 2006a. A review of the small canine piroplasms from California: *Babesia conradae* in the literature. Veterinary Parasitology 138, 112-117.

Kjemtrup, A.M., Conrad, P.A., 2000a. Human babesiosis: an emerging tick-borne disease. International Journal of Parasitology 30, 1323-1337.

Kjemtrup, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad, P.A., 2000b. There are at least three genetically distinct small piroplasms from dogs. International Journal of Parasitology 30, 1501-1505.

Kjemtrup, A.M., Thomford, J., Robinson, T., Conrad, P.A., 2000c. Phylogenetic relationships of human and wildlife piroplasm isolates in the western United States inferred from the 18S nuclear small subunit RNA gene. Parasitology 120, 487-493.

Kjemtrup, A.M., Wainwright, K., Miller, M., Penzhorn, B.L., Carreno, R.A., 2006b. *Babesia conradae,* sp. nov., a small canine *Babesia* identified in California. Veterinary Parasitology 138, 103-111.

Kocan, A.A., Kjemtrup, A., Meinkoth, J., Whitworth, L.C., Murphy, G.L., Decker, L., Lorenz, M., 2001. A genotypically unique *Babesia gibsoni*-like parasite recovered from a dog in Oklahoma. Journal of Parasitology 87, 437-438.

Kohn, B., Galke, D., Beelitz, P., Pfister, K., 2008. Clinical features of canine granulocytic anaplasmosis in 18 naturally infected dogs. Journal of Veterinary Internal Medicine doi/10.1111/j.1939-1676.2008.0180.x.

Kordick, S.K., Breitschwerdt, E.B., Hegarty, B.C., Southwick, K.L., Colitz, C.M., Hancock, S.I., Bradley, J.M., Rumbough, R., Mcpherson, J.T., MacCormack, J.N., 1999. Co-infection with multiple tick-borne pathogens in a Walker Hound kennel in North Carolina. Journal of Clinical Microbiology 37, 2631-2638.

Kriek, N., Tilley, L., Horrocks, P., Pinches, R., Elford, B.C., Ferguson, D.J., Lingelbach, K., Newbold, C.I., 2003. Characterization of the pathway for transport of the

cytoadherence-mediating protein, *PfEMP*1, to the host cell surface in malaria parasite-infected erythrocytes. Molecular Microbiology 50, 1215-1227.

Kuttler, K.L., 1988. Worldwide impact of babesiosis. In: Ristic, M. (Ed.), Babesiosis of domestic animals and man. CRC Press, Baco Raton, Florida, pp. 1-2.

Langreth, S.G., Peterson, E., 1985. Pathogenicity, stability, and immunogenicity of a knobless clone of *Plasmodium falciparum* in Colombian owl monkeys. Infectious Immunology 47, 760-766.

Levy, S.A., Magnarelli, L.A., 1992. Relationship between development of antibodies to *Borrelia burgdorferi* in dogs and the subsequent development of limb/joint borreliosis. Journal of the American Veterinary Medical Association 200, 344-347.

Lewis, B.D., Penzhorn, B.L., Lopez-Rebollar, L.M., De Waal, D.T., 1996. Isolation of a South African vector-specific strain of *Babesia canis*. Veterinary Parasitology 63, 9-16.

Losson, B., Mollet, J.J., Avez, F., Malaise, F., Mignon, B., 1999. Description of three cases of canine babesiosis in Belgium. Annales de Médecine Vétérinaire 143, 119-124.

Macintire, D.K., Vincent-Johnson, N., Dillon, A.R., Blagburn, B., Lindsay, D., Whitley, E.M., Banfield, C., 1997. Hepatozoonosis in dogs: 22 cases (1989-1994). Journal of the American Veterinary Medical Association 210, 916-22.

Mathew, J.S., Ewing, S.A., Panciera, R.J., Woods, J.P., 1998. Experimental transmission of *Hepatozoon americanum* Vincent-Johnson et al., 1997 to dogs by the Gulf Coast tick, *Amblyomma maculatum* Koch. Veterinary Parasitology 80, 1-14.

Matjila, P. T., Penzhorn, B. L., Bekker, C. P., Nijhof A. M., Jongejan F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Veterinary Parasitology 122, 119-125.

Matsuu, A., Kawabe, A., Koshida, Y., Ikadai, H., Okano, S., Higuchi, S., 2004. Incidence of canine *Babesia gibsoni* infection and sub-clinical infection among Tosa dogs in Aomori Prefecture, Japan. Journal of Veterinary Medical Science 66, 893-897.

McCully, R.M., Basson, P.A., Bigalke, R.D., De Vos, V.D., Young, E., 1975. Observations on naturally acquired hepatozoonosis of wild carnivores and dogs in the Republic of South Africa. Onderstepoort Journal of Veterinary Research 42, 117-133.

Meinkoth, J.H., Kocan, A.A., Loud, S.D., Lorenz, M.D., 2002. Clinical and hematologic effects of experimental infection of dogs with recently identified *Babesia gibsoni*-like isolates from Oklahoma. Journal of the American Veterinary Medical Association 220, 185-189.

Miller, L.H., Baruch, D.I., Marsh, K., Doumbo, O.K., 2002. The pathogenic basis of malaria. Nature 415, 673-679.

Muhlnickel, C.J., Jefferies, R., Morgan-Ryan, U.M., Irwin, P.J., 2002. *Babesia gibsoni* infection in three dogs in Victoria. Australian Veterinary Journal 80, 606-610.

Mumcuoglu, K.Y., Frish, K., Sarov, B., Manor, E., Gross, E., Gat, Z., Galun, R., 1993. Ecological studies on the brown dog tick *Rhipicephalus sanguineus* (Acari: Ixodidae) in southern Israel and its relationship to spotted fever group Rickettsiae. Journal of Medical Entomology 30, 114-121.

Nevils, M.A., Figueroa, J.V., Turk, J.R., Canto, G.J., Le, V., Ellersieck, M.R., Carson, C.A., 2000. Cloned lines of *Babesia bovis* differ in their ability to induce cerebral babesiosis in cattle. Parasitology Research 86, 437-443.

Newbold, C.I., 1999. Antigenic variation in *Plasmodium falciparum*: mechanisms and consequences. Current Opinions Microbiology 2, 420-425.

Nijhof, A.M., Bodaan, C., Postigo, M., Nieuwenhuijs, H., Opsteegh, M., Franssen, L., Jebbink, F., Jongejan, F., 2007. Ticks and associated pathogens collected from domestic animals in the Netherlands. Vector-Borne and Zoonotic Diseases 7, 585-596.

Norval, R.A., 1984. The ticks of Zimbabwe. IX. *Haemaphysalis leachi* and *Haemaphysalis spinulosa*. Zimbabwean Veterinary Journal 15, 9-17.

O'Connor, R.M., Allred, D.R., 2000. Selection of *Babesia bovis*-infected erythrocytes for adhesion to endothelial cells co-selects for altered variant erythrocyte surface antigen isoforms. Journal of Immunology 164, 2037-2045.

O'Connor, R.M., Long, J.A., Allred, D.R., 1999. Cytoadherence of *Babesia bovis*-infected erythrocytes to bovine brain capillary endothelial cells provides an *in vitro* model for sequestration. Infectious Immunology 67, 3921-3928.

Otsuka, Y., Yamasaki, M., Yamato, O., Maede, Y., 2002. The effect of macrophages on the erythrocyte oxidative damage and the pathogenesis of anemia in *Babesia gibsoni*-infected dogs with low parasitaemia. The Journal of Veterinary Medical Science 64, 221-226.

Paddock, C.D., Sumner, J.W., Shore, G.M., Bartley, D.C., Elie, R.C., McQuade, J.G., Martin, C.R., Goldsmith, C.S., Childs, J.E., 1997. Isolation and characterization of

Ehrlichia chaffeensis strains from patients with fatal ehrlichiosis. Journal of Clinical Microbiology 35, 2496-2502.

Panciera, R.J., Ewing, S.A., Mathew, J.S., Cummings, C.A., Kocan, A.A., Breshears, M.A., Fox, J.C., 1998. Observations on tissue stages of *Hepatozoon americanum* in 19 naturally infected dogs. Veterinary Parasitology 78, 265-76.

Panciera, R.J., Ewing, S.A., Mathew, J.S., Lehenbauer, T.W., Cummings, C.A., Woods, J.P., 1999. Canine hepatozoonosis: comparison of lesions and parasites in skeletal muscle of dogs experimentally or naturally infected with *Hepatozoon americanum*. Veterinary Parasitology 82, 261-72.

Pardini, A.D., 2000 (MSc. dissertation). The pathology and pathogenesis of canine cerebral babesiosis. Faculty of Veterinary Science, University of Pretoria, Onderstepoort.

Pongponratn, E., Riganti, M., Punpoowong, B., Aikawa, M., 1991. Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: a pathological study. American Journal of Tropical Medical Hygiene 44, 168-175.

Pretorius, A.M., Kelly, P.J., 1998. Serological survey for antibodies reactive with *Ehrlichia canis* and *E. chaffeensis* in dogs from the Bloemfontein area, South Africa. Journal of the South African Veterinary Association 69, 126-128.

Pretorius, A.M., Venter, T.P., Ryst, E.V.D., Kelly, P.J., 1999. Possible human ehrlichiosis in the Free State. South African Medical Journal 89, 961-961.

Rautenbach, G.H., Boomker, J., Villiers, I.L., 1991. A descriptive study of the canine population in a rural town in Southern Africa. Journal of the South African Veterinary Association 62, 158-162.

Rikihisa, Y., 1991. The tribe Ehrlichieae and ehrlichial diseases. Clinical Microbiology Review 4, 286-308.

Rikihisa, Y., Ewing, S.A., Fox, J.C., Siregar, A.G., Pasaribu, F.H., Malole, M.B., 1992. Analyses of *Ehrlichia canis* and a canine granulocytic *Ehrlichia* infection. Journal of Clinical Microbiology 30, 143-148.

Schetters, T.P., Kleuskens, J., Scholtes, N., Gorenflot, A., 1998. Parasite localization and dissemination in the *Babesia*-infected host. Annals of Tropical Medicine and Parasitology 92, 513-519.

Schetters, T.P., Moubri, K., Precigout, E., Kleuskens, J., Scholtes, N.C., Gorenflot, A., 1997. Different *Babesia canis* isolates, different diseases. Parasitology 115, 485-493.

Schetters, T.P.M., Eling, W.M.C., 1999. Can *Babesia i*nfections be used as a model for cerebral malaria? Parasitology Today 15, 492-497.

Senneville, E., Ajana, F., Lecocq, P., Chidiac, C., Mouton, Y., 1991. *Rickettsia conorii* isolated from ticks introduced to northern France by a dog. Lancet 337, 676-676.

Shakespeare, A.S., 1995. The incidence of canine babesiosis amongst sick dogs presented to the Onderstepoort Veterinary Academic Hospital. Journal of the South African Veterinary Association 66, 247-250.

Shaw, S.E., Day, M.J., Birtles, R.J., Breitschwerdt, E.B., 2001. Tick-borne infectious diseases of dogs. Trends in Parasitology 17, 74-80.

Shaw, S.E., Irwin, P., 2001. The consequences of tick infestation in dogs and cats. Walton Focus. 11, 16-23.

Sherwood, J.A., 1990. Cytoadherence of malaria-infected erythrocytes. Commentary. Progress toward understanding the pathogenesis of sequestration in falciparum malaria. Blood Cells 16, 620-628.

Shortt, H.E., 1973. *Babesia canis*: the life cycle and laboratory maintenance in its arthropod and mammalian hosts. International Journal of Parasitology 3, 119-148.

Stevens, J.R., Noyes, H.A., Schofield, C.J., Gibson, W., 2001. The molecular evolution of Trypanosomatidae. Advances in Parasitology 48, 1-56.

Uilenberg, G., Franssen, F.F., Perie, N.M., Spanjer, A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Veterinary Quarterly 11, 33-40.

Van Heerden, J., 1982. A retrospective study on 120 natural cases of canine ehrlichiosis. Journal of the South African Veterinary Association 53, 17-22.

Vincent-Johnson, N.A., Macintire, D.K., Lindsay, D.S., Lenz, S.D., Baneth, G., Shkap, V., Blagburn, B.L., 1997. A new *Hepatozoon* species from dogs: description of the causative agent of canine hepatozoonosis in North America. Journal of Parasitology 83, 1165-1172.

Weissenbock, H., Holzmann, H., 1996. Tick-borne encephalitis in Austrian dogs. Veterinary Record 139, 575-576.

Wen, B., Rikihisa, Y., Mott, J.M., Greene, R., Kim, H.Y., Zhi, N., Couto, G.C., Unver, A., Bartsch, R., 1997. Comparison of nested PCR with immunofluorescent-antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. Journal of Clinical Microbiology 35, 1852-1855.

Wormser, G.P., Dattwyler, R.J., Shapiro, E.D., Halperin, J.J., Steere, A.C., Klempner, M.S., Krause, P.J., Bakken, J.S., Strle, F., Stanek, G., Bockenstedt, L., Fish, D., Dumler, J.S., Nadelman, R.B., 2006. The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clinical Infectious Diseases 43, 1089-1134.

Wright, I.G., Goodger, B.V., Clark, I.A., 1988. Immunopathophysiology of *Babesia bovis* and *Plasmodium falciparum* infections. Parasitology Today 4, 214-218.

Zahler, M., Gothe, R., 1997. Endemic risk of *Babesia canis* by *Dermacentor reticulatus* in Germany. An epidemiologic study. Tierärztliche Praxis. Ausgabe K, Kleintiere/Heimtiere 25, 666-670.

Zahler, M., Rinder, H., Schein, E., Gothe, R., 2000. Detection of a new pathogenic *Babesia microti*-like species in dogs. Veterinary Parasitology 89, 241-248.

Zahler, M., Schein, E., Rinder, H., Gothe, R., 1998. Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. Parasitology Research 84, 544-548.

Chapter 2: Confirmation of occurrence of *Babesia vogeli* in domestic dogs in South Africa.

2.1. Abstract

The occurrence of *Babesia* infections in domestic dogs in South Africa was studied using Reverse Line Blot (RLB) and 18S rRNA gene sequence analysis. A total of 226 blood samples were collected from healthy dogs in shelters of the Society for the Prevention of Cruelty to Animals (SPCA). An additional 56 samples were obtained from dogs in need of veterinary care presented at the Onderstepoort Veterinary Academic Hospital (OVAH) and 15 samples from healthy dogs were from Keringa Kennels in Johannesburg. Blood samples (2-5ml) were collected from the cephalic vein into citrate buffered vacutainer tubes. DNA was extracted from the collected blood samples, followed by PCR/RLB assay. In order to be able to differentiate between the three large piroplasms of dogs, species-specific oligonucleotides were deduced in the amplified V4 region. RLB results showed that 18 samples from OVAH were positive for *B. rossi* and one was positive for *B. vogeli.* Twelve samples from Bloemfontein were positive for *B. vogeli* and one sample for *B. rossi*, whereas 12 samples from East London contained *B. rossi* DNA. The remaining samples from Durban, Johannesburg and Keringa Kennels were negative for *Babesia* infections. Partial sequencing of *ca* 530bp derived from samples positive for *B. vogeli* Bcv 6 (accession no. AF548006) and Bcv 28 (accession no. AF547387) were 100% identical. The occurrence of *B. vogeli* was confirmed for the first time in SA. None of the dogs sampled carried mixed infections of *B. vogeli* and *B. rossi.* The

detection of *B. vogeli* has implications with regard to the clinical manifestations of canine babesiosis in South Africa.

2.2. Introduction

Babesia species are intraerythrocytic protozoan parasites affecting a wide range of vertebrate hosts and are among the most common tick-borne pathogens of dogs in South Africa. The large piroplasms *Babesia canis* (*senso lato*) and the small piroplasm *B. gibsoni* (*senso lato*), cause canine babesiosis characterized by significant haemolytic anaemia.

The trophozoites of *B. canis* (*senso lato*), are piriform and 4-5 µm in length or amoeboid and 2-4 µm in diameter (Levine, 1985). There are currently three recognized species of the large babesias of dogs; which differ on the basis of geographical distribution, vector specificity and antigenic properties (Hauschild, Shayan and Schein, 1995; Uilenberg, Franssen, Perie and Spanjer, 1989). *Babesia canis* is transmitted by *Dermacentor reticulatus, Babesia vogeli* is transmitted by *Rhipicephalus sanguineus* and *Babesia rossi* is transmitted by *Haemaphysalis elliptica.*

Lounsbury (1901) identified *H. elliptica* as a vector of *B. canis* (*senso lato*) in South Africa. In subsequent investigations, Lewis, Penzhorn, Lopez-Rebollar and De Waal (1996) found that the South African *B. canis* (*senso lato*) isolate could not be transmitted by *R. sanguineus*, contradicting the statement by Howell, Walker and Nevill (1983) that the latter tick species was an important vector of *B. canis* (*senso lato*) in South Africa.

The species also differ in pathogenicity. *Babesia rossi* causes a frequently fatal infection in domestic dogs, even after treatment; *B. vogeli* causes a moderate often clinically inapparent infection, and *B. canis* infections result in a more variable pathogenicity intermediate between *B. rossi* and *B. vogeli* (Uilenberg et al., 1989). Further evidence provided by several authors supports the recognition of *B. rossi*, *B. vogeli* and *B. canis* as separate taxa (Schetters, Moubri, Precigout, Kleuskens, Scholtes and Gorenflot, 1997; Zahler, Schein, Rinder and Gothe, 1998).

The other species that infects dogs is *B. gibsoni* (*senso lato*), which occurs in Asia, North America, Australia, North and Eastern Africa and has also been reported in Europe (Casapulla, Baldi, Avallone, Sannino, Pazzanese and Mizzoni, 1998; Muhlnickel, Jefferies, Morgan, Ryan and Irwin, 2002). This parasite measures 1-2.5 µm in length and is either piriform or ring form (Conrad, Thomford, Yamane, Whiting, Bosma, Uno, Holshuh and Shelly, 1991). *Babesia gibsoni* (*senso lato*) can be differentiated from *B. canis* (*senso lato*) based on morphology, but not from other small piroplasms of dogs. In fact, research has revealed that there are at least three genetically distinct small piroplasms occurring in dogs in the USA, Spain and Japan (Kjemtrup, Kocan, Whitworth, Meinkoth, Birkenheuer, Cummings, Boudreaux, Stockham, Irizarry-Rovira and Conrad, 2000).

Molecular diagnosis has previously been used as a tool for the detection and characterization of *B. canis* (*senso lato*) (Birkenheuer, Levy and Breitschwerdt, 2003; Caccio, Antunovic, Moretti, Mangili, Marinculic, Baric, Slemenda and Pieniazek, 2002),

and in tick vectors (Inokuma, Yoshizaki, Shimada, Sakata, Okuda and Onishi, 2003). In this study, we report on a survey of *Babesia* parasites in dogs using molecular methods, wherein the occurrence of *B. vogeli* in naturally infected dogs was confirmed in South Africa.

2.3. Materials and Methods

2.3.1. Collection of samples

A total of 226 blood samples were collected from apparently healthy dogs in shelters of the Society for the Prevention of Cruelty to Animals (SPCA) (Table 2.1). The dogs had been abandoned by their owners or were found straying. An additional 55 samples were obtained from dogs in need of veterinary care at the Onderstepoort Veterinary Academic Hospital (OVAH) and 15 samples from apparently healthy dogs were from Keringa Kennels in Johannesburg. None of the dogs sampled displayed clinical signs of babesiosis, except for some of those sampled at OVAH (n=18) and confirmed *Babesia* positive based on smear examination. Approximately 2-4 ml of blood was collected from the cephalic vein into citrate buffered vacutainer tubes. Samples were kept cool while being transported to the Faculty of Veterinary Science, in Onderstepoort. About 200 µl of blood was aliquoted into 1.5 ml eppendorf tubes and stored at –20°C until DNA was extracted.

2.3.2. DNA extraction

DNA was extracted from 200 µl of whole blood by adding 500 µl phosphate buffered solution (PBS) to the blood, mixing and centrifuging (14,000 x g) for 5 minutes and

thereafter, the supernatant was discarded. These steps were repeated 3-5 times, until the pellet was white and the supernatant clear. The pellet was resuspended in 100 µl of lysis buffer (50 mM KCl, 0.5% Tween 20, 10 mM Tris-HCl [pH 8.0]) and 1 µl of proteinase K solution (1 µg/µl), mixed and incubated overnight at 56°C and heated at 100°C for 10 minutes to inactivate the proteinase K.

2.3.3. PCR

PCR was conducted with a set of primers that amplified 460-540bp fragment of the 18S SSU rRNA spanning the V4 region. The forward primer RLB-F2 (5′ -GAC ACA GGG AGG TAG TGA CAA G-3′) and the reverse primer RLB-R2 (biotin-5′-CTA AGA ATT TCA CCT CTG ACA GT-3′) were used for the amplification and hybridized with regions conserved for *Babesia* and *Theileria* (Gubbels, de Vos, van der Weide, Viseras, Schouls, de Vries and Jongejan, 1999). All primers were obtained from Isogen BV (Maarsen, The Netherlands). Known positive samples were used as controls.

The PCR reaction (25 µl) contained 2.5 µl of DNA template in 1 x PCR buffer (HT Biotechnology, Cambridge, England), 5 U of SuperTaq (HT Biotechnology, Cambridge, UK), 200 and 100 µM of each of the following deoxynucleoside triphosphate (dATP, dCTP, dGTP) and (dTTP, dUTP), respectively (Pharmacia Biotech, Uppsala, Sweden), 20 pmol of each primer, 5 U Taqstart Antibody (Clontech, California, USA) and 0.4 U Uracil DNA Glycosylase (Invitrogen, Breda, The Netherlands). The reactions were performed in an automated I-Cycler (Biorad, California, USA) with an initial step of 3 min at 37°C, 10 min at 94°C, 10 cycles of 94°C(20s)-67°C(30s)-72°C(30s), with

lowering of the annealing step after every second cycle with 2°C (touchdown) then followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30s and extension at 72°C for 30s.

2.3.4. Reverse line blot hybridization

Reverse Line Blot (RLB) was performed using PCR products as described by Gubbels et al. (1999). Oligonucleotide probes containing an N-terminal *N*-(trifluoracetamidohexyl-cyanoethyl,*N*,*N*-diisopropyl phosphoramidite [TFA])-C_6 amino linker (Isogen) were covalently linked to the RLB membrane using the following procedure: a Biodyne C blotting membrane (Pall Biosupport, Ann Arbor, Mich.) was activated by a 10 min incubation in 10 ml of 16% 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDAC) (Sigma, St. Louis, Mo.) at room temperature. The membrane was washed for 2 min with distilled water and placed in an MN45 miniblotter (Immunetics, Cambridge, Mass.). Specific oligonucleotides were diluted to a 200 to 1600 pmol/150 µl concentration in 500 mM $NaHCO_3$ (pH 8.4) and linked to the membrane by loading them onto the lanes of the miniblotter, followed by a 1 min incubation. After aspiration of the oligonucleotide probe solutions, the membrane was inactivated by incubation in 100 ml of a 100 mM NaOH solution for 10 min at room temperature. The membrane was washed with SSPE-0.1% sodium dodecyl sulfate (SDS) solution for 5 min at 60°C (20× SSPE contains 360 mM NaCl, 20 mM NaH_2PO_4, and 2 mM EDTA [pH 7.4]).

Before the membrane could be used, it was washed for 5 min at 42°C with 2× SSPE-0.1% SDS and placed in the miniblotter. A volume of 25 µl of PCR product was diluted

to an end volume of 150 µl of 2× SSPE-0.1% SDS, heated for 10 min at 100°C, and immediately cooled on ice. Denatured PCR samples were applied into the MN45 miniblotter slots and incubated for 60 min at 42°C. Thereafter, the PCR products were aspirated, and the membrane was washed twice at 42°C in 2× SSPE-0.5% SDS solution for 10 min followed by incubation with 10 ml 1:4,000-diluted peroxidase-labeled streptavidin (Boehringer, Mannheim, Germany) in 2× SSPE-0.5% SDS at 42°C for 30 min. The membrane was washed twice again with 2× SSPE-0.5% SDS at 42°C for 10 min and thereafter washed twice for 5 min, each time in 2× SSPE solution at room temperature. The membrane was then incubated for 1 min in 10 ml of ECL detection fluid (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) before exposure to an ECL hyperfilm (Amersham) and development. The PCR products were stripped from the membrane by two washes for 30 min each time at 80°C in 1% SDS solution. The membrane was rinsed in 20 mM EDTA (pH 8.0) for 15 min and then stored in fresh EDTA solution at 4°C for reuse (Gubbels et al., 1999).

The following modifications were included in the RLB assay: denatured PCR products were diluted in 2 x SSPE -0.1% sodium dodecyl sulphate (SDS), loaded onto the membrane and incubated at 42°C for one hour. Thereafter the membrane was washed twice at 50°C instead of 42°C.

2.3.5. Sequence analysis

In order to be able to differentiate between the three large piroplasms of dogs, species-specific oligonucleotides were deduced in the amplified V4 region (Table 2.2). The

following Genbank accession numbers of the 18S rDNA sequences were used to deduce the species-specific oligonucleotides: *B. rossi*, L19079; *B. vogeli*, AY072925 and *B. canis*, AY072926.

To confirm RLB results and to determine sequence heterogeneity of isolates, two out of 12 *B. vogeli* positive samples and one *B. rossi* positive sample from Bloemfontein were re-amplified with primers RLB-F2 and 18SEQ2 (5′-GCCCTTCCGTCAATTCCTTTAA-3′). The PCR conditions were the same as the ones described above but without dUTP and UDG and there were 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min followed by a final step of 7 min at 72°C. Partial sequences (530-540bp) from the products were sequenced (Base-Clear, Leiden, The Netherlands).

A search was performed to examine similarities among sequences in Genbank using the BLASTN program. Sequence alignment was done using the Multalin on-line interface (http://www.toulouse.inra.fr/multalin.html) and thereafter manually edited in Genedoc (version 2.6.001).

2.4. Results

Three new probes to differentiate between *B. rossi, B. canis* and *B. vogeli* were tested on known positive reference samples (Table 2.2). There were no cross-reactions between the three species-specific oligonucleotides. Screening of PCR products with the RLB showed that 18 samples from OVAH were positive for *B. rossi* and one was positive for *B. vogeli.* The latter dog was from the vicinity of Onderstepoort and had been admitted to OVAH with suspected ehrlichiosis. Twelve samples from Bloemfontein were positive

for *B. vogeli* and one sample for *B. rossi,* whereas 12 samples from East London contained *B. rossi* DNA (Table 2.1). The remaining samples from Durban, Johannesburg and Keringa Kennels were negative for *Babesia* infections (Table 2.1). Partial sequencing of approximately 530bp derived from samples positive for *B. vogeli,* Bcv 6 (accession no. AF548006) and Bcv 28 (accession no. AF547387) were 100% identical. Also there was a 99% similarity with a *B. vogeli* isolate from dogs in Europe (AY072925) and a 99% identity score with a *B. vogeli* isolate from Okinawa (AY077719). The difference of 1% between both searches was due to one insertion or deletion in the hypervariable region. The partial sequence of sample Bcr 2506 had a similarity of 99% to the known sequence of *B. rossi*, under the accession number L19079 in Genbank.

2.5. Discussion

Babesia rossi was assumed to be the cause of canine babesiosis in South Africa (Horak, 1995) based on its vector specificity (Taboada and Merchant, 1991; Uilenberg et al., 1989). This is the first report of *B. vogeli* in South Africa and an indication that it occurs less frequently than *B. rossi*. Uilenberg et al. (1989) had suggested that *B. vogeli* parasites may occur in large parts of tropical and subtropical regions on all continents, coinciding with the global distribution of *R. sanguineus* ticks. *Babesia canis*, the species reported to be endemic in Europe, was not detected in our samples, probably because the known tick vector, *D. reticulatus,* is not known to occur in South Africa. It appears that the absence of the parasite is linked to the absence of the tick vector.

The presence of tick vectors, *H. elliptica* and *R. sanguineus,* in South Africa makes it possible for the spread of *Babesia* parasites in the local dog populations. Lewis et al. (1996) reported that they had encountered strains of *B. canis* (*senso lato*) that were serologically different from strains transmitted by *H. elliptica* and suggested that these strains may be transmissible by *R. sanguineus*. Since *R. sanguineus* is the only known tick vector of *B. vogeli*, it is possible that the authors were referring to *B. vogeli* in their observations. They also reported on a preliminary epidemiological survey of *H. elliptica*-transmitted *B. canis* (*senso lato*) strains in various regions of South Africa (Lewis et al., 1996). It is possible that authors were also referring to *B. rossi* isolates in this instance.

Based on our current results, it is not clear why few collected samples at OVAH were infected with *B. vogeli* as compared to Bloemfontein. Dogs that are presented at OVAH are usually dogs that are sick and in need of veterinary care. This implies that chances of detecting *B. vogeli*-infected samples at OVAH are low, since *B. rossi* is the parasite usually associated with clinical disease. *Babesia vogeli* is mostly associated with subclinical or mild infections and infected dogs are rarely presented at OVAH. Dogs that were sampled at non-OVAH sites were asymptomatic and tick-free during sampling. This might explain why the majority of dogs from these areas were either negative or *B. vogeli* positive.

A survey conducted in resource-poor communities in North West province of South Africa found that although *H. elliptica* was present in the area, high numbers of *R. sanguineus* ticks were found on a majority of dogs (Bryson, Horak, Höhn and Louw,

2000). Both *H. elliptica* and *R. sanguineus* have overlapping distribution in South Africa and sometimes are present on the same host (Horak, 1995). A phenomenon that has been observed in South Africa is that dogs from resource-poor communities are infested with high numbers of *R. sanguineus* whereas dogs from affluent communities are likely to have high numbers of *H. elliptica* (Horak, 1995; Bryson et al., 2000). Canine babesiosis is not as common as canine ehrlichiosis in resource-poor communities (Bryson et al., 2000). Due to the high numbers of *R. sanguineus*, asymptomatic *B. vogeli* infections may be prevalent in dogs in these areas. This should be investigated. Our study samples from the SPCAs were all from apparently healthy dogs, in contrast to samples from OVAH. The indications are that the occurrence of *Babesia* species might be more widely distributed and diverse than previously thought.

2.6. Conclusion

In South Africa, babesiosis has variable clinical manifestations including haemolysis and multiorgan dysfunction. Some of these differences may be due to an excessive inflammatory response rather than to the parasite itself (Jacobson and Clark, 1994). However, we conclude that the presence of both *B. rossi* and *B. vogeli* may contribute to the varied clinical manifestations that are typical to South Africa. As far as we can ascertain, *B. vogeli* has been linked to acute cases of babesiosis and fatalities only in a study conducted in Australia (Irwin and Hutchinson, 1991). The clinical significance of *B. vogeli* still needs to be investigated in South Africa and to this end, attempts are underway to isolate *B. vogeli* in order to study its pathogenicity and also to confirm that *R. sanguineus* is the vector in the field.

2.7. Tables

Table 2.1: Dogs positive for *Babesia vogeli* and *Babesia rossi* in South Africa by reverse line blot.

Area	Number of samples	Number positive for *B. vogeli*	Number positive for *B. rossi*
Bloemfontein	41	12 (29)	1 (2)
East London	54	-	12 (22)
Durban	56	-	-
Johannesburg	75	-	-
Keringa Kennels	15	-	-
OVAH	56	1 (2)	18 (32)

Values in parentheses are percentages

Table 2.2: Species-specific oligonucleotides from large canine *Babesia* species.

Subspecies-specific oligonucleotide	Sequence (5'- 3')	*T*m (ºC)
B. rossi	CGGTTTGTTGCCTTTGTG	53.7
B. vogeli	AGCGTGTTCGAGTTTGCC	56.0
B. canis	TGCGTTGACGGTTTGAC	52.8

2.8. References

Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2003. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. Journal of Clinical Microbiology 41, 4172-4177.

Bryson, N.R., Horak, I.G., Höhn, E.W., Louw, J.P., 2000. Ectoparasites of dogs belonging to people in resource-poor communities in North West Province, South Africa. Journal of the South African Veterinary Association 71, 175-179.

Caccio, S.M., Antunovic, B., Moretti, A., Mangili, V., Marinculic, A., Baric, R.R., Slemenda, S.B., Pieniazek, N.J., 2002. Molecular characterisation of *Babesia canis canis* and *Babesia canis vogeli* from naturally infected European dogs. Veterinary Parasitology 106, 285-292.

Casapulla, R., Baldi, L., Avallone, V., Sannino, R., Pazzanese, L., Mizzoni, V., 1998. Canine piroplasmosis due to *Babesia gibsoni*: clinical and morphological aspects. Veterinary Record 142, 168-169.

Conrad, P., Thomford, J., Yamane, I., Whiting, J., Bosma, L., Uno, T., Holshuh, H.J., Shelly, S., 1991. Hemolytic anemia caused by *Babesia gibsoni* infection in dogs. Journal of the American Veterinary Medical Association 199, 601-605.

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.

Hauschild, S., Shayan, P., Schein, E., 1995. Characterization and comparison of merozoite antigens of different *Babesia canis* isolates by serological and immunological investigations. Parasitology Research 81, 638-642.

Horak, I.G., 1995. Ixodid ticks collected at the Faculty of Veterinary Science, Onderstepoort, from dogs diagnosed with *Babesia canis* infection. Journal of the South African Veterinary Association 66, 170-171.

Howell, C.J., Walker, J.B., Nevill, E.M. Ticks, mites and insects infesting domestic animals in South Africa Part 1. Description and biology. Scientic Pamphlet. vol 393. Department of Agricultural Technical Services, Pretoria. 1983. Department of Agricultural Technical Services, Pretoria.

Inokuma, H., Yoshizaki, Y., Shimada, Y., Sakata, Y., Okuda, M., Onishi, T., 2003. Epidemiological survey of *Babesia* species in Japan performed with specimens from ticks collected from dogs and detection of new *Babesia* DNA closely related to *Babesia odocoilei* and *Babesia divergens* DNA. Journal of Clinical Microbiology l41, 3494-3498.

Irwin, P.J., Hutchinson, G.W., 1991. Clinical and pathological findings of *Babesia* infection in dogs. Australian Veterinary Journal 68, 204-209.

Jacobson, L.S., Clark, I.A., 1994. The pathophysiology of canine babesiosis: new approaches to an old puzzle. Journal of the South African Veterinary Association 65, 134-145.

Kjemtrup, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad, P.A.,

2000. There are at least three genetically distinct small piroplasms from dogs. International Journal of Parasitology 30, 1501-1505.

Levine, N.D., 1985. Veterinary Protozoology. Iowa State University Press, Ames, IA..

Lewis, B.D., Penzhorn, B.L., Lopez-Rebollar, L.M., De Waal, D.T., 1996. Isolation of a South African vector-specific strain of *Babesia canis*. Veterinary Parasitology 63, 9-16.

Lounsbury C.P., 1901. Transmission of malignant jaundice of the dog by a species of tick. Agricultural. Journal of the Cape of Good Hope 714-724.

Muhlnickel, C.J., Jefferies, R., Morgan-Ryan, U.M., Irwin, P.J., 2002. *Babesia gibsoni* infection in three dogs in Victoria. Australian Veterinary Journal 80, 606-610.

Schetters, T.P., Moubri, K., Precigout, E., Kleuskens, J., Scholtes, N.C., Gorenflot, A., 1997. Different *Babesia canis* isolates, different diseases. Parasitology 115, 485-493.

Taboada, J., Merchant, S.R., 1991. Babesiosis of companion animals and man Tick-transmitted diseases. The Veterinary clinics of North America. Small Animal Practice. 21, 103-123.

Uilenberg, G., Franssen, F.F., Perie, N.M., Spanjer, A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Veterinary Quarterly 11, 33-40.

Zahler, M., Schein, E., Rinder, H., Gothe, R., 1998. Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. Parasitology Research 84, 544-548.

Chapter 3: Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa.

3.1. Abstract

A total of 1138 blood samples were collected over a of six-year period (2000-2006) from domestic dogs in South Africa. Samples from domestic dogs were obtained from the Onderstepoort Veterinary Academic Hospital (OVAH) in Pretoria, the Society for the Prevention of Cruelty to Animals (Johannesburg, Durban, East London and Bloemfontein) and private practices from 4 provinces (Gauteng, Mpumalanga, KwaZulu-Natal and Western Cape). All samples were screened for *Babesia*, *Theileria*, *Hepatozoon* and *Ehrlichia / Anaplasma* species using PCR and Reverse Line Blot (RLB) assays. On RLB, 560/1137 samples were positive for one or more parasites. Of the positive samples, 420 (75%) were infected with *Babesia rossi*; 82 (15%) dogs were infected with *Theileria* sp. (dog); 18 (3%) dogs were infected with *Babesia vogeli* and 14 (3%) samples were infected with *Ehrlichia canis*. Mixed infections were also found: *B. rossi* and *E. canis* were detected in 12 (2%) samples; *B. vogeli* and *E. canis* occurred in 7 (1%) samples; *Theileria* sp. (dog) and *E. canis* in 3 (0.5%) samples; *B. rossi* and *B. vogeli* in one sample. *Babesia rossi, B. vogeli* and *E. canis* occurred simultaneously in one dog. There was also one finding of a dog positive for *Trypanosoma congolense*. The results indicate that a wide range of tick-borne pathogens are circulating in the canine populations in South Africa.

3.2. Introduction

Ticks transmit a wide variety of pathogens, including protozoa, bacteria and viruses. The most important tick-transmitted infections that cause severe clinical illness in South African dogs are babesiosis (Collett, 2000) and ehrlichiosis (Rautenbach, Boomker and Villiers, 1991; Van Heerden, 1982). *Babesia* parasites of dogs can be grouped into two groups based on morphology (Kuttler, 1988): the large babesias, known as the *B. canis* (*senso lato*) group (Uilenberg, Franssen, Perie and Spanjer, 1989) and the small babesias, which include the *B. gibsoni* (*senso lato*) group, *B. conradae* and *Theileria annae* (Kjemtrup, Kocan, Whitworth, Meinkoth, Birkenheuer, Cummings, Boudreaux, Stockham, Irizarry-Rovira and Conrad, 2000; Kjemtrup, Wainwright, Miller, Penzhorn and Carreno 2006; Zahler, Rinder, Schein and Gothe, 2000). The large babesias of dogs have a wide distribution which includes South Africa (Uilenberg et al., 1989), while the small babesias of dogs occur in South-East Asia, North-East Africa, Spain and the USA (Kjemtrup et al., 2000; Kjemtrup et al., 2006; Zahler, Schein, Rinder and Gothe, 1998). Canine babesiosis is an economically important disease of dogs in South Africa (Collett, 2000), hence the main focus of our study was to screen blood samples for *Babesia* sp. infections.

Ehrlichia and / or *Anaplasma* species infecting humans and dogs have also been documented in South Africa (Pretorius and Kelly, 1998; Pretorius, Venter, Ryst and Kelly, 1999) although the results of these reports were based only on serological diagnostic assays. It is currently unknown whether the South African domestic dog population carries tick-borne infectious agents of human importance, which can be

detected using molecular techniques. To date we are aware of one report where a new *Anaplasma* species closely related to *Anaplasma phagocytophilum* was detected from canine blood (Inokuma, Oyamada, Kelly, Jacobson, Fournier, Itamoto, Okuda, and Brouqui, 2005). Therefore the second focus of this report was to search for zoonotic *Ehrlichia* and *Anaplasma* species and thirdly, to evaluate the degree of co-infection with multiple tick-borne pathogens. Additionally we aimed to identify tick-borne pathogens that were incidental or previously unknown pathogens of dogs.

3.3. Materials and Methods

3.3.1. Collection of samples

A total of 1138 (Table 3.1) domestic-dog blood samples were collected from four inland provinces (Gauteng, North West, Mpumalanga and Free State) and three coastal provinces (KwaZulu-Natal, Eastern Cape and Western Cape) (Fig 3.1) out of the nine provinces of South Africa. Except for samples from Mpumalanga, which were collected from known *Babesia*-positive animals, all samples were collected from domestic dogs that were available during our sampling days. Blood samples were obtained from dogs at the Onderstepoort Veterinary Academic Hospital (OVAH), the Society of the Prevention of Cruelty to Animals (SPCA) and private veterinary clinics. The OVAH is situated in the northern part of Gauteng, close to the North West Province border. Therefore, blood samples collected from OVAH (n=527) could also represent samples from North West Province. All samples came from dogs presented with clinical disease requiring veterinary care. An additional 90 blood samples were collected in southern Gauteng. Samples from apparently healthy dogs were obtained from the Johannesburg SPCA,

while samples from sick dogs were obtained from private practices. Blood samples (n=38) collected from Mpumalanga were obtained from sick dogs at a private practice in White River. Blood samples (n=129) collected from the Free State were obtained from the Bloemfontein SPCA from apparently healthy dogs. A total 253 blood samples were obtained from KwaZulu-Natal's two major cities: 56 were from the Durban SPCA from apparently healthy dogs, 4 from sick dogs at a private practice in Durban and 192 were collected from private practices in the Pietermaritzburg area from healthy dogs involved in a study of tick-repellent impregnated dog collars. An additional sample confirmed positive on blood smear for *Trypanosoma* was obtained from a private clinic in Hluhluwe, northern KwaZulu-Natal. Blood samples (n=54) collected from Eastern Cape were obtained from the East London SPCA from apparently healthy dogs and blood samples (n=47) obtained from the Western Cape were collected from sick dogs at private practices in Cape Town. Blood samples were collected from the cephalic vein into EDTA tubes.

3.3.2. DNA extraction

Once collected, the blood samples were sent to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. DNA was extracted from 200 µl of each blood sample. The QIAmp blood and tissue extraction kit (Qiagen, Hilden, Germany) was used for DNA extractions, following the manufacturer's protocols.

3.3.3. PCR

The *Babesia / Theileria / Hepatozoon* PCR was performed with 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′) amplifying a fragment of 460-540bp from the 18S rRNA gene spanning the V4 region (Gubbels, de Vos, Van der Weide, Viseras, Schouls, de Vries and Jongejan, 1999; Matjila, Penzhorn, Bekker, Nijhof and Jongejan, 2004). The *Ehrlichia / Anaplasma* PCR was performed with the forward primer Ehr-F (5'GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') and Ehr-R (5'-Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3') amplifying a fragment of 460 to 520 bp from the V1 hypervariable region of the 16S SSU rRNA gene (Bekker, de Vos, Taoufik, Sparagano and Jongejan, 2002; Nijhof, Pillay, Steyl, Prozesky, Stoltsz, Lawrence, Penzhorn and Jongejan, 2005). The conditions for the PCR included an initial step of 3 min at 42º C, 10 min at 94ºC, 10 cycles of 94ºC (20s)- 67 ºC (30s)- 72º C (30s), with lowering of annealing step after every second cycle with 2º C (touchdown PCR). The reaction was then followed by 40 cycles of denaturation at 94º C for 30s, annealing at 57º C for 30s and extension at 72º C for 30s. The PCR and restriction fragment length polymorphism assay (RFLP) for the species identification of the *Trypanosoma* infected sample was done as described by Delespaux, Ayral, Geysen and Geerts (2003). The amplification for this nested PCR was first done on the 18S gene using the forward primer 18STnF2 (CAA CGA TG-ACA CCC ATG AAT TGG GGA) and 18STnR3 (TGC GCG ACC AAT AAT TG-CAA TAC) (Geysen, Delespaux, and Geerts, 2003). The second amplification was done using the forward primer 18STnF2 (CAA CGA TG-ACA CCC ATG AAT TGG GGA) of the first amplification with the reverse primer 18STnR2 (GTG TCT TGT TCT

CAC TGA CAT TGT AGT G). Nested products were then analysed using the RLFP for species identification as described by Delespaux et al. (2003). Known positive samples were used as controls.

3.3.4. Reverse line blot hybridisation

RLB was subsequently conducted on amplified products (*Babesia*, *Theileria*, *Hepatozoon*, *Anaplasma* and *Ehrlichia*) as previously described (Matjila et al., 2004). The list of probes and their sequences used for detecting pathogen DNA are listed in Table 3.2.

3.4. Results

The majority of samples collected from OVAH (382/527; 72%) were infected with at least one pathogen (Table 3.3). Most samples (65%) from this area were infected with *B. rossi*. A newly identified species (*Theileria* sp. dog) was detected in 3 samples (Matjila, Leisewitz, Oosthuizen, Jongejan and Penzhorn, 2008). Mixed infections were also detected: 8 samples were co-infected with *B. rossi* and *E. canis*; 7 samples were co-infected with *B. vogeli* and *E. canis*; one sample was co-infected with *B. rossi* and *B. vogeli* and one sample had a triple infection of *B. rossi*, *B. vogeli* and *E. canis*.

Almost all of the samples collected from Mpumalanga (37/38; 97%) were positive for *B. rossi*. Forty percent (101/253) of samples collected from KwaZulu-Natal were positive for at least one parasite. Sixteen samples from this area were infected with *B. rossi*, 1 sample with *E. canis* and 79 samples with *Theileria* sp. dog. Mixed infections were

detected in four samples, one of which was co-infected with *B. rossi* and *E. canis* and 3 samples were co-infected *Theileria* sp. dog and *E. canis*. One sample collected from the northern part of KwaZulu-Natal (Hluhluwe) was confirmed positive for *Trypanosoma congolense*. Twenty-two percent (12/54) of samples collected from the Eastern Cape were positive, with only 12 infected *B. rossi* samples. Sixteen percent (21/129) of samples collected from the Free State were positive: 7 samples were *B. rossi* positive, 13 were *B. vogeli* positive and only 1 sample was *E. canis* positive. Mixed infections were not detected in samples collected from this area. Only 6% (3/47) of samples collected from Western Cape were positive: 2 samples were positive for *B. rossi* and 1 sample was co-infected with *B. rossi* and *E. canis*. Finally, only 4% (4/90) of samples collected from southern Gauteng were positive: 2 samples were *B. rossi* positive and 1 sample was co-infected with *B. rossi* and *E. canis*. We were also able to detect *B. gibsoni* in a sample collected at a private practice in southern Gauteng from a pit-bull pup recently imported into South Africa (Matjila, Penzhorn, Leisewitz, Bhoora and Barker, 2007). No *Hepatozoon* infections were detected in domestic dogs.

3.5. Discussion

Samples obtained from the OVAH were infected with a wide variety of pathogens. Our findings seem to correlate with a study on ixodid ticks collected from *B. canis* (*senso lato*) infected dogs presented at OVAH (Horak, 1995). A majority of *Babesia*-infected dogs (41%) were only infested with *H. elliptica* (previously regarded as synonymous with *H. leachi*) (Apanaskevich, Horak and Camica, 2007), the only known vector of *B. rossi* (Lewis, Penzhorn, Lopez-Rebollar and De Waal, 1996). An additional 35 % of dogs

were infested with *H. elliptica* as well as other ticks species. Based on the current understanding, the high incidence of *B. rossi* infections in our sampled dogs correlates with the high incidence of the tick-vector collected from dogs with *Babesia* infections presented at OVAH. In the study by Horak (1995), *Rhipicephalus sanguineus* ticks were collected from 15% of the dogs as pure infestations and a further 22% of dogs had mixed infestations including *R. sanguineus*. Since *R. sanguineus* is the known vector of *E. canis* (Groves, Dennis, Amyx and Huxsoll, 1975) and *B. vogeli* (Uilenberg et al., 1989), it came as no surprise that *E. canis* and *B. vogeli* were detected, albeit in less than 3% of our sampled dogs. *Rhipicephalus sanguineus* and *H. elliptica* have overlapping distributions and have been collected from the same host (Horak, 1995). Since these ticks transmit *B. vogeli* / *E. canis* and *B. rossi,* respectively, it follows that we were able to detect mixed infections of *B. rossi* and *E canis* in 8 dog samples and *B. vogeli* and *E. canis* in 7 dog samples. It remains unclear, however, why there were so few dogs co-infected with both *B. vogeli* and *B. rossi*. We detected only one sample co-infected with *B. rossi* and *B. vogeli* and we also detected only one sample co-infected with *B. rossi*, *B. vogeli* and *E. canis*. Of interest was the detection of a *Theileria* species from 3 samples collected at OVAH, as we are not aware of any *Theileria* species ever having been isolated from dogs in South Africa (Matjila et al., 2008).

Babesia rossi was the most common species detected from all sampled areas. *Haemaphysalis elliptica* is present in the eastern half and southern regions of South Africa, even in lowveld and other arid areas where the average rainfall might seem too low for the tick's survival (Norval, 1984). Since the areas sampled correlated with the

distribution of *H. elliptica*, it would be logical to associate parasite occurrence with the distribution of the tick vector. The samples collected from Mpumalanga were 97% *B. rossi* positive, as sampling in this area was biased to only *Babesia*-positive animals based on blood smear examination. Even though all the Mpumalanga samples were from dogs diagnosed with babesiosis, we could not amplify DNA from one sample. We also detected co-infections of *B. rossi* and *E. canis* in all our sampled areas except in the Free State and Eastern Cape. This could indicate that *H. elliptica* and *R. sanguineus* have overlapping distribution and also feed on the same hosts in those areas where *B. rossi* and *E. canis* occurred as co-infections. Currently, *B. vogeli* infections have only been detected in samples collected from Free State and OVAH (Matjila et al., 2004), even though *R. sanguineus* has a wider distribution in South Africa (Horak, 1995). This implies that *B. vogeli* infections are not as widely spread as *B. rossi* infections in South Africa. The same *Theileria* species isolated from 3 samples collected at OVAH was detected in 79 samples collected from Pietermaritzburg, KwaZulu-Natal. This species was also isolated in three samples co-infected with *E. canis*. The vector of this *Theileria* species is still unknown. Additionally, *Trypanosoma congolense* infection was detected from a sample collected at Hluhluwe, northern KwaZulu-Natal. A recent report indicated that *Trypanosoma* infections isolated from cattle and buffalos are prevalent in the Hluhluwe-iMfolozi area (Van Den Bossche, Esterhuizen, Nkuna, Matjila, Penzhorn, Geerts and Marcotty, 2006). To our knowledge this is the first report of a confirmed *T. congolense* infection from a dog originating from Hluhluwe.

Currently the two tick-transmitted *Ehrlichia* / *Anaplasma* species known to cause human disease are *E. chaffeensis* (Dumler, Chen, Asanovich, Trigiani, Popov and Walker, 1995; Paddock, Sumner, Shore, Bartley, Elie, McQuade, Martin, Goldsmith and Childs, 1997) and *Anaplasma phagocytophilum* (Wormser, Dattwyler, Shapiro, Halperin, Steere, Klempner, Krause, Bakken, Strle, Stanek, Bockenstedt, Fish, Dumler and Nadelman, 2006). Our RLB assay had probes that could detect and differentiate between the two zoonotic species (Table 3.2), but our results were negative. The reported detection of *E. chaffeensis* in dogs and in a human being from Bloemfontein (Pretorius et al., 1999), was based on serological assay and not on the detection of parasite DNA. Serological cross-reactivity between *Ehrlichia* infections is known to occur (Parola, Inokuma, Camicas, Brouqui and Raoult, 2001) which suggests that molecular assays should be used to support serological evidence. On the other hand, a species closely related to *A. phagocytophilum* has been identified from 3 dog samples in South Africa (Inokuma et al., 2005). A more directed study considering *Ehrlichia*-infected dogs only may elucidate the existence of potentially zoonotic *Ehrlichia* species.

3.6. Conclusion

Our current results indicate that the abundance of tick vectors on domestic dog hosts encourages the cyclical transmission of tick-borne pathogens in the country. Molecular diagnostic techniques allow for previously unknown species to be identified. There is no doubt that if the current momentum of research is maintained, various other important pathogens will be discovered which will in turn influence our understanding of the epidemiology, control and treatment of tick-borne pathogens of domestic dogs. These

findings also underpin the need for effective control measures to prevent transmission of tick-borne pathogens to domestic dogs in South Africa.

3.7. Figures and Tables

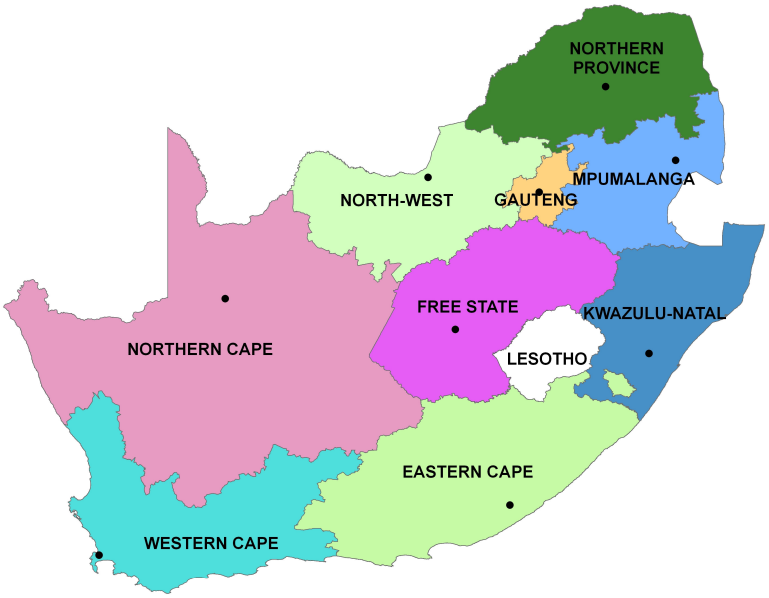


Figure 3.1: Map of South Africa, indicating provinces where blood samples were collected.

Table 3.2: The number (n=1138) of samples collected from dogs, by province and locality, in South Africa.

Localities	Gauteng and North West	Mpumalanga	Free State	KwaZulu-Natal	Eastern Cape	Western Cape
OVAH	527					
Johannesburg SPCA	90					
White River private clinic		38				
Bloemfontein SPCA			129			
Durban SPCA				56		
Durban private practice				4		
Pietermaritzburg private practice				192		
Hluhluwe private practice				1		
East London SPCA					54	
Cape Town private practice						47
Total numbers of samples	**617**	**38**	**129**	**253**	**54**	**47**

Table 3.2: List of organisms and their corresponding probe sequences used to detect pathogen DNA.

Anaplasma centrale	TCG AAC GGA CCA TAC GC
Anaplasma marginale	GAC CGT ATA CGC AGC TTG
Anaplasma ovis	ACC GTA CGC GCA GCT TG
Anaplasma phagocytophilum 1	TTG CTA TAA AGA ATA ATT AGT GG
Anaplasma phagocytophilum 3	TTG CTA TGA AGA ATA ATT AGT GG
Anaplasma phagocytophilum 5	TTG CTA TAA AGA ATA GTT AGT GG
Anaplasma phagocytophilum 7	TTG CTA TAG AGA ATA GTT AGT GG
Ehrlichia / Anaplasma catch-all	GGG GGA AAG ATT TAT CGC TA
Ehrlichia canis / Ehrlichia ovina	TCT GGC TAT AGG AAA TTG TTA
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG
Ehrlichia sp. (Omatjenne)	CGG ATT TTT ATC ATA GCT TGC
Hepatozoon catch-all	GCT TTG TAA TTG GAA TGA TAG A
Theileria / Babesia catch-all	TAA TGG TTA ATA GGA RCR GTT G
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG
Theileria annulata	CCT CTG GGG TCT GTG CA
Theileria bicornis	GCG TTG TGG CTT TTT TCT G
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT
Theileria catch-all	ATT AGA GTG CTC AAA GCA GGC
Theileria equi	TTC GTT GAC TGC GYT TGG
Theileria parva	GGA CGG AGT TCG CTT TG
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
Theileria sp. (duiker)	CAT TTT GGT TAT TGC ATT GTG G
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C
Theileria taurotragi	TCT TGG CAC GTG GCT TTT

Theileria velifera	CCT ATT CTC CTT TAC GAG T
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT
Babesia canis	TGC GTT GAC CGT TTG AC
Babesia canis 2	TGG TTG GTT ATT TCG TTT TCG
Babesia catch-all 1	ATT AGA GTG TTT CAA GCA GAC
Babesia catch-all 2	ACT AGA GTG TTT CAA ACA GGC
Babesia felis	TTA TGC GTT TTC CGA CTG GC
Babesia gibsoni Japan	TAC TTG CCT TGT CTG GTT T
Babesia gibsoni USA	CAT CCC TCT GGT TAA TTT G
Babesia microti	GRC TTG GCA TCW TCT GGA
Babesia ovis	TGC GCG CGG CCT TTG CGT T
Babesia rossi	CGG TTT GTT GCC TTT GTG
Babesia vogeli	AGC GTG TTC GAG TTT GCC

Table 3.3: Pathogen species detected from domestic dogs using the RLB.

Pathogen	OVAH* (n = 527)	Southern Gauteng (n = 90)	Mpumalanga (n = 38)	Free State (n = 129)	KwaZulu-Natal (n = 253)	Eastern Cape (n = 54)	Western Cape (n = 47)
B. rossi	345	2	36	7	16	12	2
B. vogeli	5	-	-	13	-	-	-
E. canis	12	-	-	1	1	-	-
Theileria sp. dog	3	-	-	-	79	-	-
B. gibsoni	-	1	-	-	-	-	-
B. rossi & *E. canis*	8	1	1	-	1	-	1
B. vogeli & *E. canis*	7	-	-	-	-	-	-
Theileria sp. dog & *E. canis*	-	-	-	-	3	-	-
B. rossi & *B. vogeli*	1	-	-	-	-	-	-
B. rossi & *B. vogeli* & *E. canis*	1	-	-	-	-	-	-
T. congolense	-	-	-	-	1	-	-
Total number of positives	**382 (72%)**	**4 (4%)**	**37 (97%)**	**21 (16%)**	**101 (40%)**	**12 (22%)**	**3 (6%)**

* Onderstepoort Veterinary Academic Hospital (Northern Gauteng / North West Province)

3. 8. References

Apanaskevich D.A., Horak, I.G., Camica J.-L., 2007. Redescription of *Haemaphysalis* (*Rhipistoma*) *elliptica* (Koch, 1844), an old taxon of the *Haemaphysalis* (*Rhipistoma*) *leachi* group from East and Southern Africa, and of *Haemaphysalis* (*Rhipistoma*) *leachi* (Audouin, 1826) (Ixodida, Ixodidae). Onderstepoort Journal of Veterinary Research 74, 181-208.

Bekker, C.P.J., de Vos, S., Taoufik, A., Sparagano, O.A.E., Jongejan, F., 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. Veterinary Microbiology 89, 223-238.

Collett, M.G., 2000. Survey of canine babesiosis in South Africa. Journal of the South African Veterinary Association 71, 180-186.

Delespaux, V., Ayral, F., Geysen, D., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification: applicability for the diagnosis of mixed infections with different trypanosome species in cattle. Veterinary Parasitology 117, 185-193.

Dumler, J.S., Chen, S.M., Asanovich, K., Trigiani, E., Popov, V.L., Walker, D.H., 1995. Isolation and characterization of a new strain of *Ehrlichia chaffeensis* from a patient with nearly fatal monocytic ehrlichiosis. Journal of Clinical Microbiology 33, 1704-1711.

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.

Groves, M.G., Dennis, G.L., Amyx, H.L., Huxsoll, D.L., 1975. Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). American Journal of Veterinary Research 36, 937-940.

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.

Horak, I.G., 1995. Ixodid ticks collected at the Faculty of Veterinary Science, Onderstepoort, from dogs diagnosed with *Babesia canis* infection. Journal of the South African Veterinary Association 66, 170-171.

Inokuma, H., Oyamada, M., Kelly, P.J., Jacobson, L.A., Fournier, P.E., Itamoto, K., Okuda, M., Brouqui, P., 2005. Molecular detection of a new *Anaplasma* species closely related to *Anaplasma phagocytophilum* in canine blood from South Africa. Journal of Clinical Microbiology 43, 2934-2937.

Kjemtrup, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad, P.A., 2000. There are at least three genetically distinct small piroplasms from dogs. International Journal of Parasitology 30, 1501-1505.

Kjemtrup, A.M., Wainwright, K., Miller, M., Penzhorn, B.L., Carreno, R.A., 2006. *Babesia conradae*, sp. nov., a small canine *Babesia* identified in California. Veterinary Parasitology 138, 103-111.

Kuttler, K.L., 1988. Worldwide impact of babesiosis. In: Ristic, M. (Ed.), Babesiosis of domestic animals and man. CRC Press, Baco Raton, Florida, pp. 1-2.

Lewis, B.D., Penzhorn, B.L., Lopez-Rebollar, L.M., De Waal, D.T., 1996. Isolation of a South African vector-specific strain of *Babesia canis*. Veterinary Parasitology 63, 9-16.

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, 123-127.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P., Nijhof, A.M., Jongejan, F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Veterinary Parasitology 122, 119-125.

Matjila, P.T., Penzhorn, B.L., Leisewitz, A.L., Bhoora, R., Barker, R., 2007. Molecular characterization of *Babesia gibsoni* infection from a pit-bull terrier pup recently imported into South Africa. Journal of the South African Veterinary Association 78, 2-5.

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., 1984. The ticks of Zimbabwe. IX. *Haemaphysalis leachi* and *Haemaphysalis spinulosa*. Zimbabwe Veterinary Journal 15, 9-17.

Paddock, C.D., Sumner, J.W., Shore, G.M., Bartley, D.C., Elie, R.C., McQuade, J.G., Martin, C.R., Goldsmith, C.S., Childs, J.E., 1997. Isolation and characterization of *Ehrlichia chaffeensis* strains from patients with fatal ehrlichiosis. Journal of Clinical Microbiology 35, 2496-2502.

Parola, P., Inokuma, H., Camicas, J.L., Brouqui, P., Raoult, D., 2001. Detection and identification of spotted fever group Rickettsiae and Ehrlichiae in African ticks. Emerging Infectious Diseases 7, 1014-1017.

Pretorius, A.M., Kelly, P.J., 1998. Serological survey for antibodies reactive with *Ehrlichia canis* and *E. chaffeensis* in dogs from the Bloemfontein area, South Africa. Journal of the South African Veterinary Association 69, 126-128.

Pretorius, A.M., Venter, T.P., Ryst, E.V.D., Kelly, P.J., 1999. Possible human ehrlichiosis in the Free State. South African Medical Journal 89, 961-961.

Rautenbach, G.H., Boomker, J., Villiers, I.L., 1991. A descriptive study of the canine population in a rural town in Southern Africa. Journal of the South African Veterinary Association 62, 158-162.

Uilenberg, G., Franssen, F.F., Perie, N.M., Spanjer, A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Veterinary Quarterly 11, 33-40.

Van Den Bossche, P., Esterhuizen, J., Nkuna, R., Matjila, T., Penzhorn, B., Geerts, S., Marcotty, T., 2006. An update of the bovine trypanosomosis situation at the edge of Hluhluwe-iMfolozi Park, KwaZulu-Natal Province, South Africa. Onderstepoort. Journal of Veterinary Research 73, 77-79.

Van Heerden, J., 1982. A retrospective study on 120 natural cases of canine ehrlichiosis. Journal of the South African Veterinary Association 53, 17-22.

Wormser, G.P., Dattwyler, R.J., Shapiro, E.D., Halperin, J.J., Steere, A.C., Klempner, M.S., Krause, P.J., Bakken, J.S., Strle, F., Stanek, G., Bockenstedt, L., Fish, D., Dumler, J.S., Nadelman, R.B., 2006. The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis:

clinical practice guidelines by the Infectious Diseases Society of America. Clinical Infectious Diseases 43, 1089-1134.

Zahler, M., Rinder, H., Schein, E., Gothe, R., 2000. Detection of a new pathogenic *Babesia microti*-like species in dogs. Veterinary Parasitology 89, 241-248.

Zahler, M., Schein, E., Rinder, H., Gothe, R., 1998. Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. Parasitology Research 84, 544-548.

Chapter 4: Preliminary evaluation of the *BrEMA*1 gene as a tool for correlating *Babesia rossi* genotypes and clinical manifestation of canine babesiosis.

4.1. Abstract

Babesia rossi, an intra-erythrocytic protozoan, causes a severe, often life-threatening, disease of domestic dogs. Dogs treated early for *B. rossi* infection usually recover from the disease, but dogs left untreated or treated at a later stage of infection seldom survive. Dogs infected with *B. rossi* have varied clinical manifestations that can be categorised as uncomplicated (with a good prognosis) or complicated (with a poor prognosis). One hundred and twenty one (121) blood samples were collected from dogs presented to the Onderstepoort Veterinary Academic Hospital (OVAH) and diagnosed with babesiosis on thin blood smear. An additional twenty (20) samples were obtained from private clinics around the Onderstepoort, Johannesburg, Durban, White River and Cape Town areas. The samples were screened by PCR targeting the *Babesia rossi* Erythrocyte Membrane Antigen (*BrEMA*1) gene and sequencing of the polymorphic region (i.e. a variable number of hexapeptide repeats). Analysis of PCR products revealed 11 different gene profiles, visualised by gel electrophoresis. Twelve distinct *BrEMA*1 genotypes were identified by sequencing, however, of which numbers of hexapeptide repeats varied from 6 to 31 (classified as genotype6 to genotype31). The genotypes were retrospectively compared to the clinical case data. The most frequently encountered *B. rossi* parasites were those attributed to genotype19 (36.2%), genotype28 and 29 (20.6% each) and genotype11 (12.7%). These genotypes were also the only ones associated with poorest prognosis. This

preliminary finding suggests clinically important differences between the various *B. rossi* genotypes identified.

4.2. Introduction

The large *Babesia* parasites, *Babesia rossi* and *Babesia vogeli,* are two of the most frequently encountered blood parasites of dogs in South Africa (Böhm, Leisewitz, Thompson and Schoeman, 2006; Matjila, Leisewitz, Jongejan and Penzhorn, 2008; Matjila, Penzhorn, Bekker, Nijhof and Jongejan, 2004). *Babesia rossi* is the most frequently encountered species detected in dogs presented with clinical babesiosis at the Outpatients Clinic of the Onderstepoort Veterinary Academic Hospital (OVAH). *Babesia vogeli* infections are reported to be rare and less virulent than *B. rossi* infections (Böhm et al., 2006; Uilenberg, Franssen, Perie and Spanjer, 1989). Canine babesiosis caused by *B. rossi* is clinically classified as either being uncomplicated or complicated. Clinical hallmarks of *Babesia* infections in dogs always include fever and splenomegaly. The disease is said to be uncomplicated if the clinical changes could be attributed directly to a mild or moderate anaemia with no clinical evidence of organ dysfunction or failure (Jacobson and Clark, 1994). Dogs with mild anaemia are usually treated with an antibabesial drug and discharged. Dogs with moderate anaemia may receive a blood transfusion and are then discharged. The survival rate in this manifestation of disease is almost 100% (Jacobson, 2006). Examples of complicated cases of the disease are those where clinical presentation is complicated by evidence of non-solid organ failure (non-solid organ complication [SOC]) characterized by severe anaemia and haemoconcentration or organ dysfunction or failure (solid-organ complications, [SOC]). Examples of complicated disease include acute renal failure (ARF), acute respiratory distress syndrome (ARDS), cerebral

involvement, coagulopathy, icterus and hepatopathy, immune-mediated haemolytic anaemia, peracute manifestations, haemoconcentration, pancreatitis, rhabdomyolysis and shock (Jacobson and Lobetti, 1996; Jacobson, 2006). These dogs all require admission to an intensive-care facility where advanced medical treatment is essential. The mortality rate in this group is around 15%, irrespective of the nature of the treatment administered (Shakespeare, 1995).

The mechanisms that result in *B. rossi* parasites being associated with such a range of diverse clinical signs and severe disease in the host are still unknown. As has been suggested for *B. canis* (*senso stricto*), there might be genotypic differences among *B. rossi* strains (Bourdoiseau, 2006) that could be associated with (and / or explain) variable virulence. A polymorphic phosphoprotein localised on the cytoplasmic surface of *B. rossi*-infected red blood cell has recently been characterised and named *Babesia rossi* Erythrocyte Membrane Antigen 1 (*BrEMA*1) (B. Carcy, personal communication). Analysis of the *BrEMA*1 genes of various laboratory strains of *B. rossi* revealed that these code for polymorphic proteins that contain variable numbers of repetitive hexapeptide motifs. We used this gene as a genetic marker to classify *B. rossi* isolates obtained from South Africa and analysed the relationship between particular genotypes and the occurrence and severity of clinical manifestations of *B. rossi*-induced canine babesiosis. The molecular and the biochemical characterization of the *BrEMA*1 will be reported elsewhere by B. Carcy, University of Montpellier.

4.3. Materials and Methods

4.3.1. Sample origin and grouping

Canine blood samples (n=121) were obtained from the Outpatients Clinic of the Onderstepoort Veterinary Academic Hospital (OVAH). These dogs were diagnosed with canine babesiosis based on clinical signs and microscopic confirmation of infected red blood cells on a blood smear. Blood samples were collected prior to treatment, into EDTA tubes from the cephalic vein. If the presence of *B. rossi* was confirmed in our laboratory, clinical data of a specific dog were retrieved from patient files. A further 20 blood samples were obtained from geographically dispersed private clinics (Onderstepoort area, Johannesburg, Durban, White River and Cape Town) from dogs diagnosed with canine babesiosis and sent to our laboratory for molecular characterisation of the infection. Dogs sampled from the non-OVAH sites were not included in the clinical part of the study. These samples were used only for establishing parasite genotypes. Dogs sampled at OVAH were grouped according to their clinical presentation and outcome as follows: Group H (=Home): Dogs that were treated with an antibabesial drug during consultation and discharged immediately; Group A (=Admitted): Dogs admitted for treatment and that survived until discharged; Group D (=Died): Dogs that died despite treatment or that were euthanised owing to poor prognosis (Böhm et al., 2006). Follow-up examinations were conducted on dogs in Group H that were sent home, to confirm that these dogs had indeed recovered completely. Canine babesiosis cases were diagnosed and treated in a similar manner by all clinicians at the OVAH. The diagnostic criteria used for the diagnoses of the various complications include: In-saline positive agglutination (ISA) for immune-mediated haemolytic anaemia; respiratory distress as evidenced by hyperpnoea or blood-gas evidence of lung

oedema for the diagnosis of ARDS; a rise in haematocrit above 50% or fall below 10% (identifying haemoconcentration and severe anaemia, respectively); the development of any central nervous system signs that could not be attributed to any other cause (especially hypoglycaemia) for the diagnosis of cerebral disease; clinical collapse for the diagnosis of shock; the rise of blood-creatinine levels above 150 µmol/L or anuria for the diagnosis of ARF and hypoglycaemia (glucose < 3.3 mmol/L). Based on the above criteria, dogs that were admitted with complications were then separated into two groups, i.e. those with solid-organ complication (SOC) and those without SOC. Finally, dogs from both groups were separated into dogs that survived until discharged and dogs that died despite treatment or that were euthanized owing to poor prognosis.

Two additional samples that were known positives of *B. canis* and *B. vogeli* were obtained from The Netherlands and Bloemfontein, respectively, for the purpose of evaluating whether *BrEMA*1 could be detected in these species.

4.3.2. DNA extraction and PCR

Once collected, the blood samples were sent to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. DNA was extracted from 200 µl of each blood sample using the QIAmp® blood and tissue extraction kit (Qiagen, Hilden, Germany), following the manufacturer's protocols. As a first step for the molecular diagnosis of *Babesia* species infecting each dog, a PCR was performed with Reverse Line Blot (RLB) primers 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′). The conditions for the PCR included an initial step of 3 min at

37º C, 10 min at 94ºC, 10 cycles of 94ºC (20s)- 67 ºC (30s)- 72º C (30s), with lowering of annealing step after every second cycle by 2º C (touchdown PCR). The reaction was then followed by 40 cycles of denaturation at 94º C for 30s, annealing at 57º C for 30s and extension at 72º C for 30s. The PCR-amplified products, a 460-540bp fragment from the 18S rRNA gene spanning the V4 region (Gubbels, de Vos, van der Weide, Viseras, Schouls, de Vries and Jongejan, 1999; Matjila et al., 2004) were tested with the RLB, as previously described (Matjila et al., 2004).

The *B. rossi* genetic diversity was then analysed on genomic DNA from samples that tested positive for *B. rossi* on the RLB. These samples were re-amplified with primers Frep*BrEMA*1 (5'-CCA ACA TTG ATG ATG ACA A-3') and Rrep*BrEMA*1 (5'-CTG CAT GTC AGC TTC ATC A-3'). These primers were designed from the *B. rossi BrEMA*1 gene (Accession number AJ416994). They amplified an 18-nucleotide repetitive sequence whose number (from 16 to 31, i.e., size fragment from 375 to 645bp) and sequence were initially shown to be variable between *BrEMA*1 genes from 3 laboratory strains (B. Carcy, personal communication). To test whether the *BrEMA*1 gene was unique to *B. rossi* isolates, two additional samples positive for *B. vogeli* and *B. canis* were included. PCR amplification was done with 2.5 µl of extracted DNA in a final 25 µl of PCR reaction containing 0.5 µl of each primer (10 µM), 4 µl of dNTP mix (1.25mM each), 2.5 µl of 10X PCR buffer and Taq DNA polymerase (1U). The conditions of the PCR included in the program were 1 cycle of 5 min at 94°C, 30 cycles of amplification (94°C 30s, 55°C 30s and 72°C 1 min) and 1 cycle of 7 min at 72°C.

4.3.3. Sequencing, phylogenetic and statistical analysis

PCR products (5µl) were loaded and visualised on a 1.5% agarose gel. Samples with distinct bands were purified with a QIAquick PCR purification kit (Qiagen, Germany). The sequencing reaction was prepared with 3.2 pmol of the primer Frep*BrEMA*1 and Rrep*BrEMA*1. Sequencing was performed in the Genetics Section of the Faculty of Veterinary Science. Sequence data for the full *BrEMA*1 sequences were assembled using the GAP 4 of the Staden package (Version 1.6.0 for Windows). Sequence alignments were manually edited using Bioedit (version 5.0.9). DNA sequences were translated into amino acid sequences and genotyping was done according to the number of hexapeptide repeats. Sequence alignment of the amino acid sequences and phylogenetic trees were generated using the website: www.genebee.msu.su/services/malign_reduce.html. The Cluster and Topological algorithm methods were used for the construction of phylogenetic trees (Phylip, multiline) into the phylogram format from the alignment sequences. The *B. rossi BrAK* (adenosine kinase) sequence (Carret, Walas Carcy, Grande, Precigout, Moubri, Schetters and Gorenflot, 1999) was used as an outgroup. Genotype frequencies were compared using a two-tailed binomial test. Proportions were compared using a two-tailed Fisher's exact test. Analyses were done using Stata 8.2 (StataCorp, College Station, TX, U.S.A.).

4.3.4. Nucleotide sequence accession numbers

The *BrEMA*1 gene nucleotide sequences (designated genotype6, 11, 12, 13, 16g1, 16g2, 18, 19g1, 19g2, 22, 28, 29 and 31) have been submitted to EMBL, GenBank and DDBJ Nucleotide Sequence databases and can be retrieved under the accession numbers FM164393 to FM164405 respectively.

4.4. Results

4.4.1. Genetic analysis B. rossi isolates

All the blood samples (n=141) were positive for *B. rossi* on the PCR/RLB assay. These samples were further analysed to determine the genetic heterogeneity of the *BrEMA*1 gene.

4.4.1.1. Diversity of BrEMA1 genotypes

PCR amplification of the polymorphic region of the *BrEMA*1 gene revealed a total of 11 distinct profiles by examining the size of the amplicons which could be visualised on gel electrophoresis. The profile of the amplicons seemed to correlate to a specific genotype. The smallest amplified product was 200bp (lane 8) and the largest *ca* 645bp (lane 15&17) (Fig. 4.1). Sequencing results of the amplicons showed 13 different consensus sequences that ranged in size between 6 and 31 hexapedtide repeats, which in fact reflected 12 distinct genotypes (Table 4.1). Samples with a repetitive region composed of 16 or 19 hexapeptide repeats, respectively, were subdivided into two groups (designated g1 or g2). Genotype16g1 and genotype16g2 corresponded with two distinct sequences, indicating that they are genetically distant. In contrast, genotype19g1 and genotype19g2 differed by a single conservative amino acid mutation, A or V, in the hexapeptide KS (A/V) ASV. Finally, sequence analysis of genotype11 and genotype16g2 indicated that they might be genetically related. This is based on the fact that they are the only genotypes containing the motif ASPGSV and 2 amino acid substitutions (IP rather than VL) downstream from the repetitive sequence of *BrEMA*1 (Table 4.1).

The two positive *B. vogeli* and *B. canis* samples could not be amplified with the *BrEMA*1 primers and were therefore negative for the *BrEMA*1 gene.

4.4.1.2. Phylogenetic relationship of BrEMA1 sequences

The low bootstrap values obtained at certain nodes, especially those <50, strongly suggest that the phylogenetic analysis of *BrEMA*1 genotypes presented in our study is incomplete (Fig. 4.2). Based on the 13 *BrEMA*1 genotype sequences available to date, it follows that the genotypes could belong to 6 distinct monophyletic groups: (i) group A which includes genotype6; (ii) group B which includes genotype11; (iii) group C which includes genotype12, genotype13 and genotype16g1; (iv) group D which includes geno16g2; (v) group E which includes genotype18, genotype19 (i.e., 19g1 and 19g2) and genotype22; and lastly (vi) group F which includes genotype28, genotype29 and genotype31 (Fig. 4.2).

4.4.1.3. Occurrence of BrEMA1 genotypes among all samples

So far, the total analysis of the 141 blood samples revealed 12 different *BrEMA*1 genotypes that clustered in 6 monophyletic groups (see above). The most abundant were genotype19 (36%, i.e., 22% genotype19g1 and 14% genotype19g2); genotypes 28 and 29 (both at *ca* 20%) and genotype11 (12.8%) (Fig. 4.3). The remaining genotypes represented 10% of the cases. Genotype16g2 represented 3.5% of cases, whereas each of the remaining genotypes represented less than 1.5% of the cases (data not shown).

4.4.2. Occurrence of BrEMA1 genotypes among OVAH samples

Results revealed that there were 4 frequently occurring genotypes: genotype19 represented *ca* 40% of cases (i.e., 25% genotype19g1 and 15% genotype19g2); genotype28 and 29 both represented *ca* 22% and 23%, respectively, and genotype11 represented *ca* 12% (Fig. 4. 4a). The other genotypes occurred less frequently, representing only 3.3% of the cases. Using the two-tailed binomial test, genotype19 was more frequent than genotype11 ($P<0.001$), genotype28 ($P=0.02$), genotype29 ($P=0.03$) and the remaining genotypes ($P<0.001$).

4.4.2.1. Analysis of BrEMA1 genotype and clinical parameters

The analysis of the association of *BrEMA*1 genotypes with clinical parameters was performed only on the dogs that were seen at the OVAH and followed up (n=121). Of these, 86 dogs (71%) were admitted with complications (non-SOC or SOC; Table 4.2). The remaining 35 dogs (29%) were treated and sent home immediately after consultation. There were no significant differences in the *BrEMA*1 frequencies of admitted dogs versus dogs that were sent home immediately after consultation ($P=0.48$, Fisher's exact test). The percentage of dogs diagnosed with SOC was 24% and the mortality rate was estimated at 16.5%.

4.4.2.2. Occurrence of BrEMA1 genotypes in complicated cases

The distribution of the various *B. rossi* genotypes in the complicated cases (n=86) was compared to that in the entire group (Fig. 4.4b; linear regression, $R^2=0.99$, $p<0.05$). There was no specific association of particular *BrEMA*1 genotype with complicated disease.

4.4.2.3. Occurrence of BrEMA1 genotypes in SOC cases

Regarding the distribution of the various *BrEMA*1 genotypes, significant differences (*P=0.01*) were found using Fisher's exact test, between dogs with non-SOC (n=57) and SOC (n=29) amongst admitted dogs. *Babesia rossi* genotypes 19, 28, 29 and 11 were all associated with SOC (29 dogs; Table 4.2). *Babesia rossi* genotype19 represented 62% of SOC cases (38% genotype19g1 and 24% genotype19g2). *Babesia rossi* genotypes 28 and 29 were associated with *ca* 30% of SOC cases (20% and 7% respectively) and genotype11 with 10% (Fig. 4.4c). In the SOC group, significantly more dogs were infected with genotype19 than with the other genotypes combined (*P=0.02*, Fisher's exact test).

4.4.2.4. Occurrence of BrEMA1 genotypes in fatal cases

The number of dogs infected with genotype19 that died of complications (n=13) was significantly higher than the number of fatal cases (n=7) of dogs infected with the remaining genotypes (Fisher's exact test; p=*0.01*) (Fig. 4.4d). The distribution of *B. rossi* genotypes in the fatal cases (n=20) approximated to the distribution of *B. rossi* genotypes in the SOC group (n=29) (Fig. 4.5). This suggested an association between SOC and fatal outcome. This correlation was statistically significant (linear regression, R^2=0.95; p<0.05).

4.4.2.5. Correlation between BrEMA1 genotypes and clinical signs

Frequency of clinical signs and associated genotypes were analysed among the 29 dogs diagnosed with SOC and among the 19 dogs that died from SOC-induced death (Table 4.3). Genotypes 19 and 28 were associated with most of the clinical signs. The two most frequently encountered clinical signs, ARDS and shock, were found

preferentially associated with genotype19 (Table 4.3). Genotype19 was associated with >70% (11/16 amongst SOC and 8/10 amongst fatal cases) of ARDS cases (against *ca* 10% for genotype28) and with >60% (6/9 amongst SOC and 5/8 amongst fatal cases) of shock cases (against *ca* 20% for genotype28).

4.5. Discussion

It should be noted that a relative large number of disease phenotypes and parasite genotypes were studied in a relative small population. This means that associations between disease phenotype and parasite genotype, although interesting, requires a much larger prospective study to elucidate them further.

This study identified the existence of 12 *B. rossi* genotypes, the most common being genotype19. This genotype was also associated with high numbers of dogs that were admitted at OVAH suffering form *B. rossi*-induced canine babesiosis. Furthermore, our data have shown that *B. rossi* genotype19 is associated with most of the severe clinical signs diagnosed among SOC and fatal cases. Although not unique to genotype19, ARDS was the most common clinical sign associated with genotype19. Since genotype19 appears to be more virulent, this might suggest a correlation between this genotype and severe clinical signs in admitted cases of canine babesiosis.

Statistical analysis appears to suggest that SOC can be a precursor to fatal outcome. In agreement to published data (Shakespeare, 1995), we estimated that once a dog is *B. rossi* infected, its chance to develop SOC would be *ca* 24% with a 16.5% chance of dying from these complications.

Phylogenetic analysis of the *BrEMA*1 sequences identified 6 monophyletic groups. The low bootstrap values (<50) obtained at certain nodes of the *BrEMA*1 phylogenetic tree imply that the diversity of *B. rossi* in the field is higher than described in this report. These data, however, indicate that genotypes19, 28, 29 and 11 are the most prevalent amongst the 12 identified genotypes. These 4 genotypes belong to 3 monophyletic groups (i.e. genotype19, genotype 28/29 and genotype11), in agreement with their separation into 3 distinct groups of genotypes on the basis of their prevalence and virulence. In comparison, genotype19 appears highly prevalent and virulent, genotypes28/29 appear moderate in prevalence and virulence, whereas the prevalence and virulence of genotype11 appears low.

Our analysis also indicates that the *BrEMA*1 gene might be a good genetic marker for investigating *B. rossi* virulent genotypes in endemic areas, especially since the less pathogenic species, *B. canis* and *B. vogeli,* were found not to have the *BrEMA*1 gene. This finding is likened to related studies through which specific genes have been found to be unique to virulent protozoal species. For example, the virulence of *Plasmodium falciparum* and *Babesia bovis* is influenced by their ability to adhere to capillary endothelium, a phenomenon known as sequestration (Allred and Al-Khedery, 2004; Miller, Baruch, Marsh and Doumbo, 2002; O'Connor, Long and Allred, 1999). Sequestration has been linked to proteins expressed on the surface of infected red blood cells of both *P. falciparum* and *B. bovis*, which seem to alter the adhesive properties of these cells (Cooke, Mohandas and Coppel, 2001; O'Connor et al., 1999). Preliminary evidence has suggested that the expression of parasite-derived antigens (with adhesive properties) on the extra-cellular surface of *B. rossi*-infected red blood cells may be responsible for their ability to sequester *in vivo* and to

aggregate *in vitro* (Schetters and Eling, 1999). This has not been proven, however. Furthermore, there is no evidence related to the involvement of the *BrEMA*1 in sequestration. It has been demonstrated, though, that both adhesive and mechanical properties of *P. falciparum* or *B. bovis*-infected red blood cells play a pivotal role in their ability to sequester and consequently in the virulence of these parasites (Cooke et al., 2001; Hutchings, Li, Fernandez, Fletcher, Jackson, Molloy, Jorgensen, Lim and Cooke, 2007). If virulence is indeed directly related to cytoadhesion, as suggested in the *B. bovis* model (Allred and Al-Khedery, 2004; Allred and Al-Khedery, 2006), then more work should be undertaken to correlate the relationship between the virulent *BrEMA*1 genotypes and cytoadhesion or to identify auxiliary genes that may play a role in this phenomenon.

4.6. Conclusion

The significant suggestion of this work is that different parasite genotypes may cause differing host responses to infection (i.e. there could be a relationship between parasite genotypes and disease pathogenesis). Changing disease outcome through treatment depends heavily on understanding of disease pathogenesis. In this sense, this preliminary data may eventually prove clinically significant.

4.7. Figures and Tables

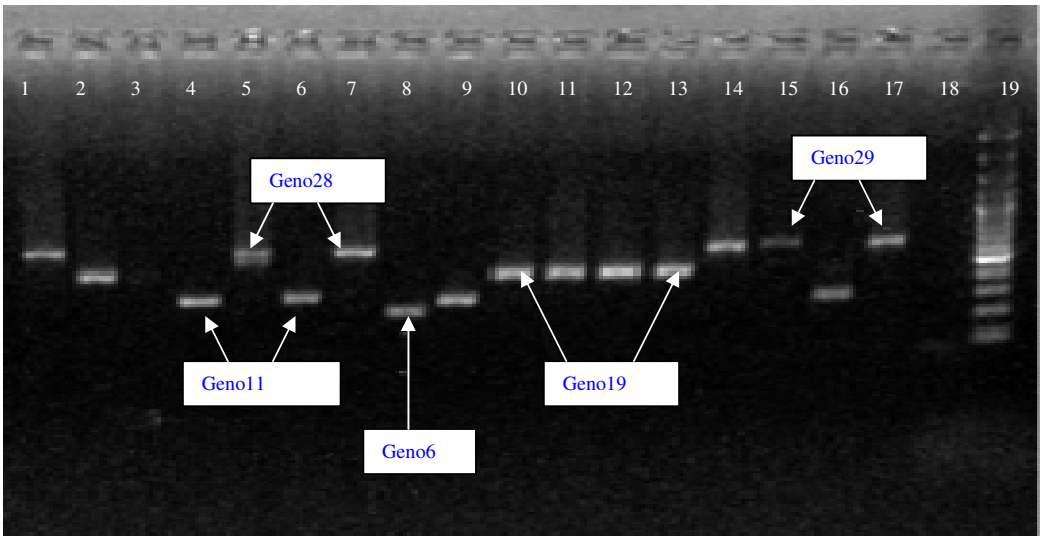


Figure 4.1**:** Field polymorphism of *B. rossi* strains in South Africa evidenced by the PCR amplification of *BrEMA*1 repeats region with primers Frep*BrEMA*1 / Rrep*BrEMA*1. PCR products were loaded on a 1.5% agarose gel. PCR profiles of the most encountered genotypes (11, 19, 28&29) and that of genotype6 are indicated on the picture as follows: lanes 15 and 17 represent genotype29; lanes 1, 5, 7 and 14 represents genotype28; lanes 2, 10, 11, 12 and 13 represents genotype19; lanes 4, 6, 9 and 16 represents genotype11; lane 8 represents genotype6 and lane 19 is the 100bp marker.

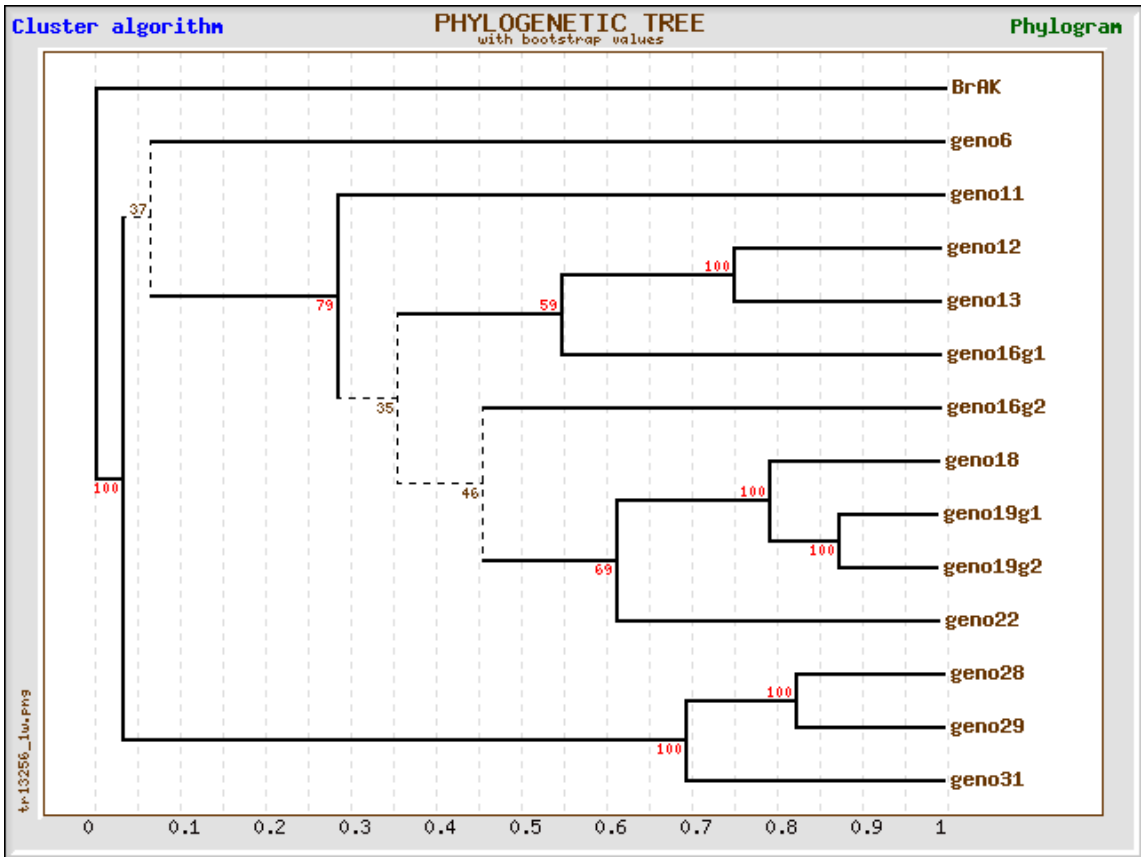


Figure 4.2: Cluster algorithm tree, showing the phylogenetic relationship between various genotypes based on the *BrEMA*1 gene sequences. *BrAK* (Adenosine kinase from *B. rossi;*AJ223322) was used as an outgroup.

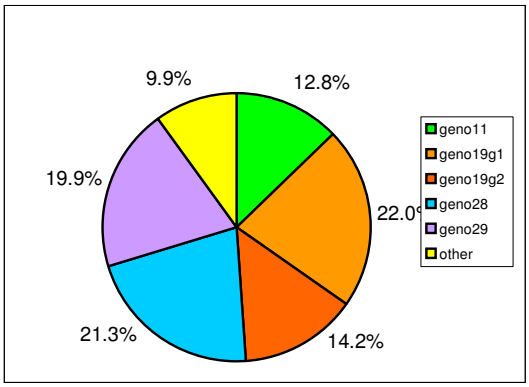
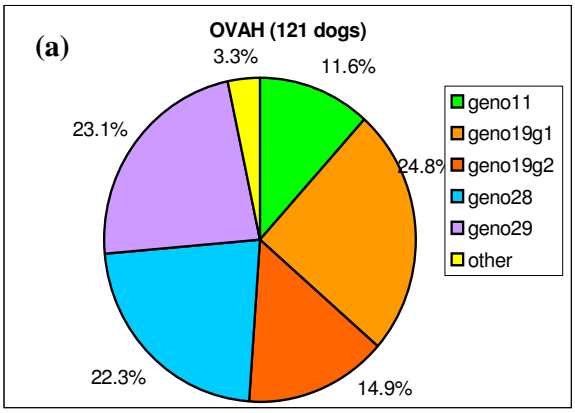
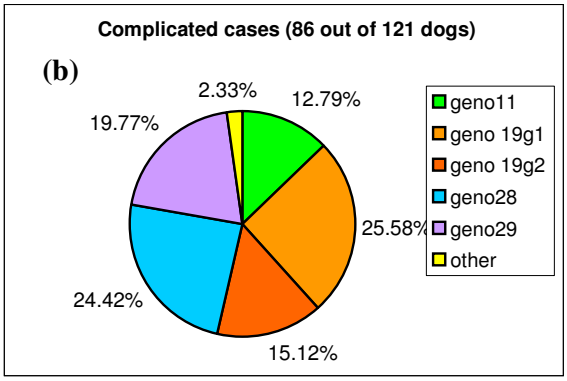
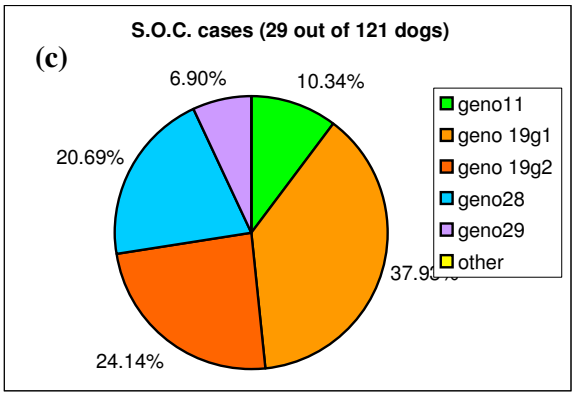


Figure 4.3: Occurrence of *B. rossi BrEMA*1 genotypes among all samples (141 dogs).







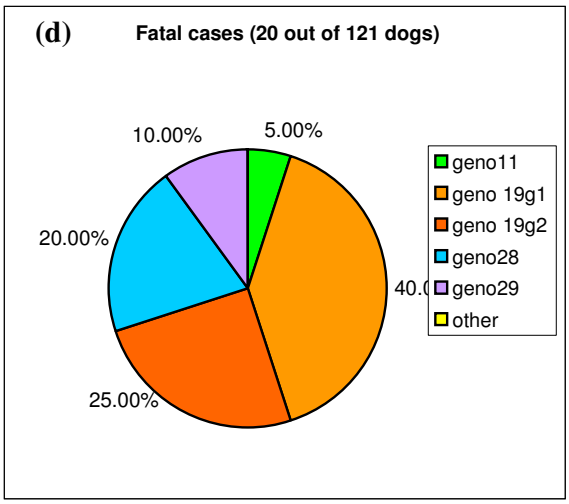


Figure 4.4: Occurrence of *B. rossi BrEMA*1 genotypes among (a) total (b) complicated (c) Solid organ complications (S.O.C). and (d) fatal cases.

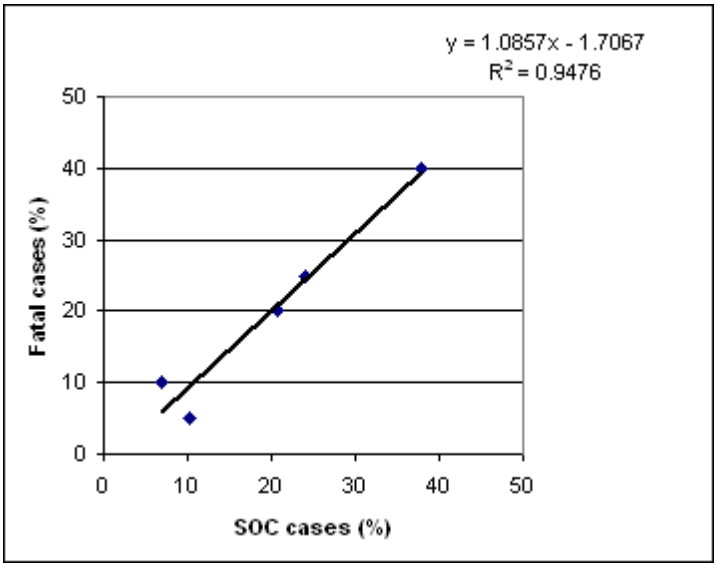


Figure 4.5: Relationship between occurrence of fatalities (n=20) and occurrence of SOC-cases (n=29).

Table 4.1: Consensus amino acid sequence of the 13 *BrEMA*1 genotypes**.** Sequencing of the repetitive region of the gene was performed on 141 dogs diagnosed with *B. rossi* infections.

Genotype		**sample origin**	**Consensus amino acid sequence**
Geno6		OVAH (1)	NIDDDKASV KSADSL KSAGSA KSVASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ 64
Geno11		OVAH (14) Ondersterpoort (4)	NIDDDKASV KSAGSV [RSADSV ASPGSV KSAASV]**2** RSADSV ESVASV RSADAD**IP**HDTHLDEADMQ 94
Geno12		OVAH (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSVASV [RSADSV ESAGSA KSVASV]**2** RSADADVLHDTHLDEADMQ 100
Geno13		Onderstepoort (1)	NIDDDKASV KSAASL KSADSA KSVASV RSADSV ESAGSA KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSAASV RSADADVLHDTHLDEADMQ 106
Geno16	Geno16g1 (**Repeated region from sequence AJ416994**)	Johannesburg (1) OVAH (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAASV KSAASV RSADSV ESAASA KSAASV [RSADSV ESAGSA KSVASV]**2** RSADADVLHDTHLDEADMQ 124
	Geno16g2	Durban (1) White River (4)	NIDDDKASV KSAGSV [KSAASV RSADSV ASPGSV]**4** KSAASV RSADSV ESVASV RSADAD**IP**HDTHLDEADMQ 124
Geno18		OVAH (1) Onderstepoort (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA [KSVASV RSADSV ESAASA]**3** KSVASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ 136
Geno19	Geno19g1 (**Repeated region from sequence AJ416997**)	OVAH (30) Onderstepoort (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KS**A***ASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ 142
	Geno19g2	OVAH (18) Onderstepoort (1) White River (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KS**V***ASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ 142
Geno22		Onderstepoort (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAASV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSAASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ 160
Geno28		OVAH (27) Onderstepoort (3)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA [KSVASV RSADSV ESAGSA]**5** KSVASV RSADADVLHDTHLDEADMQ 196

Geno29	OVAH (28)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA [KSVASV RSADSV ESAGSA]**5** KSVASV RSADADVLHDTHLDEADMQ 202
Geno31 **(Repeated region from sequence AJ416996)**	Cape Town (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV [KSAASV RSADSV ESAGSA]**2** [KSVASV RSADSV ESAGSA]**6** KSVASV RSADADVLHDTHLDEADMQ 214

( ): number of samples sequenced
*Differences in a single conservative amino acid mutation

Table 4.2: Frequency of *B. rossi BrEMA*1 genotype identified from blood samples and clinical outcomes of dogs presented at OVAH.

	FOLLOW-UP(OVAH)						
	Complicated cases (admitted)				Home	Total	
	S.O.C.		Non S.O.C.				
	Died	Survived	Died	Survived			
geno11	1	2		8	3	14	14
geno19g1 (AJ416997)	8	3		11	8	30	48
geno19g2	5	2		6	5	18	
geno28	4	2		15	6	27	55
geno29	1	1	1	14	11	28	
geno6					1	1	4
geno12				1		1	
geno13						0	
geno18				1		1	
geno16g1 (AJ416994)					1	1	
geno16g2						0	
geno22						0	
geno31 (AJ416996)						0	
Total	*19*	*10*	*1*	*56*	*35*	*121*	

S.O.C.: Solid-Organ Complication

Table 4.3: Clinical signs from complicated cases with solid-organ complications and their associated *BrEMA*1 genotypes.

Sample No	Genotype	Outcome	Complication
ES15	11	S	ARDS
BC214(*)	11	S	ARDS
ES80	19g1	S	ARDS
ES90	19g1	S	ARDS
ES46	19g2	S	ARDS
BC232(*)	19g1	D	ARDS
BC289	19g1	D	ARDS
BC298(*)	19g1	D	ARDS
ES78	19g1	D	ARDS
BC238	19g1	D	ARDS
BC228 (*)	19g1	D	ARDS
BC265	19g2	D	ARDS
ES53	19g2	D	ARDS
ES1	28	S	ARDS
BC302 (*)(h)	28	D	ARDS
BC303(*)	29	D	ARDS
BC232(*)	19g1	D	Shock
BC298(*)	19g1	D	Shock
BC228(*)	19g1	D	Shock
ES10	19g2	D	Shock
ES76	19g2	D	Shock
BC138	19g2	S	Shock
BC302 (*)(h)	28	D	Shock
BC251 (*)(h)	28	D	Shock
BC303(*)	29	D	Shock
ES19	11	D	ARF
ES84 (*)	19g1	D	ARF
BC288 (h)	28	S	ARF
BC251 (*)(h)	28	D	ARF
BC303 (*)	29	D	ARF
BC268(*)	28	D	Icterus and hypoglycaemia

BC168(*)	28	D	Icterus and hypoglycaemia
BC228 (*)	19g1	D	Neurological signs
ES84 (*)	19g1	D	Pancreatitis
BC214(*)	11	S	Splenomegaly
BC211(*)	19g1	S	Icterus and ISA
BC217(*)	29	S	Icterus and ISA
ES63 (**)	29	D	Haemoconcentration

(*) Sample associated with two distinct clinical signs; (h) Sample associated with haemolysis; (S) Survived; (D) Died; (**) Sample associated with non-solid organ complications
ARDS: Acute respiratory distress syndrome
ARF: Acute renal failure

4.8. References

Allred, D.R., Al-Khedery B., 2004. Antigenic variation and cytoadhesion in *Babesia bovis* and *Plasmodium falciparum*: different logics achieve the same goal. Molecular and Biochemical Parasitology 134, 27-35.

Allred, D. R., Al-Khedery B., 2006. Antigenic variation as an exploitable weakness of babesial parasites. Veterinary Parasitology 138, 50-60.

Böhm, M., Leisewitz, A.L., Thompson, P.N., Schoeman, J. P., 2006. Capillary and venous *Babesia canis rossi* parasitaemias and their association with outcome of infection and circulatory compromise. Veterinary Parasitology 141, 18-29.

Bourdoiseau, G., 2006. Canine babesiosis in France. Veterinary Parasitology 138, 118-125.

Carret, C., Walas F., Carcy, B., Grande, N., Precigout, E., Moubri, K., Schetters T. P., Gorenflot, A., 1999. *Babesia canis canis*, *Babesia canis vogeli, Babesia canis rossi:* differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small subunit ribosomal RNA genes. The Journal of Eukaryotic Microbiology 46, 298-303.

Cooke, B.M., Mohandas, N., Coppel R.L., 2001. The malaria-infected red blood cell: structural and functional changes. Advances in Parasitology 50, 1-86.

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.

Hutchings, C.L., Li, A., Fernandez, K.M., Fletcher, T., Jackson, L.A., Molloy, J. B., Jorgensen, W.K., Lim, C.T., Cooke, B.M., 2007. New insights into the altered

adhesive and mechanical properties of red blood cells parasitized by *Babesia bovis*. Molecular Microbiology 65, 1092-1105.

Jacobson, L.S., Clark, I.A., 1994. The pathophysiology of canine babesiosis: new approaches to an old puzzle. Journal of the South African Veterinary Association 65, 134-145.

Jacobson, L.S., Lobetti R.G., 1996. Rhabdomyolysis as a complication of canine babesiosis. The Journal of Small Animal Practice. 37, 286-291.

Jacobson, L.S., 2006. The South African form of severe and complicated canine babesiosis: Clinical advances 1994-2004. Veterinary Parasitology 138, 126-139.

Matjila, P.T., Leisewitz, A.L., Jongejan, F., Penzhorn, B.L., 2008. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. Veterinary Parasitology 155, 152-157.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P., Nijhof, A.M., Jongejan F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Veterinary Parasitology 122, 119-125.

Miller, L. H., Baruch, D. I., Marsh, K., Doumbo, O. K., 2002. The pathogenic basis of malaria. Nature 415, 673-679.

O'Connor, R.M., Long, J.A., Allred, D.R., 1999. Cytoadherence of *Babesia bovis*-infected erythrocytes to bovine brain capillary endothelial cells provides an *in vitro* model for sequestration. Infectious Immunology 67, 3921-3928.

Schetters, T. P., Eling W.M., 1999. Can *Babesia* infections be used as a model for cerebral malaria? Parasitology Today 15, 492-497.

Shakespeare, A.S., 1995. The incidence of canine babesiosis amongst sick dogs presented to the Onderstepoort Veterinary Academic Hospital. Journal of the South African Veterinary Association 66, 247-250.

Uilenberg, G., Franssen, F. F., Perie, N.M., Spanjer A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Veterinary Quarterly 11, 33-40.

Chapter 5: Autochthonous canine babesiosis in the Netherlands.

5.1. Abstract

Outbreaks of autochthonous babesiosis, caused by *Babesia canis*, occurred in the Netherlands in the spring and autumn of 2004 affecting 23 dogs. Blood samples were taken from dogs that had been brought to the clinics with signs and symptoms associated with clinical babesiosis. The samples originated from the surroundings of the two cities in the Netherlands, The Hague and Arnhem. These samples were sent to the Department of Companion Animal Medicine and to the Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University for diagnostic purposes. Samples were tested for *Babesia* and *Ehrlichia* parasites by blood or buffy coat smear investigation, Indirect flourescent antibody test (IFAT), Polymerase chain reaction (PCR) followed by Reverse Line Blot (RLB). Twenty-three cases of autochthonous babesiosis were confirmed by a combination of clinical history, positive blood smears, IFAT and PCR/RLB. Nineteen animals recovered after treatment, whereas 4 dogs died. Adult *Dermacentor reticulatus* ticks collected from these dogs indicate that canine babesiosis could become endemic in the Netherlands.

5.2. Introduction

During the spring of 2004, outbreaks of babesiosis occurred in dogs simultaneously in two different residential areas in the Netherlands. Previously, canine babesiosis had only been diagnosed from dogs returning from endemic areas outside the Netherlands. The only known autochthonous cases of canine babesiosis associated with *Dermacentor reticulatus* were recorded in 1985 from five dogs (Uilenberg, Top, Arends, Kool, van Dijk, van Schieveen and Zwart, 1985). In the current study, twenty dogs, which had not been outside the Netherlands, were affected; 13 in the municipality of Rijkerswoerd, situated just south of Arnhem (51º56' N; 005º53' E) (Fig. 5.1) in the east of the Netherlands, and another 7 dogs acquired the infection in a recreational area in The Hague (52º05' N; 004º17' E) (Fig. 5 1) further to the west of the country. There were four fatal cases; two in each locality, which had not been recognised as babesiosis and hence had been left untreated. Typical symptoms in each of these fatal cases (dog no. 5, 7, 9, and 17) were lethargy, anorexia, fever, haemoglobinuria/dark urine, and vomiting. Haematological abnormalities included anaemia and thrombocytopenia. Moreover, bilirubinuria, icterus, petechiae and splenomegaly were also noticed. *Babesia* infection was only confirmed after the dogs had died and no post-mortem examinations were carried out.

A subsequent notice to all veterinary clinicians in the Netherlands through the Royal Dutch Veterinary Association, concerning outbreaks of canine babesiosis of an autochthonous origin, prevented any further casualties. Another 16 dogs exhibited clinical symptoms associated with an acute haemolytic disease typical for babesiosis

(Boozer and Macintire, 2003). Symptoms included lethargy, anorexia, fever, anaemia and abdominal tenderness. Blood smear examination revealed the presence of a large *Babesia* species in erythrocytes. All animals were successfully treated with imidocarb dipropionate (Imizol®), Schering-Plough, Utrecht, the Netherlands. Two partly fed ticks and one almost engorged female of *D. reticulatus* were collected from three different dogs in both areas.

During the autumn of 2004, three additional clinical cases of babesiosis (dog no. 21, 22 and 23, see Table 5.1) were diagnosed in dogs that had also not been outside the Netherlands. These dogs were frequent visitors of the same recreational area in The Hague, where the previous outbreak had occurred. This time typical symptoms were recognised early and the dogs were successfully treated with imidocarb dipropionate.

5.3. Materials and Methods

5.3.1. Collection of samples

Twenty samples were collected in the spring and three in the autumn of 2004. Samples were collected from 10 dogs involved in the outbreaks in The Hague and from 13 dogs involved in the outbreak near Arnhem. Blood smears, serum samples as well as EDTA blood samples for PCR were collected from each dog. In addition, 13 dogs were sampled again between 4 and 10 weeks after treatment to obtain a second blood sample for PCR to determine the carrier status of the animals after treatment. Blood smears were stained with Giemsa and examined for *Babesia* parasites. The indirect fluorescent antibody test (IFAT) was carried out as described previously by Uilenberg, Franssen, Perie and Spanjer

(1989). PCR and reverse line blotting for simultaneously detection and differentiation of *Babesia* sp. was essentially performed as described previously (Gubbels, de Vos, Van der Weide, Viseras, Schouls, de Vries and Jongejan, 1999; Matjila, Penzhorn, Bekker, Nijhof and Jongejan, 2004) with some modifications.

5.3.2. DNA extraction

DNA was extracted from 200 µl of each blood sample, from three collected *D. reticulatus* ticks, as well as from the eggs produced by the engorged *D. reticulatus* female tick which had been kept at 27 ºC in an incubator at 80 % relative humidity for oviposition. The QIAmp blood and tissue extraction kit (Qiagen, Hilden, Germany) was used for DNA extractions, following the manufacturer's protocols. DNeasy tissue kit was used for the extraction of DNA from the ticks with the following modifications. Surface sterilised ticks were cut into small pieces and triturated in liquid nitrogen. The material was subsequently lysed at 55º C and further treated according to the manufacturer's protocols.

5.3.3. PCR

PCR was performed with primers RLB-F2 and RLB-R2 amplifying a fragment of 460-540bp from the 18S rRNA gene spanning the V4 region (Gubbels et al., 1999; Matjila et al., 2004). The conditions for the PCR included an initial step of 3 min at 37º C, 10 min at 94ºC, 10 cycles of 94ºC (20s)- 67 ºC (30s)- 72º C (30s), with lowering of annealing step after every second cycle with 2º C (touchdown PCR). The reaction was then followed by 40 cycles of denaturation at 94º C for 30s, annealing at 57º C for 30s and extension at 72º C for 30s.

5.3.4. Reverse line blot hybridisation

RLB was subsequently conducted on amplified products as previously described (Matjila et al., 2004). The method was improved by the addition of a novel plasmid control, which was used as an internal positive control to check whether all *Babesia* species-specific oligonucleotides were correctly attached to the RLB membrane and functioning properly. Three fragments (A, B & C), each containing four RLB-probe sequences flanked by a restriction enzyme recognition sequence for sticky end cloning and their reverse complement, were synthesized by Isogen-Lifescience (Maarssen, the Netherlands) (Table 5.2 and 5.3). The oligonucleotides were diluted in MilliQ to a working solution of 20 pmol/µl. Equal volumes of fragment A and its reverse complement were allowed to hybridise for 15 min at room temperature and subsequently ligated in cloning vector pUC19 digested with the accompanying restriction enzymes EcoRI and KpnI using standard techniques. The ligated plasmid was transferred to competent *E. coli* cells by electroporation. Transformants were checked for successful incorporation of the fragment by performing a PCR using biotin labelled M13 primers and subsequent testing of the PCR product on a RLB membrane. Positive clones were then digested with BamHI and SalI and ligated with hybridised fragment B. Using the same methods, positive clones for fragment A and B were digested with PstI and HindIII to ligate hybridised fragment C. All fragments were successfully incorporated (Fig. 5 2).

5.4. Results

Twenty-three cases of autochthonous babesiosis were confirmed by a combination of clinical history, positive blood smears, PCR/RLB hybridization and detection of *B. canis*-

specific antibodies. Eighteen out of 23 cases were confirmed to be caused by *B. canis* only by PCR/RLB (Fig. 5 2). Although DNA samples were not available for the remaining 5 animals, dogs numbered 1, 4, and 6 were shown to be infected indirectly by a high IFAT antibody titre against *B. canis* (Table 5.1). Dog no. 10 was confirmed positive by a combination of clinical history, positive response to anti-*Babesia* treatment and a *B. canis* positive PCR test conducted by another laboratory. Finally, dog no. 19 was the only dog that was found positive based on blood smears only in combination with the clinical history and positive response to treatment. Thirteen dogs were retested within 4 to 10 weeks after treatment. All were found negative on blood smear examination as well as in the PCR/RLB assay, and titres had either declined (dog 6, 8, 14, 16) or disappeared (dog 1). Three *D. reticulatus* ticks collected from the dogs were also positive for *B. canis* only (Fig. 5.2). The egg progeny derived from the engorged *D. reticulatus* female was also positive (Fig. 5.2).

DNA from the four fatal cases was PCR-amplified using primers RLB-F2 and RLB-R2 and subsequently sequenced (BaseClear, Leiden, The Netherlands). The resulting sequences were submitted to Genbank (AY703070, AY703071, AY703072 and AY703073) and were 100% homologous with *B. canis* 18S sequences deposited in Genbank.

5.5. Discussion

In the spring of 2004, thirteen dogs in Arnhem and 7 dogs in The Hague were involved in outbreaks of babesiosis, whereas in the autumn another three dogs acquired the infection

in The Hague. Pathogen DNA isolated from 18 cases (including four fatalities) was demonstrated to be *B. canis* only (Fig. 5.2). This is based on the observation that no hybridization with any other *Babesia* species was found in the RLB (Fig. 5.2). Although additional tick-borne parasites have been reported in dogs, for instance *B. gibsoni* (Casapulla, Baldi, Avallone, Sannino, Pazzanese and Mizzon, 1998), *Theileria annae* (Zahler Rinder, Schein and Gothe 2000a) and a large *Babesia* recently discovered (Birkenheuer, Neel, Ruslander, Levy and Breitschwerdt, 2004), the catch-all probe would hybridize with all *Babesia* or *Theileria* species or variants for which no species-specific probe is included in the RLB. The cloned plasmid control that was added to the assay, considerably enhanced the reproducibility of the assay and eliminated the need to include pathogen DNA from clinical cases as positive controls (Fig. 5.2). In addition to 18 confirmed *B. canis* cases using PCR/RLB, material from 5 other cases was insufficient to conduct a full analysis. However, babesiosis could be confirmed either by serological means or by clinical and parasitological evidence (Table 5.1).

However, of critical importance here was to obtain confirmation that these dogs had indeed not been outside the Netherlands. Each owner was contacted in order to confirm this; with the exception of four dogs (nos. 10, 11, 12, and 16), no dogs had been out of the country. Dog no. 10 had been in France once in 1995, dog no. 11 had been to Turkey in 2000, dog no. 12 visited France in August 2003 and dog 16 had been to Switzerland in 2001. None of these dogs had ever been treated or diagnosed with canine babesiosis prior to the current outbreak. It is unlikely that dog no's 10, 11, or 16 had contributed to the current outbreak either as asymptomatic carriers or as carriers for ticks, since at least

three years had elapsed since these visits took place. Finally, dog no. 12 was diagnosed with babesiosis in the outbreak in April 2004, ten months after the animal returned from France. The fact that it was negative on IFAT at the onset of the disease (Table 5.1) indicates that the infection had been acquired in the Netherlands.

After the first 4 fatal cases occurred in the spring of 2004, a subsequent warning to all clinicians in the Netherlands prevented any further casualties. It can also be derived from Table 5.1 that the duration between the onset of clinical signs and subsequent treatment became progressively shorter after this warning had been issued. All remaining dogs exhibiting clinical symptoms typical for babesiosis were successfully treated. It is important to note that 13 dogs that were retested several weeks after treatment were all negative in the RLB (Table 5.1). This would indicate that the infection had been cleared, although in theory organisms could still be present under the detection limit of the assay. According to the literature, a single dose (7.5 mg/kg) of imidocarb dipropionate (Penzhorn, Lewis, De Waal and Lopez Rebollar, 1995; Boozer and Macintire, 2003) or two doses (7mg/kg) with a 14-day interval (Brandao, Hagiwara and Myiashiro, 2003) will sterilize the infection. In addition to the life-saving ability of the drug, sterilising the infection in dogs in the Netherlands would render the dogs non-infective for ticks. Moreover, it would also ease matters with respect to blood-component therapy, which has become more accessible in veterinary practice over the recent years (Reine, 2004).

On the other hand, it has been shown that sterilization of infection by imidocarb creates higher susceptibility to re-infection and that untreated animals are more resistant to

homologous challenge (Penzhorn et al., 1995; Lewis, Penzhorn and Lopez Rebollar, 1995). Vaccination, which would sufficiently reduce clinical symptoms but not prevent infection, would be the preferred preventive measure in an endemic area (Schetters, Kleuskens, Scholtes, Pasman and Goovaerts, 1997). The future policy for the control of babesiosis in the Netherlands would depend upon the establishment of the vector tick.

In 1985, *Dermacentor reticulatus* ticks were reported in the Netherlands associated with the first five autochthonous cases of babesiosis (Uilenberg et al., 1985). In several non-endemic European countries including Switzerland, Germany and Belgium (Pfister, Schwalbach, Chuit, Liz and Aeschlimann, 1993; Zahler and Gothe, 1997; Losson, Mollet, Avez, Malaise and Mignon, 1999; Zahler, Steffen, Lutz, HShnel, Rinder and Gothe, 2000b; Duh, Tozon, Petrovec, Strasek and Avsic-Zupanc, 2004) outbreaks of *B. canis* have been reported associated with *D. reticulatus* ticks. It is interesting to note that localized populations of *D. reticulatus* do occur in southwest England, Belgium and Germany, in similar ecological conditions as are present in the Netherlands (Hillyard, 1996; Zahler et al., 2000b). However, there is a lack of detailed distribution maps for *Dermacentor* ticks in North-western Europe, as recently shown by Trotz-Williams and Trees (2003).

Dermacentor reticulatus is a three-host tick of which only the adult stage feeds on dogs; larvae and nymphs feed primarily on small mammals and also on birds (Estrada-Peňa, Bouattour, Camicas and Walker, 2004). It is likely that *Dermacentor* ticks are spread in Europe on dogs traveling to and from endemic areas. In addition, an estimated 1500 dogs

are imported every year into the Netherlands, the majority originating from southern Europe, where *D. reticulatus* occurs (Estrada-Peña et al., 2004). In theory, several wildlife species and migrating birds could also play a role. Without horizontal transfer of adult *Dermacentor* ticks between dogs, engorged female ticks would have to be introduced into each area in the year preceding the current outbreaks. After immature stages had successfully fed on small mammals, subsequent adult ticks emerged in the spring of 2004. Evidence to support this theory is the demonstrated transovarial passage of *B. canis* through the eggs of *D. reticulatus* (Fig. 5.2).

5.6. Conclusion

Although the second, smaller outbreak in the autumn of 2004 was predicted based on the seasonal dynamics of the tick in France (Martinod and Gilot, 1991) it remains to be shown whether further outbreaks should be anticipated. In order to clarify this situation, a survey was initiated to identify ticks from dogs presented at veterinary clinics combined with a survey for *B. canis* using PCR/RLB. Preliminary data from this survey showed that although *Ixodes ricinus* was the predominant tick found on dogs (510 specimens), *Ixodes hexagonus* (33 ticks), *Rhipicephalus sanguineus* (20 ticks) and one additional *D. reticulatus* were also found. These findings would suggest including other pathogens, such as *Ehrlichia canis* transmitted by *R. sanguineus* ticks, in future surveys. In fact, *E. canis* has already been found in dogs introduced into the Netherlands either alone or in combination with *B. canis* (Zandvliet, Teske and Piek, 2004). All clinical cases of babesiosis included in this case report were negative for *E. canis* (data not shown). The establishment of *D. reticulatus* in the Netherlands would also mean that *Rickettsia*

slovaca, causing TIBOLA in humans, would have to be taken into consideration (Raoult, Lakos, Fenollar, Beytout, Brouqui and Fournier, 2002).

In conclusion, future strategies to control ticks and tick-borne diseases in companion animals in the Netherlands need to be reassessed in case populations of *D. reticulatus* ticks become established in the Netherlands. To this date, a recent survey has shown that *Dermacentor* ticks have established themselves at several locations in the Netherlands and in large numbers (Nijhof, Bodaan, Postigo, Nieuwenhuijs, Opsteegh, Franssen, Jebbink and Jongejan, 2007).

5.7. Figures and Tables

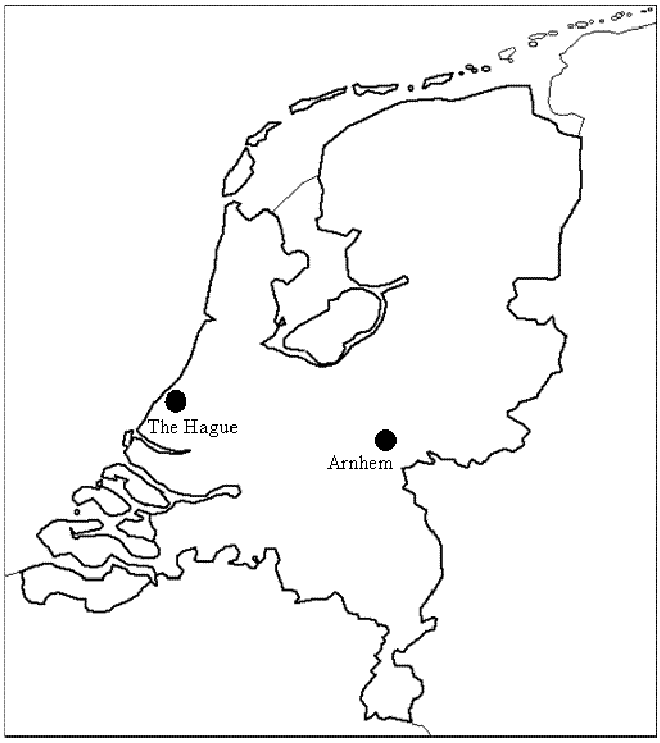


Figure 5.1: Map of the Netherlands indicating the two locations where cases of canine babesiosis were detected.

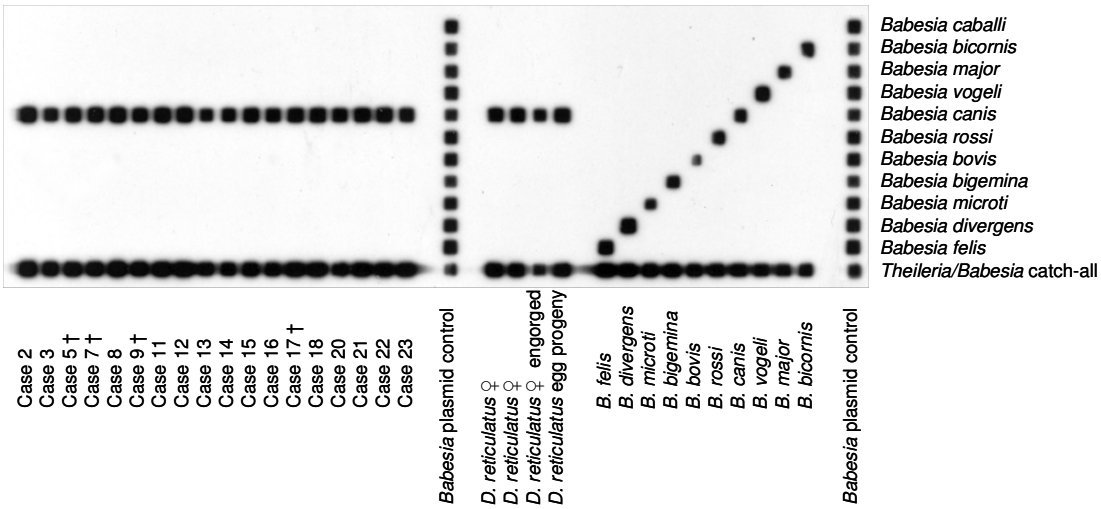


Figure 5.2: RLB results displaying 11 species-specific oligonucleotides of the 18S rRNA gene in the horizontal lanes and PCR products in the vertical lanes. From left to right are shown: 18 canine blood samples from clinical cases, *Babesia* plasmid positive control, 4 tick specimens, 11 positive *Babesia* sp. DNA controls and a second *Babesia* plasmid positive control.

Table 5.1: Twenty-three confirmed cases of autochthonous babesiosis caused by *Babesia canis* in the Netherlands in 2004.

Dog	Location	Onset of disease	1st Imizol treatment	Date blood collection	Blood Smear	PCR / RLB	IFAT
1	Arnhem	23 Feb	2 Apr	2 Apr	NI	NI	1:640
				10 Jun	-	-	-
2	Arnhem	4 Mar	1 Apr	30 Mar	-	+	NI
				29 Jun	-	-	-
3	Arnhem	9 Mar	16 Apr	2 Apr	NI	+	1:640
4	Arnhem	19 Mar	8 Apr	25 Mar	NI	NI	1:1280
				10 Jun	-	-	NI
5 [a]	The Hague	21 Mar	None	21 Mar	+	+	-
6	Arnhem	23 Mar	2 Apr	2 Apr	NI	NI	1:1280/2560
				10 Jun	-	-	1:80/160
7 [a]	Arnhem	24 Mar	None	29 Mar	NI	+	-
8	Arnhem	25 Mar	1 Apr	30 Mar	+	+	1:2560
				28 Jun	-	-	1:320
9 [a]	Arnhem	26 Mar	None	26 Mar	NI	+	NI
10	The Hague	30 Mar	7 Apr	2 Apr	NI	+	NI
				3 Jun	-	-	NI
11	The Hague	5 Apr	5 Apr	6 Apr	NI	+	1:80/160
				9 Jun	-	-	NI
12	The Hague	6 Apr	9 Apr	8 Apr	+	+	-
				9 Jun	-	-	NI
13	The Hague	7 Apr	13 Apr	13 Apr	+	+	1:1280
14	Arnhem	10 Apr	20 Apr	19 Apr	-	+	1:1280
				28 Jun	-	-	1:320
15	Arnhem	14 Apr	14 Apr	14 Apr	+	+	NI
				28 Jun	-	-	-
16	Arnhem	27 Apr	10 May	16 Apr	-	+	1:640
				29 Jun	-	-	1:160
17 [a]	The Hague	28 Apr	29 Apr	29 Apr	+	+	-
18	The Hague	29 Apr	29 Apr	29/Apr	+	+	-
19	Arnhem	4 May	7 May	5 May	+	NI	-
				10 Jun	-	-	NI
20	Arnhem	5 May	26 May	13 May	+	+	1:640
21	The Hague	13 Oct	16 Oct	16 Oct	+	+	NI
22	The Hague	23 Oct	8 Nov	8 Nov	+	+	NI
23	The Hague	2 Nov	4 Nov	4 Nov	+	+	NI

RLB: Reverse line blot
IFAT: Indirect fluorescent antibody test
NI: not investigated
[a] Indicates a fatal case

Table 5.2: Composition of the *Babesia* plasmid control with three fragments (A, B and C) each containing four RLB-probe sequences flanked by the restriction enzyme recognition sequence for sticky-end cloning.

Fragment	**Synthesized oligonucleotides**	**Restriction enzymes**
A	5'-AATTC(sequence probe 1 to 4)GGTAC-3' 5'-C(reverse complement probe 4 to 1)G-3'	EcoRI and KpnI
B	5'-GATCC(sequence probe 5 to 8)G-3' 5'-TCGAC(reverse complement probe 8 to 5)G-3'	BamHI and SalI
C	5'-G(sequence probe 9 to 12)A-3' 5'-AGCTT(reverse complement probe 12 to 9)CTGCA-3'	PstI and HindIII

Table 5.3: RLB-probes incorporated in the *Babesia* plasmid control.

Probe	Oligonucleotide probe specificity	Oligonucleotide sequence (5'→3')
1	*Babesia* catch-all	TAATGGTTAATAGGA(AG)C(AG)GTT
2	*Babesia bicornis*	TTGGTAAATCGCCTTGGT
3	*Babesia bigemina*	CGTTTTTTCCCTTTTGTTGG
4	*Babesia bovis*	CAGGTTTCGCCTGTATAATTGAG
5	*Babesia canis*	TGCGTTGACGGTTTGAC
6	*Babesia rossi*	CGGTTTGTTGCCTTTGTG
7	*Babesia vogeli*	AGCGTGTTCGAGTTTGCC
8	*Babesia felis*	TTATGCGTTTTCCGACTGGC
9	*Babesia major*	TCCGACTTTGGTTGGTGT
10	*Babesia caballi*	GCTTGATTTTCGCTTCGCTT
11	*Babesia divergens*	ACT(AG)ATGTCGAGATTGCAC
12	*Babesia microti*	GACTTGGCATCTTCTGGA

5.8. References

Birkenheuer, A.J., Neel, J., Ruslander, D., Levy, M.G., Breitschwerdt, E.B., 2004. Detection and molecular characterization of a novel large *Babesia* species in a dog. Veterinary Parasitology 124, 151-160.

Boozer, A.L., Macintire, D.K., 2003. Canine babesiosis. The Veterinary clinics of North America. Small Animal Practice 33, 885-904.

Brandao, L.P., Hagiwara, M.K., Myiashiro, S.I., 2003. Humoral immunity and reinfection resistance in dogs experimentally inoculated with *Babesia canis* and either treated or untreated with imidocarb dipropionate. Veterinary Parasitology 114, 253-265.

Casapulla, R., Baldi, L., Avallone, V., Sannino, R., Pazzanese, L., Mizzoni, V., 1998. Canine piroplasmosis due to *Babesia gibsoni*: clinical and morphological aspects. Veterinary Record 142, 168-169.

Duh, D., Tozon, N., Petrovec, M., Strasek, K., Avsic-Zupanc, T., 2004. Canine babesiosis in Slovenia: molecular evidence of *Babesia canis canis* and *Babesia canis vogeli*. Veterinary Research 35, 363-368.

Estrada-Peňa, A., Bouattour, A., Camicas, J.-L., Walker, A.R., 2004. Ticks of domestic animals in the Mediterranean region, a guide to the identification of species. University of Zaragoza, Spain. (Book,Monograph).

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.

Hillyard, P.D., 1996. Ticks of North-West Europe; keys and notes for identification of species. Synopses of the British Fauna, New Series, 52, 176-176.

Lewis, B.D., Penzhorn, B.L., Lopez Rebollar, L.M., 1995. Immune responses to South African *Babesia canis* and the development of a preliminary vaccine. Journal of the South African Veterinary Association 66, 61-65.

Losson, B., Mollet, J.J., Avez, F., Malaise, F., Mignon, B., 1999. Description of three cases of canine babesiosis in Belgium. Annales de Médecine Vétérinaire 143, 119-124.

Martinod, S., Gilot, B., 1991. Epidemiology of canine babesiosis in relation to the activity of *Dermacentor reticulatus* in southern Jura (France). Experimental and Applied Acarology 11, 215-222.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P., Nijhof, A.M., Jongejan, F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Veterinary Parasitology 122, 119-125.

Nijhof, A.M., Bodaan, C., Postigo, M., Nieuwenhuijs, H., Opsteegh, M., Franssen, L., Jebbink, F., Jongejan, F., 2007. Ticks and associated pathogens collected from domestic animals in the Netherlands. Vector-Borne and Zoonotic Diseases 7, 585-596.

Penzhorn, B.L., Lewis, B.D., De Waal, D.T., Lopez Rebollar, L.M., 1995. Sterilisation of *Babesia canis* infections by imidocarb alone or in combination with diminazene. Journal of the South African Veterinary Association 66, 157-159.

Pfister, K., Schwalbach, B., Chuit, P.A., Liz, J., Aeschlimann, A., 1993. Präliminäre Untersuchungen zur endemischen Ausbreitung von *Babesia canis* und der Zecke *Dermacentor reticulatus* in der Schweiz. Mitteilungen der Österreichischen Gesellschaft für Tropenmedizin und Parasitologie 1-6.

Raoult, D., Lakos, A., Fenollar, F., Beytout, J., Brouqui, P., Fournier, P.E., 2002. Spotless rickettsiosis caused by *Rickettsia slovaca* and associated with *Dermacentor* ticks. Clinical Infectious Diseases 34, 1331-1336.

Reine, N.J., 2004. Infection and blood transfusion: a guide to donor screening. Clinical Techniques in Small Animal Practice 19, 68-74.

Schetters, T.P.M., Kleuskens, J.A.G.M., Scholtes, N.C., Pasman, J.W., Goovaerts, D., 1997. Vaccination of dogs against *Babesia canis* infection. Veterinary Parasitology 73, 35-41.

Trotz-Williams, L.A., Trees, A.J., 2003. Systematic review of the distribution of the major vector-borne parasitic infections in dogs and cats in Europe. Veterinary Record 152, 97-105.

Uilenberg, G., Franssen, F.F., Perie, N.M., Spanjer, A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Veterinary Quarterly 11, 33-40.

Uilenberg, G., Top, P.D., Arends, P.J., Kool, P.J., van Dijk, J.E., van Schieveen, P.B., Zwart, D., 1985. Autochthonous babesiosis in dogs in the Netherlands? Tijdschrift voor Diergeneeskunde 110, 93-98.

Zahler, M., Gothe, R., 1997. The risk of *Babesia canis* infection, transmitted by *Dermacentor reticulatus* becoming endemic in Germany. Tierärztliche Praxis. Ausgabe K, Kleintiere/Heimtiere 25, 666-670.

Zahler, M., Rinder, H., Schein, E., Gothe, R., 2000a. Detection of a new pathogenic *Babesia microti*-like species in dogs. Veterinary Parasitology 89, 241-248.

Zahler, M., Steffen, T., Lutz, S., HShnel, W.C., Rinder, H., Gothe, R., 2000b. *Babesia canis* and *Dermacentor reticulatus* in Munich: a new endemic focus in Germany. Tierärztliche Praxis. Ausgabe K, Kleintiere/Heimtiere 28, 116-120.

Zandvliet, M.M., Teske, E., Piek, C.J., 2004. *Ehrlichia* and *Babesia* infections in dogs in The Netherlands. Tijdschrift voor Diergeneeskunde 129, 740-745.

Chapter 6: Detection of *Theileria* sp. infections in dogs in South Africa.

6.1. Abstract

A *Theileria* sp. was detected by PCR in blood samples collected from dogs in the Pietermaritzburg area and also found in dogs presented at the Outpatients Clinic of the Onderstepoort Veterinary Academic Hospital (OVAH) in the Pretoria area, South Africa. In the Pietermaritzburg area 79/192 samples were positive, while 3/527 of the Onderstepoort samples were positive. Three positive samples from Pietermaritzburg were co-infected with *Ehrlichia canis*. Parasite DNA was detected and identified by PCR and Reverse Line Blot (RLB), followed by sequence analysis. Phylogenetic analysis of the 18S rRNA full-length sequences of one sample from Pietermaritzburg (VT12: Accession number EU053201) and two samples from OVAH (BC281, Accession number: EU053199 and BC295, Accession number: EU053200) revealed that the isolates were closely related to the published isolates of *Theileria* sp. (sable) (AY74862) and *Theileria* sp. (sable) (L19081). Clinical signs of the dogs that were examined at OVAH and from which the three blood samples were collected were not consistent with theileriosis. All dogs had a similar immune-mediated condition with severe thrombocytopenia. These findings identify a *Theileria* sp. in dogs for the first time in South Africa and add yet another microorganism to the growing list of haemoprotozoan parasites infecting dogs. The clinical significance of this infection in dogs is poorly resolved.

6.2. Introduction

Canine babesiosis, a haemolytic disease of significant economic importance, is the most frequently encountered tick-borne protozoal infection of dogs in South Africa (Shakespeare, 1995; Collett, 2000). The parasites associated with canine babesiosis in South Africa are *Babesia rossi* and *Babesia vogeli* (Matjila, Penzhorn, Bekker, Nijhof and Jongejan, 2004). *Babesia rossi* which causes a severe disease that can be life-threatening, is the most prevalent species isolated from dogs presented at Onderstepoort Veterinary Academic Hospital (OVAH) (Böhm, Leisewitz, Thompson and Schoeman, 2006). The clinical signs and pathology of the disease may include pyrexia, splenomegaly, anaemia, haemolysis and haemoglobinuria, icterus, circulatory collapse, multiple organ failure and neurological signs (Jacobson and Clark, 1994). The clinical signs of infection caused by *B. vogeli* infection has not been well documented in South Africa (Bohm et al., 2006), although *B. vogeli* has been detected in dogs diagnosed with clinical babesiosis presented at the Outpatients Clinic, OVAH (Böhm et al., 2006). Elsewhere *B. vogeli* infections have been reported to cause only a mild disease in dogs (Uilenberg, Franssen, Perie and Spanjer, 1989).

Recent publications have reported on previously unknown pathogens that infect dogs and cause a haemolytic syndrome. A novel large *Babesia* sp. has been identified in dogs in North America (Birkenheuer, Neel, Ruslander, Levy and Breitschwerdt, 2004). The parasite identified was isolated from the bone marrow as well as the blood of a dog with haematological abnormalities consistent with babesiosis (Birkenheuer et al., 2004). *Rangelia vitalli*, a blood parasite causing a disease characterized by anaemia, jaundice,

fever, splenomegaly, lymphadenopathy, haemorrhage in the gastrointestinal tract and persistent bleeding from the nose, has been described in Brazil (Loretti and Barros, 2005). *Rangelia vitalli* is suspected to be tick-transmitted and the authors have stated that the parasite is a protozoan of the phylum Apicomplexa, although different from *Babesia*, since it has an intra-endothelial stage. The authors did not report on any molecular comparisons, which limited determination of the phylogenetic relationship to other blood protozoan parasites. Small babesias with similar morphology to *B. gibsoni* (*senso lato*) have been described lately (Kjemtrup, Kocan, Whitworth, Meinkoth, Birkenheuer, Cummings, Boudreaux, Stockham, Irizarry-Rovira and Conrad, 2000; Kjemtrup, Wainwright, Miller, Penzhorn and Carreno, 2006). Although similar in morphology to *B. gibsoni* (*senso lato*), the parasites are genetically distinct and include an Asian isolate, a Spanish isolate and a Californian isolate (Kjemtrup et al., 2000). Recent molecular research has shown the Californian isolate to be genotypically and phenotypically different from the *B. gibsoni* (*senso lato*) group. It has thus been named *Babesia conradae* (Kjemtrup et al., 2006).

Until recently there had not been any reports on pathogenic *Theileria* sp. in dogs. *Theileria annae* was the first *Theileria* species to be associated with a haemolytic disease of dogs (Zahler Rinder, H., Schein, E., Gothe, 2000; Camacho, Pallas, Gestal, Guitian, Olmeda, Goethert and Telford, 2001; Camacho, Guitian, Pallas, Gestal, Olmeda, Goethert, Telford and Spielman, 2004) . A number of authors cited Goethert and Telford (2003) when referring to this parasite as *Babesia annae*. Goethert and Telford (2003) did not propose the name *B. annae*, however, but disputed the use of *Theileria* as a genus

name since no evidence was presented by Zahler et al. (2000) for a pre-erythrocytic or lymphocyte-infecting stage, nor was there any evidence for the absence of transovarial transmission in ticks (Goethert and Telford, 2003). Current molecular evidence based on the analysis of the 18S rRNA gene justifies the naming of *T. annae* for this parasite (instead of *B. annae*) and it will be referred to as such in our report.

Other *Theileria* sp. that have been detected in dogs are *Theileria annulata* (Criado Martinez, Buling, Barba, Merino, Jefferies and Irwin, 2006) and *Theileria equi* (Criado-Fornelio Martinez-Marcos, Buling-Sarana and Barba-Carretero, 2003a). *Theileria annulata* was detected from an asymptomatic dog (Criado et al., 2006) whereas *Theileria equi* was detected from three asymptomatic dogs and one symptomatic dog (Criado-Fornelio et al., 2003a). These findings were followed up, however, and as far as we know none of these *Theileria* parasites have subsequently been isolated from clinically reacting dogs. This report describes a *Theileria* sp. isolated from dogs originating from two localities in South Africa, namely Pietermaritzburg (KwaZulu-Natal) and the Onderstepoort district of Pretoria (Gauteng). The *Theileria* sp. was first detected from samples collected in Pietermaritzburg in 2004. The DNA of this organism was later detected in two clinical samples collected from two dogs presented at the Outpatients Clinic of the OVAH in 2005. The same DNA found in this organism was detected in a third clinical sample, collected from a dog presented at the Outpatients Clinic in January 2007.

6.3. Materials and Methods

6.3.1. Collection of samples

Blood samples (n=192) were collected monthly over a six-month period from the Pietermaritzburg area, during the early summer months of 2004, and late summer months of 2005. The samples were collected routinely from dogs involved in a study of tick-repellent impregnated dog collars. Blood samples (n=527) were collected from dogs in need of veterinary care presented at the Outpatients Clinic, OVAH from January 2002 to January 2007. Blood-smear examinations were done by the attending clinicians on all samples. Blood smear examinations did not always reveal piroplasms. Blood samples were then collected into EDTA Vacutainer® (Franklin Lakes, USA) tubes and sent to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, for molecular detection of parasites.

6.3.2. DNA extraction

DNA was extracted from 200 µl of each blood sample using the QIAmp® blood and tissue extraction kit (Qiagen, Hilden, Germany), following the manufacturer's protocols.

6.3.3. PCR

PCR was performed with 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′) amplifying a fragment of 460-540bp from the 18S rRNA gene spanning the V4 region (Gubbels, de Vos, Van der Weide, Viseras, Schouls, de Vries and Jongejan, 1999; Matjila et al., 2004). The conditions for the PCR included an initial step of 3 min at 37º C, 10 min at 94ºC, 10

cycles of 94ºC (20s)- 67 ºC (30s)- 72º C (30s), with lowering of annealing step after every second cycle by 2º C (touchdown PCR). The reaction was then followed by 40 cycles of denaturation at 94º C for 30s, annealing at 57º C for 30s and extension at 72º C for 30s.

6.3.4. Reverse line blot hybridisation

PCR-amplified products were tested with the RLB, as previously described (Matjila et al., 2004). An additional plasmid control was used as an internal positive control to ensure that all *Babesia* species–specific probes were correctly bound to the RLB membrane and that they were functional (Matjila, Nijhof, Taoufik, Houwers, Teske, Penzhorn, de Lange and Jongejan, 2005).

6.3.5. Sequencing

PCR products that did not hybridize to any of the species-specific probes but hybridized to the *Theileria* genus-specific probe were selected from the samples collected in Pietermaritzburg and Onderstepoort. The RLB was repeated using a new membrane which included *Theileria* probes described by Nijhof, Pillay, Steyl, Prozesky, Stoltsz, Lawrence, Penzhorn and Jongejan (2005). Samples, VT4, VT9, VT12 and VT17 collected from Pietermaritzburg and samples BC285 and BC295 and BC610 collected from the OVAH were partially sequenced (400-540bp) using primers RLB F2 and RLB R2. These samples were selected for sequencing based on the quality and quantity of their genomic DNA. A BLAST search was performed with the obtained sequences using the BLASTn algorithm and compared with sequences deposited in GenBank. The full-

length 18S rRNA gene of sample VT12 (Pietermaritzburg) and the two clinical samples, BC281 and BC295 (OVAH) were amplified using 20 pmol of primers Nbab 1F (5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3') and TB Rev (5'-AAT AAT TCA CCG GAT CAC TCG-3') to give a PCR amplicon of *ca* 1800 base pairs that was subsequently visualized by gel electrophoresis.

These PCR products were purified with the QIAmp® PCR purification kit (Qiagen, Hilden, Germany), and sent for sequencing at the Genetics Section of the Faculty of Veterinary Science. The full-length 18S rRNA gene was sequenced in parts using 3.2 pmol of the following primers: Nbab1F (5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3') (Oosthuizen, Zweygarth Collins, Troskie and Penzhorn, 2008), TB Rev (5'-AAT AAT TCA CCG GAT CAC TCG-3'), BT 2R (5'-CCC GTG TTG AGT CAA ATT AAG CCG-3'), BT 3F (5'-GGG CAT TCG TAT TTA ACT GTC AGA GG-3'), (Oosthuizen et al., 2008), Nbab 4F (5'-CCG TTA ACG GAA CGA GAC CTT AAC C-3') and Nbab 4R (5'-GGT AGG CCA ATA CCC TAC CG-3').

DNA amplicons of sample VT12, BC281 and BC295 were also cloned into the pGem T easy vector (Promega, Leiden, The Netherlands) following the manufacturer's instructions. Twelve clones of each sample containing the amplified product were then sequenced using primers SP6 (5'-TAA ATC CAC TGT GAT ATC TTA TG-3') and T7 (5'-TAT GCT GAG TGA TAT CCC GCT-3').

6.3.6. Phylogenetic analysis

Sequence data for the full-length 18S rRNA gene were assembled and edited to a total length of 1627 bp using GAP 4 of the Staden package (Version 1.6.0 for Windows) (Bonfield, Smith and Staden, 1995; Staden, 1996; Staden, Beal and Bonfield, 2000), and deposited in GenBank. The sequences were aligned with sequences of related genera using ClustalX (Version 1.81 for Windows). The alignment was manually truncated to the size of the smallest sequence (~1368 bp). As only partial sequences (415bp) of *T. annae* were available in Genbank (AY150068 and AY150069), *T. annae* was omitted from further phylogenetic analysis. The two-parameter model of Kimura and the Jukes and Cantor correction model for multiple base changes were used to construct similarity matrices (Jukes and Cantor, 1969; Kimura, 1980). Neighbor-joining (Saitou and Nei, 1987) and the maximum parsimony methods were used for the construction of phylogenetic trees using the Mega 3.0 software package (Kumar, Tamura and Nei, 2004). The methods above were used in combination with the bootstrap method (Felsenstein, 1985)(1000 replicates/tree for distance methods and 100 replicates/tree for parsimony methods).

6.4. Results

Some of the processed samples were negative on blood-smear examination for piroplasms but were suspected to be *Babesia* positive. Initial processing of blood samples using the RLB assay revealed that 76 of the 192 blood samples (Table 6.1) from Pietermaritzburg were positive for a *Theileria* sp. by hybridizing with a *Theileria/Babesia* genus-specific catchall probe as well as the *Theileria* genus-specific catchall probe.

Three of the 527 samples collected from the Outpatients Clinic, OVAH, were positive for a *Theileria* sp. by also hybridizing with the same *Theileria*/*Babesia* genus-specific probe as well as the *Theileria* genus-specific catchall probe. Selection and partial sequencing (400-500bp) of samples VT4, 9, 12 and 17 from Pietermaritzburg and samples BC281, 295 and 610 from OVAH revealed that the samples were similar to the previously described *Theileria* sp. characterized from sable antelope (*Hippotragus niger*) (Stoltsz and Dunsterville, 1992). Repeated testing of all the samples on the RLB membrane that had species–specific probes that included *Theileria* sp. (greater kudu), *Theileria* sp. (grey duiker), *Theileria* sp. (sable) (Nijhof et al., 2005) and *Theileria annae* (CCG AAC GTA ATT TTA TTG ATT TG) revealed that all the previously *Theileria* genus-specific positive samples hybridized with the *Theileria* sp. (sable) probe. Three further blood samples from Pietermaritzburg were concurrently infected with *Theileria* sp. and *Ehrlichia canis*, as detected by the RLB. Blood-smear examinations of Pietermaritzburg and OVAH samples did not show any *Theileria*-infected leukocytes and / or red blood cells, but there were other important haemoparasites (including *B. rossi*, *E. canis* and mixed infections of *B. rossi* and *E. canis*) of dogs detected in blood samples by light microscopy and PCR/RLB, collected from Pietermaritzburg and from OVAH (Matjila, Leisewitz, Jongejan and Penzhorn, 2008).

Full-length 18S rRNA gene sequences of samples VT12 (EU053201) from Pietermaritzburg and two samples from OVAH BC281 (EU053199) and BC295 (EU053200) were compared with sequences of related genera. The BLAST search revealed highest similarities (~99%) with a *Theileria* sp. (AY748462) isolated from a

sable antelope originating from Malelane (southern Kruger National Park area of South Africa), and a *Theileria* sp. (L19081) that was also isolated from a sable antelope and later described and named: *Theileria* sp. (sable) (Allsopp, Cavalier-Smith, De Waal, and Allsopp, 1994). Samples VT12, BC281 and 295 also showed ~98% similarity with two *Theileria* sp. isolated from Texas (USA) dama gazelle (AY735116 and AY735115) and with *Theileria separata* (AY260175). These similarities were confirmed by both neighbor-joining and maximum parsimony phylogenetic approaches. No significant changes in the topology of the trees, or in the bootstrap values, were found when using any of the phylogenetic analysis procedures. The representative tree obtained by the neighbor-joining method with the Kimura two-parameter distance calculation (Kimura, 1980), is based on a 1368 bp region of the 18S rRNA gene (Fig. 6.1). In the aligned region, isolates VT12, BC281 and BC295 showed a one bp difference with *Theileria* sp. (sable) (AY748462) and four bp differences and a deletion with *Theileria* sp. (sable) (L19081).

6.5. Discussion

The only *Theileria* sp. currently known to cause disease in domestic dogs is the *B-microti*-like, *T. annae* (Zahler et al., 2000; Camacho et al., 2004; Garcia, 2006), which has only been reported to occur in Spain. *Theileria equi* and *T. annulata* have also been isolated from dogs in Spain (Criado et al., 2006; Criado-Fornelio et al., 2003a). *Theileria annae* has been reported to cause a disease characterized by apathy, fever, and anaemia (Zahler et al., 2000). Severe regenerative anaemia and thrombocytopenia have been reported to be a constant characteristic of *T. annae* infection (Garcia, 2006). The level of

parasitaemia is also usually low and not statistically related to the severity of the anaemia or renal failure (Garcia, 2006).

In our study we used molecular techniques to identify a *Theileria* species of dogs associated with a haemolytic disease. No other causes of clinical signs could be identified in the affected dogs. The Pietermaritzburg samples were part of an independent private group's commercial study on acaricide-impregnated dog collars used as a prophylactic measure against canine babesiosis. This made it difficult for us to obtain the exact histories of dogs that tested positive for the *Theileria* sp. and / or *E. canis*. However, from the brief histories of samples that we received from dog samples VT5, 6, 14, 17 and 21, we gathered the following information. The dog yielding sample VT5 had a history of anaemic episodes, which seemed to respond well to steroid treatment. This could be indicative of any inflammatory or an immune-mediated disease. At the time of collecting sample VT6 (from a 4-year-old dog), the dog had a depressed habitus, anorexia, fever, abdominal pain and respiratory difficulty. No piroplasms were seen during smear examination and the dog was suspected to have an immune-mediated condition. Sample VT14 was collected from a dog with abdominal pain and suspected colitis, whereas sample VT17 was collected from a five-year-old German Shepherd dog presented with weight loss and a fever. Smear examination of VT17 showed suspected *Babesia*-infected erythrocytes and a regenerative anaemia. However, this sample was PCR/RLB negative for *Babesia*. Further details were not provided. Finally (5) sample VT21 was collected from a two-year-old emaciated dog with heavy hook-worm infection and thrombocytopenia. With the exception of VT14, findings were consistent with

canine babesiosis (fever, anorexia, anaemia and thrombocytopenia) or similar to those described in dogs diagnosed with *T. annae* infection (fever, anaemia, and thrombocytopenia).

Detailed clinical histories were obtained from the three *Theileria*-positive samples (BC281, 295 and 610) collected at the Outpatients Clinic (OVAH). Sample BC281 was collected from a four-year-old Doberman Pinscher diagnosed with chronic-active necrotic superficial dermatitis and deep cellulitis of uncertain cause, anaemia and severe thrombocytopenia. Diagnostic ultrasound of the abdomen indicated that there was a mild iliac lymph node enlargement. The dog was again seen three months later, when it was diagnosed with nasal trauma and severe thrombocytopenia. PCR/RLB analysis of the blood sample revealed that the dog was infected with a *Theileria* sp. No *Ehrlichia* and / or *Anaplasma* infections were detected from sample BC281.

Sample BC295 was collected at the Outpatients Clinic, OVAH, from a two-and-a-half-month-old Miniature Schnauzer. On clinical examination the dog had a fever and bloody diarrhoea. The dog was diagnosed with parvovirus infection, based on clinical signs. PCR/RLB tests confirmed a *Theileria* sp. infection only. A month later, the dog was brought back to the Outpatients Clinic and was diagnosed with distemper and parvovirus, infection based on clinical signs. Blood samples taken on this second occasion again indicated a *Theileria* sp. infection by PCR/RLB tests.

Sample BC610 was collected from a dog admitted for splenomegaly diagnosed at the Outpatient Clinic, OVAH. Haematology revealed severe thrombocytopenia and abdominal ultrasound demonstrated an enlarged spleen. The dog's condition worsened and an emergency splenectomy was performed. The thrombocyte count returned to normal the following day. It was thus suspected that the thrombocytopenia was as a result of sequestration or immune-mediated destruction of thrombocytes. PCR/RLB tests confirmed a *Theileria* sp. infection and no *Ehrlichia* and / or *Anaplasma* infection. Smear examinations of the three OVAH samples (BC281, 295 and 610) did not show any piroplasm infections. It should be borne in mind, however, that the parasite density may have been below the level of detection for routine light microscopy as often encountered in *T. annae* infections (Garcia, 2006).

Although the pathophysiology of the detected *Theileria* sp. in dogs is unknown, it is apparent from the few cases described here that anaemia (possibly haemolytic), splenomegaly and a possible immune-mediated syndrome may be associated with this organism. Similar clinical signs are normally seen in dogs infected with *T. annae* (Garcia, 2006) including haematological disorders such as thrombocytopenia, which is a common finding in the absence of *Ehrlichia* infection in 75% of dogs infected with *T. annae* (Garcia 2006). Phylogenetic analysis (Fig. 6.1) of the *Theileria* sp. in dogs characterized in this study (BC281: Accession number: EUO53199; BC295: Accession number: EUO53200; and VT12: Accession number: EUO53201) showed a close similarity with one base pair difference to *Theileria* sp. (sable) (AY748462) and four base differences to *Theileria* sp. (sable) (L19081). Both *Theileria* (sable) species cause

mortalities in sable antelopes (Nijhof et al., 2005). To our knowledge none of the dogs that the *Theileria* sp. was isolated from died as a result of the infection. As previously suggested, this may indicate evidence of a chronic established host-parasite relationship (Ebert, 1998), or it may indicate that the parasite is not as virulent in dogs as it is in sable antelopes (Nijhof et al., 2005) . It has been shown that parasites that are known to be virulent in their typical hosts may infect incidental host without causing disease (Criado-Fornelio, Martinez-Marcos, Buling-Sarana and Barba-Carretero, 2003b).

6.6. Conclusion

We can therefore currently only speculate on the clinical relevance of the detected *Theileria* sp. in our sampled dogs. Our findings identify a *Theileria* sp. in dogs for the first time in South Africa and add yet another microorganism to the list of haemoprotozoans infecting dogs. More clinical samples and data will need to be collected and analysed to understand the importance of the *Theileria* sp. We will therefore refer to this parasite as "*Theileria* sp. (dog)" which we found in South Africa.

6.7. Figures and Tables

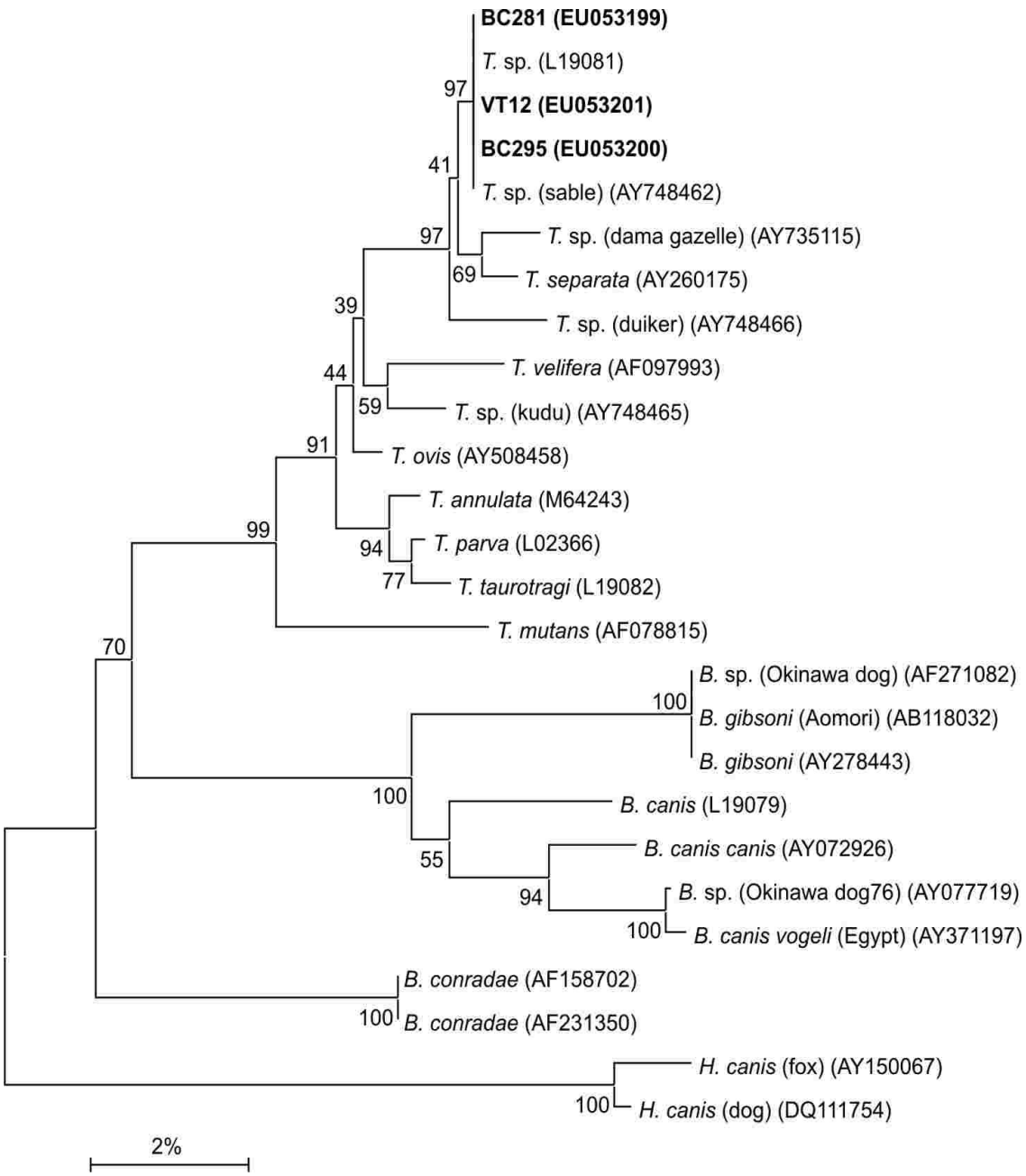


Figure 6.1: Neighbor-joining tree, with the Kimura two-parameter distance (Kimura, 1980) calculation showing the phylogenetic relationship of BC281, 295 & VT12 to related species based on the 18S rRNA gene sequences. Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. The scale bar represents the % nucleotide difference. The GenBank accession numbers are indicated in parentheses.

Table 6.1: Reverse line blot hybridization results of dogs positive for only *Theileria* sp. and for mixed infections of *Theileria* sp. and *E. canis*.

Location	Total number of collected samples	Number of samples positive for *Theileria* sp.	Number of samples positive for *Theileria* sp. and *E. canis*
Pietermaritzburg	192	76	3
OVAH	527	3	-

NB: Samples from both localities were positive for other important blood parasite. The results of these are reported in chapter 3

6.8. References

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

Birkenheuer, A.J., Neel, J., Ruslander, D., Levy, M.G., Breitschwerdt, E.B., 2004. Detection and molecular characterization of a novel large *Babesia* species in a dog. Veterinary Parasitology 124, 151-160.

Böhm, M., Leisewitz, A.L., Thompson, P.N., Schoeman, J.P., 2006. Capillary and venous *Babesia canis rossi* parasitaemias and their association with outcome of infection and circulatory compromise. Veterinary Parasitology 141, 18-29.

Bonfield, J.K., Smith, K., Staden, R., 1995. A new DNA sequence assembly program. Nucleic Acids Research 23, 4992-4999.

Camacho, A.T., Guitian, E.J., Pallas, E., Gestal, J.J., Olmeda, A.S., Goethert, H.K., Telford, S.R., III, Spielman, A., 2004. Azotemia and mortality among *Babesia microti*-like infected dogs. Journal of Veterinary Internal Medicine 18, 141-146.

Camacho, A.T., Pallas, E., Gestal, J.J., Guitian, F.J., Olmeda, A.S., Goethert, H.K., Telford, S.R., 2001. Infection of dogs in north-west Spain with a *Babesia microti*-like agent. Veterinary Record 149, 552-555.

Collett, M.G., 2000. Survey of canine babesiosis in South Africa. Journal of the South African Veterinary Association 71, 180-186.

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

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carretero, J.C., 2003b. Presence of *Mycoplasma haemofelis*, *Mycoplasma haemominutum* and piroplasmids in cats from southern Europe: a molecular study. Veterinary Microbiology 93, 307-317.

Ebert, D., 1998. Experimental evolution of parasites. Science (Washington) 282, 1432-1435.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783-791.

Garcia, A.T.C., 2006. Piroplasma infection in dogs in northern Spain. Veterinary Parasitology 138, 97-102.

Goethert, H.K., Telford, S.R.I., 2003. What is *Babesia microti*? Parasitology 127, 301-309.

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.

Jacobson, L.S., Clark, I.A., 1994. The pathophysiology of canine babesiosis: new approaches to an old puzzle. Journal of the South African Veterinary Association 65, 134-145.

Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro, H.N. (Ed.), Mammalian Protein Metabolism. Academic Press, New York, pp. 21-132.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16, 111-120.

Kjemtrup, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad, P.A., 2000. There are at least three genetically distinct small piroplasms from dogs. International Journal of Parasitology 30, 1501-1505.

Kjemtrup, A.M., Wainwright, K., Miller, M., Penzhorn, B.L., Carreno, R.A., 2006. *Babesia conradae*, sp. nov., a small canine *Babesia* identified in California. Veterinary Parasitology 138, 103-111.

Kumar S, Tamura K, Nei M, 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinformatics 5, 150-163.

Loretti, A.P., Barros, S.S., 2005. Hemorrhagic disease in dogs infected with an unclassified intraendothelial piroplasm in southern Brazil. Veterinary Parasitology 134, 193-213.

Matjila, P.T., Leisewitz, A.L., Jongejan, F., Penzhorn, B.L, 2008. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. Veterinary Parasitology 155, 152-157.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P., Nijhof, A.M., Jongejan, F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Veterinary Parasitology 122, 119-125.

Matjila, T.P., Nijhof, A.M., Taoufik, A., Houwers, D., Teske, E., Penzhorn, B.L., De Lange, T.D., Jongejan, F., 2005. Autochthonous canine babesiosis in The Netherlands. Veterinary Parasitology 131, 23-29.

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, 5907-11.

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

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular and Biological Evolution 4, 406-425.

Shakespeare, A.S., 1995. The incidence of canine babesiosis amongst sick dogs presented to the Onderstepoort Veterinary Academic Hospital. Journal of the South African Veterinary Association 66, 247-250.

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.

Stoltsz, W.H., Dunsterville, M.T., 1992. In vitro establishment and cultivation of a *Cytauxzoon* sp. (*Theileria* sp.) from a sable antelope (*Hippotragus niger*, Harris 1838). Journal of the South African Veterinary Association 63, 182 (Abstract).

Uilenberg, G., Franssen, F.F., Perie, N.M., Spanjer, A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Veterinary Quarterly. 11, 33-40.

Zahler, M., Rinder, H., Schein, E., Gothe, R., 2000. Detection of a new pathogenic *Babesia microti*-like species in dogs. Veterinary Parasitology 89, 241-248.

Chapter 7: Molecular characterization of *Babesia gibsoni* infection from a pit-bull terrier pup recently imported into South Africa.

7.1. Abstract

Canine babesiosis caused by *Babesia gibsoni* was diagnosed in a three-month-old pit-bull pup during a routine clinical examination. The dog was a three-month old pit-bull puppy imported from Philadelphia, USA. On physical examination, the dog had no typical signs of clinical babesiosis, except for a temperature of 39°C. PCR targeting the 18S rRNA gene was conducted followed by the Reverse line blot (RLB) assay. *Babesia gibsoni* infection was confirmed by way of smear examination, PCR, Reverse Line Blot (RLB) and sequence analysis. Sequence analysis revealed that the sequence had a 100% homology with the Asian genotype (accession no. AF205636). Treatment was initiated with diminazene aceturate (Berenil RTU®) followed by 2 doses of imidocarb diproprionate (Forray-65®) 3 days and 14 days later, respectively. *Babesia gibsoni* DNA was still detectable two weeks post-treatment on the PCR/RLB test. A ten-day course of combination drug therapy using atovaquone and azithromycin was initiated. Blood samples taken on Day 1 and Day 40 after completion of treatment were negative for *B. gibsoni* DNA on PCR/RLB test. Increased travel and movement of animals into South Africa has increased the possibility of new tick-borne pathogens being introduced in non-endemic areas. Without vigilant surveillance and stringent import control, the presence of potential tick vectors increases the risk of this pathogen becoming established in South Africa.

7.2. Introduction

Canine babesiosis is caused by large or small *Babesia* parasites. The large piroplasms form the *B. canis* (*senso lato*) group comprising three species: *B. canis* endemic in Southern Europe, *B. rossi* endemic in Sub-Saharan Africa and *B. vogeli* endemic in tropical and subtropical areas (Uilenberg, Franssen, Perie and Spanjer, 1989; Oyamada, Davoust, Boni, Dereure, Bucheton, Hammad, Itamoto, Okuda and Inokuma, 2005).

The small piroplasms, previously regarded as *Babesia gibsoni* (*senso lato*), have been reported to be endemic in Asia, North America, North and Eastern Africa, Europe and Australia (Yamane, Thomford, Gardner, Dubey, Levy and Conrad, 1993; Casapulla, Baldi, Avallone, Sannino, Pazzanese and Mizzoni, 1998; Muhlnickel, Jefferies, Morgan-Ryan and Irwin, 2002). There are at least three genetically distinct entities in this group: the Asian, North American (California) and Spanish isolates, respectively (Kjemtrup, Kocan, Whitworth, Meinkoth, Birkenheuer, Cummings, Boudreaux, Stockham, Irizarry-Rovira and Conrad, 2000). In the United States the most reported *B. gibsoni* infections are those of the Asian genotype (Irizarry-Rovira, Stephens, Christian, Kjemtrup, DeNicola, Widmer and Conrad, 2001; Kocan, Kjemtrup, Meinkoth, Whitworth, Murphy, Decker and Lorenz, 2001; MacIntire, Boudreaux, West, Bourne, Wright and Conrad, 2002; Birkenheuer, Levy and Breitschwerdt, 2003). The Spanish isolate was provisionally named *Theileria annae* (Zahler, Rinder, Schein and Gothe, 2000; Goethert and Telford, III, 2003) and the Californian isolate has been named *Babesia conradae* (Kjemtrup, Wainwright, Miller, Penzhorn and Carreno, 2006).

Clinical disease associated with *B. gibsoni* (*senso lato*) may range from being a mild to a severe infection (Birkenheuer, Levy, Savary, Gager and Breitschwerdt, 1999; Macintire et al., 2002). The disease may follow a hyper-acute, acute or chronic course. The acute course, is characterized by fever, lethargy, anaemia, thrombocytopenia, lymphadenopathy and splenomegaly (Irizarry-Rovira et al., 2001; Matsuu, Kawabe, Koshida, Ikadai, Okano and Higuchi, 2004). The hyper-acute state is characterized by shock and extensive tissue damage (Conrad, Thomford, Yamane, Whiting, Bosma, Uno, Holshuh and Shelly, 1991; Freeman, Kirby, Panciera, Henik, Rosin and Sullivan, 1994). The chronic form has been reported in Australia and the USA (Hughes and Oz, 1995; Kjemtrup et al., 2000), and although its difficult to diagnose chronic *Babesia canis* (*senso lato*) infections, PCR can be a useful tool in confirming chronic infections (Tuttle, Birkenheuer, Juopperi, Levy and Breitschwerdt, 2003).

Definitive diagnosis of *Babesia* infections in dogs depends on the demonstration of infected erythrocytes on Romanowsky-stained blood smears (Uilenberg et al., 1989). Although smear examination is useful, chances of false negatives are high in cases where parasitaemias are low. Indirect immunofluorescent antibody test (IFAT) can also be used for such cases (Uilenberg, Verdiesen and Zwart, 1981; Vercammen, De Deken and Maes, 1995). Also new technologies that include Polymerase Chain Reaction (PCR) and Reverse Line Blot (RLB) can be used to confirm *Babesia* infections (Matjila, Penzhorn, Bekker, Nijhof and Jongejan, 2004; Matjila, Nijhof, Taoufik, Houwers, Teske, Penzhorn, Lange and Jongejan, 2005).

Dogs with clinical babesiosis normally improve within 24 hours of treatment with an anti-babesial drug (Jacobson, Reyers, Berry and Viljoen, 1996). The drugs of choice in South Africa against *B. rossi* are diminazene aceturate and / or imidocarb diproprionate. A single dose of imidocarb at 7.5 mg/kg or a single dose of diminazene at 3.5 mg/kg followed by a dose of 6 mg/kg imidocarb the following day have been shown to clear *B. rossi* infections (Penzhorn, Lewis, De Waal and Lopez Rebollar, 1995). Diminazene and / or imidocarb are ineffective in treating *B. gibsoni* (Asian type) infections in dogs. The only therapy currently reported to successfully treat *B. gibsoni* infections in dogs is a combination of azithromycin and atovaquone (Birkenheuer, Levy and Breitschwerdt, 2004).

7.2.1. Case history

A three-month-old pit-bull terrier pup from the USA, imported into South Africa, was presented for routine microchip implantation and deworming. The owner indicated that the dog had good appetite and habitus. On physical examination, the dog had temperature of 39 4°C. A capillary blood smear was made and stained with Diff-Quick (Scientific Products Co., McGaw Park, USA). Numerous small piroplasms were seen on the smear (Fig. 7.1) which also revealed thrombocytopenia and a marked reticulocytosis. The dog was treated by subcutaneous injection of diminazene aceturate (Berenil RTU®) at a dose of 3.5 mg/kg.

Two days later the dog was returned to the clinic. On examination the dog had a rectal temperature of 38.6°C. A capillary blood smear was made and examined, and small

piroplasms were still present, although the parasitaemia was reduced. Based on blood smear examination thrombocyte numbers had increased and marked reticulocytosis persisted. On the 6th day, the dog's temperature was 38°C. A single infected erythrocyte was observed during smear examination as well as normal thrombocyte numbers, reticulocytosis and a monocytosis had developed. The dog was injected subcutaneously with imidocarb diproprionate (Forray-65®) at 6 mg/kg and the owner was requested to return the dog for a follow-up injection after 14 days.

Fourteen days later the dog had a rectal temperature of 39°C. Large numbers of small piroplasms were observed during smear examination, as well as a decrease in the number of thrombocytes and a marked increase in reticulocytes. A jugular blood sample was taken at this presentation and this was also the only point at which haematology was assessed. The same sample was also assessed using PCR/RLB test. The dog was again treated with imidocarb (Forray-65®) at a dose of 6 mg/kg.

The dog was examined again 14 days later. Blood smear investigations showed that the piroplasm parasitaemia had persisted. Treatment was initiated with azithromycin (10 mg/kg) given orally once daily for ten days and atovaquone (13.3 mg/kg) twice daily for ten days. Molecular evaluation of blood parasite DNA was carried out only on Day 1 and Day 40 after completion of treatment; on both occasions blood smears were examined and jugular blood samples were collected.

7.3. Materials and Methods

7.3.1. Collection of samples

Three blood samples collected in EDTA tubes at various intervals were sent to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, for PCR and RLB tests. A blood sample in EDTA was also sent to a private veterinary clinical pathology laboratory for a full haematological examination. The first sample for PCR/RLB test and the only sample subjected to a haematological examination was collected two weeks before treatment with azithromycin and atovaquone began. The two other samples were taken on Day 1 and Day 40 after completion of treatment with azithromycin and atovaquone.

7.3.2. DNA extraction

DNA was extracted from 200 µl of each blood sample using the QIAmp® blood and tissue extraction kit (Qiagen, Hilden, Germany), following the manufacturer's protocols.

7.3.3. PCR

PCR was performed with 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′) amplifying a fragment of 460-540bp from the 18S rRNA gene spanning the V4 region (Gubbels, de Vos, Van der Weide, Viseras, Schouls, de Vries and Jongejan, 1999; Matjila et al., 2004). The conditions for the PCR included an initial step of 3 min at 37º C, 10 min at 94ºC, 10 cycles of 94ºC (20s)- 67 ºC (30s)- 72º C (30s), with lowering of annealing step after every second cycle by 2º C (touchdown PCR). The reaction was then followed by 40

cycles of denaturation at 94º C for 30s, annealing at 57º C for 30s and extension at 72º C for 30s.

7.3.4. Reverse Line Blot hybridisation

PCR-amplified products were tested with the RLB, as previously described (Matjila et al., 2004). An additional plasmid control was used as an internal positive control to check whether all *Babesia* species–specific probes were correctly attached to the RLB membrane and functioning properly (Matjila et al., 2005).

7.3.5. Sequencing

Extracted DNA was PCR-amplified with the primers RLB-F2 and RLB-R2 and partial segments (400-540bp) of the PCR product were sequenced (Inqaba-Biotec, Pretoria, South Africa).

7.3.6. Phylogenetic analysis

Phylogenetic analysis was done to confirm the relationship between our positive *B. gibsoni* sample and other *Babesia* species (Fig. 7.2). Sequence data were assembled to a total length of 413bp using GAP4 of the Staden package (Version 1.6.0 for Windows). The sequences were aligned with sequences of related genera using ClustalX (Version 1.81 for Windows). The two parameter model of Kimura and the Jukes and the Cantor correction model for multiple base changes were used to construct similarity matrices (Jukes and Cantor, 1969; Kimura, 1980). Neighbor-joining (Saitou and Nei, 1987) and the maximum parsimony methods were used for the construction of phylogenetic trees

using the Mega 3.0 software package (Kumar, Tamura and Nei, 2004). The above methods were used in combination with the bootstrap method (Felsenstein, 1985).

7.4. Results

The blood sample collected prior to treatment with a combination drug therapy of atovaquone and azithromycin was confirmed positive for *B. gibsoni* by smear examination (Fig. 7.1), PCR and RLB. Sequence analysis (413bp) revealed that the sequence had a 100% homology with at least two full sequences (± 1600bp) of two genotypes: *B. gibsoni* (Japan, Aomori, AB118032) and *Babesia* sp. (Oklahoma, AF205636). Phylogenetic analysis procedures showed that the *B. gibsoni* (pit-bull terrier) sequence was closely related to *B. gibsoni* (Japan, Aomori, AB118032) and *Babesia* sp. (Oklahoma, AF205636) (Fig. 7.2). These procedures also showed that there were no significant changes in topology of trees or in bootstrap values using either the neighbor-joining or the maximum parsimony methods. Haematology results showed that the dog had a low PCV, a reduced erythrocyte count and a thrombocytopenia (Table 7.1).

The blood samples collected on Day 1 and Day 40 after completion of the combination drug therapy were negative on PCR/RLB test. All samples were negative for *Ehrlichia* infection, using PCR/RLB tests at all time points.

7.5. Discussion

In this study molecular techniques proved to be invaluable in confirming *B. gibsoni* infection in the imported dog. Prior to the blood samples being sent for PCR/RLB test it

was not clear which piroplasm was parasitizing the erythrocytes. Although the dog had no obvious signs of clinical babesiosis, microscopic examination of the capillary blood smears revealed the presence of piroplasms in the erythrocytes. Prior to the atovaquone / azithromycin therapy, infected erythrocytes could still be observed on capillary blood smears on all five occasions that the dog was examined. This was true for up to one month after treatment with diminazene and imidocarb. A number of researchers have reported that treatment with diminazene and / or imidocarb is ineffective against *B. gibsoni* (Asian genotype) infection (Birkenheuer et al., 1999; Stegeman, Birkenheuer, Kruger and Breitschwerdt, 2003). After completion of the atovaquone / azithromycin therapy, no infected erythrocytes were observed on capillary blood smear examination. PCR/RLB results were also negative. This treatment regimen was therefore successful in either clearing the infection or reducing the parasite load to below the detection limit of our PCR/RLB assay. Some dogs that had been treated with this combination drug therapy were negative on PCR assay up to120 days post treatment (Birkenheuer et al., 2004).

The dog was moderately anaemic and thrombocytopenic. Dogs sub-clinically infected with *B. gibsoni* might be anaemic as a result of the host response to the parasite, immune-mediated erythrocyte destruction, or a combination of the host immune-mediated intravascular and extravascular haemolysis (Conrad et al., 1991; Adachi, Tateishi, Horii, Nagatomo, Shimizu and Makimura, 1995). Thrombocytopenia with variable leukocyte change is also a common feature of dogs infected with *B. gibsoni* (Conrad et al., 1991; Wozniak, Barr, Thomford, Yamane, McDonough, Moore, Naydan, Robinson and

Conrad, 1997; Birkenheuer et al., 1999; Wilkerson, Shuman, Swist, Harkin, Meinkoth and Kocan, 2001; Fukumoto, Suzuki, Igarashi and Xuan, 2005) .

Dogs imported into South Africa are subject to pre-import blood tests, including *B. gibsoni*, or exporting countries certifying freedom of disease, as this is a controlled disease in South Africa. Countries declaring freedom from *B. gibsoni* and other diseases are not required to do pre-importation blood testing. Only dogs with negative test results or those coming from *B. gibsoni*-free countries may be imported. In addition, dogs imported from certain countries are subjected to post-arrival quarantine and repeat serological blood testing on arrival in South Africa. *B. gibsoni* does occur in the USA and thus dogs from that country are tested prior to import but are not subjected to post-arrival quarantine. As this pit-bull terrier had tested negative in the USA within 30 days prior to importation, it was allowed to enter the country. The owner alleged that the dog had been in South Africa two weeks before it was taken to the veterinarian. The chances of the infection having been acquired locally are negligible since *B. gibsoni* is not endemic in South Africa. The translocation of infected dogs into *B. gibsoni*-free areas has been implicated as an important element in the spread of this parasite (Conrad et al., 1991).

Bull terrier-type dog breeds have a higher incidence of sub-clinical *B. gibsoni* infection than other breeds (Birkenheuer et al., 1999; Kocan et al., 2001). American pit-bull terriers are reported to be the most commonly reported *B. gibsoni*-infected breed in the USA, the reasons for this however remain unclear (Birkenheuer et al., 1999; Kocan et al.,

2001; MacIntire et al., 2002). Although transmission experiments have not yet proved which tick species is the vector for *B. gibsoni*, *Haemaphysalis bispinosa*, *H. longicornis*, *H. leachi* and *R. sanguineus* have been implicated in the transmission of the parasite (Wozniak et al., 1997; Kjemtrup et al., 2000). Both *H. elliptica* and *R. sanguineus* are endemic to South Africa (Horak, 1995) and they could be suitable vectors should *B. gibsoni* become established in South Africa.

Increased travel and movement of animals into South Africa have increased the possibility of new tick-borne pathogens being introduced in non-endemic areas. The risk of *B. gibsoni* becoming established in South Africa is limited, due to the requirements of pre-import testing of all dogs from countries that are not free of *B. gibsoni*. It is also crucial that blood testing should always include serological and molecular testing. Without vigilant surveillance and stringent import control, the presence of potential tick vectors increases the risk of this pathogen becoming established in South Africa. Furthermore, it has been suggested in Japan that in the absence of the tick vector, there is a possibility that *B. gibsoni* might be transmitted through blood contamination during dog fights (Matsuu et al., 2004). The dog reported in this case report was lost to further clinical follow up, as the dog had allegedly died of unknown causes.

7.6. Conclusion

This dog should not have been treated, as *B. gibsoni* is a controlled disease in South Africa and treatment is not allowed. Private veterinarians should be aware of possible diagnosis of similar diseases, which do not usually occur in South Africa, especially in

imported dogs. The diagnosis of any controlled disease in South Africa must be reported to the Veterinary Administration (National Department of Agriculture), and these diseases may only be managed and treated with express permission and under direction of the Senior Manager Animal Health, Department of Agriculture.

7.7. Figures and Tables

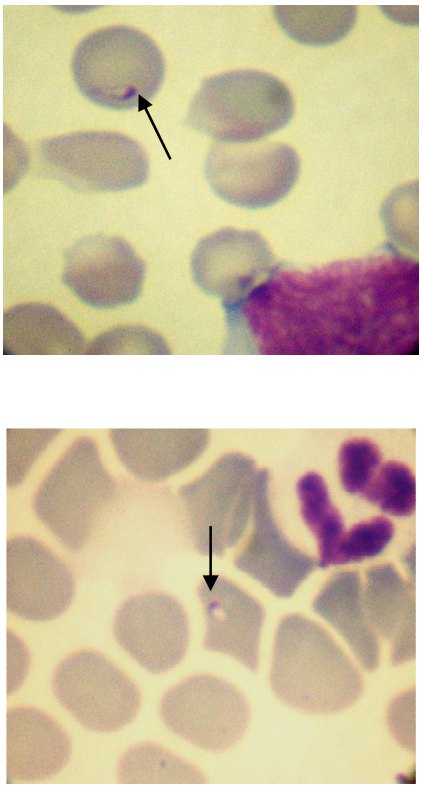


Figure 7.1: Giemsa-stained blood smears showing infected erythrocytes (magnification, x 1000).

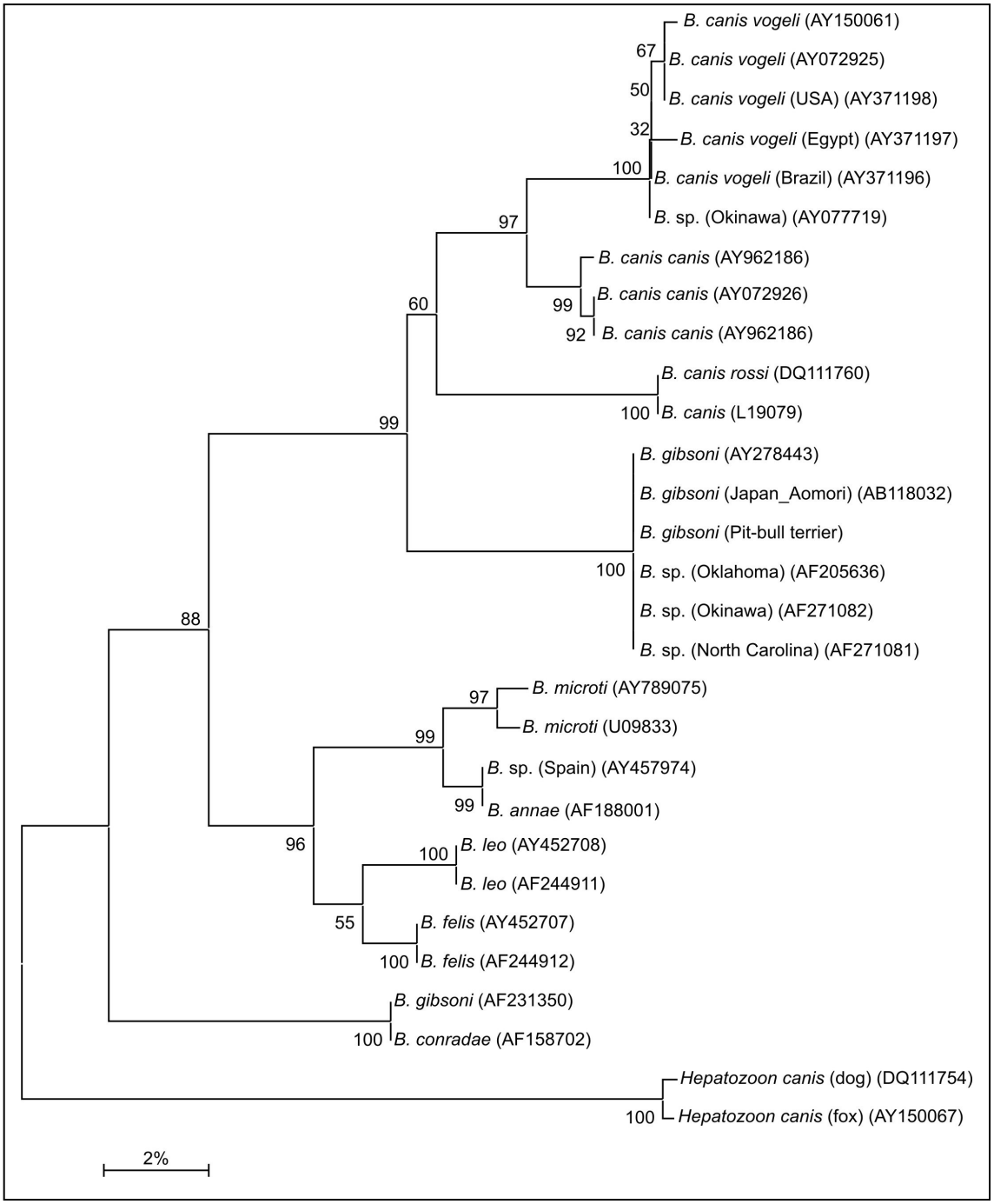


Figure 7.2: Neighbor-joining tree, based on the Kimura two-parameter distance calculation, showing the phylogenetic relationship of *B. gibsoni* (pit-bull terrier) to other *Babesia* sp. Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. The scale bar represents the % nucleotide difference. *Hepatozoon canis* was used as an outgroup.
NB: *B. gibsoni* (AF231350): This sequence is another isolate of *B. conradae* (Kjemtrup et al., 2006).

Table 7.1: Haematological report indicating full blood counts 2 weeks prior to treatment of the dog with a combination of atovaquone and azithromycin.

		Normal range	
Red blood cell count	4.2 x 10^{12} /l	5.5	8.5
Haemoglobin	9.55 g/l	12	18
Haematocrit (PCV)	29.00%	37	55
Mean cell volume (MCV)	69.00 fl	60	77
Mean cell haem cons (MCHC)	33.00 fl	32	36
White cell count	7.32 x 10^{9} /l	6	15
Platelet counts	83.00 x 10^{9} /l	200	500
Differential count			
Segmented neutrophils %	64	60.0	80
Band neutrophils %	2.00	0	3
Lymphocytes %	17.00	12	30
Monocytes %	15.00	3	10
Eosinophils %	1.00	2	10
Basophils%	1.00	0	3
Segmented neutrophils (abs)	4.68 x 10^{9} /l	3	1.5
Band neutrophils (abs)	0.15 x 10^{9} /l	0	.03
Lymphocytes (abs)	1.24 x 10^{9} /l	1	4.8
Monocytes (abs)	1.10 x 10^{9} /l	0.1	1.4
Eosinophils (abs)	0.07 x 10^{9} /l	0	1.2
Basophils (abs)	0.07 x 10^{9} /l	0	.02

7.8. References

Adachi, K., Tateishi, M., Horii, Y., Nagatomo, H., Shimizu, T., Makimura, S., 1995. Immunologic characteristics of anti-erythrocyte membrane antibody produced in dogs during *Babesia gibsoni* infection. Journal of Veterinary Medical Science 57, 121-123.

Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2004. Efficacy of combined atovaquone and azithromycin for therapy of chronic *Babesia gibsoni* (Asian genotype) infections in dogs. Journal of Veterinary Internal Medicine 18, 494-498.

Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2003. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. Journal of Clinical Microbiology 41, 4172-4177.

Birkenheuer, A.J., Levy, M.G., Savary, K.C., Gager, R.B., Breitschwerdt, E.B., 1999. *Babesia gibsoni* infections in dogs from North Carolina. Journal of the American Animal Hospital Association 35, 125-128.

Casapulla, R., Baldi, L., Avallone, V., Sannino, R., Pazzanese, L., Mizzoni, V., 1998. Canine piroplasmosis due to *Babesia gibsoni*: clinical and morphological aspects. Veterinary Record 142, 168-169.

Conrad, P., Thomford, J., Yamane, I., Whiting, J., Bosma, L., Uno, T., Holshuh, H.J., Shelly, S., 1991. Hemolytic anemia caused by *Babesia gibsoni* infection in dogs. Journal of the American Animal Hospital Association 199, 601-605.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 783-791.

Freeman, M.J., Kirby, B.M., Panciera, D.L., Henik, R.A., Rosin, E., Sullivan, L.J., 1994. Hypotensive shock syndrome associated with acute *Babesia canis* infection in a dog. Journal of the American Animal Hospital Association 204, 94-96.

Fukumoto, S., Suzuki, H., Igarashi, I., Xuan, X., 2005. Fatal experimental transplacental *Babesia gibsoni* infections in dogs. International Journal of Parasitology 35, 1031-1035.

Goethert, H.K., Telford, S.R., III, 2003. What is *Babesia microti*? Parasitology 127, 301-309.

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.

Horak, I.G., 1995. Ixodid ticks collected at the Faculty of Veterinary Science, Onderstepoort, from dogs diagnosed with *Babesia canis* infection. Journal of the South African Veterinary Association 66, 170-171.

Hughes, W.T., Oz, H.S., 1995. Successful prevention and treatment of babesiosis with atovaquone. Journal of Infectious Diseases 172, 1042-1046.

Irizarry-Rovira, A.R., Stephens, J., Christian, J., Kjemtrup, A., DeNicola, D.B., Widmer, W.R., Conrad, P.A., 2001. *Babesia gibsoni* infection in a dog from Indiana. Veterinary Clinical Pathology 30, 180-188.

Jacobson, L.S., Reyers, F., Berry, W.L., Viljoen, E., 1996. Changes in haematocrit after treatment of uncomplicated canine babesiosis: a comparison between diminazene and trypan blue, and an evaluation of the influence of parasitaemia. Journal of the South African Veterinary Association 67, 77-82.

Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro, H.N. (Ed.), Mammalian Protein Metabolism. Academic Press, New York, pp. 21-132.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16, 111-120.

Kjemtrup, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad, P.A., 2000. There are at least three genetically distinct small piroplasms from dogs. International Journal of. Parasitology 30, 1501-1505.

Kjemtrup, A.M., Wainwright, K., Miller, M., Penzhorn, B.L., Carreno, R.A., 2006. *Babesia conradae*, sp. nov., a small canine *Babesia* identified in California. Veterinary Parasitology 138, 103-111.

Kocan, A.A., Kjemtrup, A., Meinkoth, J., Whitworth, L.C., Murphy, G.L., Decker, L., Lorenz, M., 2001. A genotypically unique *Babesia gibsoni*-like parasite recovered from a dog in Oklahoma. Journal of Parasitology 87, 437-438.

Kumar S, Tamura K, Nei M, 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinformatics 5, 150-163.

MacIntire, D.K., Boudreaux, M.K., West, G.D., Bourne, C., Wright, J.C., Conrad, P.A., 2002. *Babesia gibsoni* infection among dogs in the south-eastern United States. Journal of the American Veterinary Medical Association 220, 325-329.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P., Nijhof, A.M., Jongejan, F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Veterinary Parasitology 122, 119-125.

Matjila, T.P., Nijhof, A.M., Taoufik, A., Houwers, D., Teske, E., Penzhorn, B.L., Lange, T.D., Jongejan, F., 2005. Autochthonous canine babesiosis in The Netherlands. Veterinary Parasitology 131, 23-29.

Matsuu, A., Kawabe, A., Koshida, Y., Ikadai, H., Okano, S., Higuchi, S., 2004. Incidence of canine *Babesia gibsoni* infection and sub-clinical infection among Tosa dogs in Aomori Prefecture, Japan. Journal of Veterinary Medical Science 66, 893-897.

Muhlnickel, C.J., Jefferies, R., Morgan-Ryan, U.M., Irwin, P.J., 2002. *Babesia gibsoni* infection in three dogs in Victoria. Australian Veterinary Journal 80, 606-610.

Oyamada, M., Davoust, B., Boni, M., Dereure, J., Bucheton, B., Hammad, A., Itamoto, K., Okuda, M., Inokuma, H., 2005. Detection of *Babesia canis rossi*, *B. canis vogeli*, and *Hepatozoon canis* in dogs in a village of eastern Sudan by using a screening PCR and sequencing methodologies. Clinical Diagnostic Laboratory Immunology 12, 1343-1346.

Penzhorn, B.L., Lewis, B.D., De Waal, D.T., Lopez Rebollar, L.M., 1995. Sterilisation of *Babesia canis* infections by imidocarb alone or in combination with diminazene. Journal of the South African Veterinary Association 66, 157-159.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406-425.

Stegeman, J.R., Birkenheuer, A.J., Kruger, J.M., Breitschwerdt, E.B., 2003. Transfusion-associated *Babesia gibsoni* infection in a dog. Journal of the American Veterinary Medical Association 222, 959-963.

Tuttle, A.D., Birkenheuer, A.J., Juopperi, T., Levy, M.G., Breitschwerdt, E.B., 2003. Concurrent bartonellosis and babesiosis in a dog with persistent thrombocytopenia. Journal of the American Veterinary Medical Association 223, 1306-1310.

Uilenberg, G., Franssen, F.F., Perie, N.M., Spanjer, A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Veterinary Quarterly 11, 33-40.

Uilenberg, G., Verdiesen, P.A., Zwart, D., 1981. Imidocarb: a chemoprophylactic experiment with *Babesia canis.* Veterinary Quarterly 3, 118-123.

Vercammen, F., De Deken, R., Maes, L., 1995. Clinical and serological observations on experimental infections with *Babesia canis* and its diagnosis using the IFAT. Parasite 2, 407-410.

Wilkerson, M.J., Shuman, W., Swist, S., Harkin, K., Meinkoth, J., Kocan, A.A., 2001. Platelet size, platelet surface-associated IgG, and reticulated platelets in dogs with immune-mediated thrombocytopenia. Veterinary Clinical Pathology 30, 141-149.

Wozniak, E.J., Barr, B.C., Thomford, J.W., Yamane, I., McDonough, S.P., Moore, P.F., Naydan, D., Robinson, T.W., Conrad, P.A., 1997. Clinical, anatomic, and immunopathologic characterization of *Babesia gibsoni* infection in the domestic dog (*Canis familiaris*). Journal of Parasitology 83, 692-699.

Yamane, I., Thomford, J.W., Gardner, I.A., Dubey, J.P., Levy, M., Conrad, P.A., 1993. Evaluation of the indirect fluorescent antibody test for diagnosis of *Babesia gibsoni* infections in dogs. American Journal of Veterinary Research. 54, 1579-1584.

Zahler, M., Rinder, H., Schein, E., Gothe, R., 2000. Detection of a new pathogenic *Babesia microti*-like species in dogs. Veterinary Parasitology 89, 241-248.

Chapter 8: General discussion

8.1. General discussion

In this thesis several tick-borne pathogens that occur in dogs, particularly in South Africa, are identified and characterized. Parasite occurrence, clinical relevance, molecular diagnosis and, to some extent, the genetic diversity of a number of protozoan tick-borne pathogens was investigated. In this concluding chapter, the most important findings of the preceding chapters are summarized and discussed. Specific recommendations for the control of tick-borne diseases in dogs are given and, finally, opportunities for future research are suggested.

8.1.1. Babesia rossi

The most common haemoparasite species detected in domestic dogs in all areas sampled was *B. rossi* (Chapter 3). Interestingly, there appeared to be a correlation between the clinical manifestation of the disease and the *B. rossi* genotype based on the *BrEMA*1 gene. In particular, genotype11, 19 and 28/29 were mostly associated with complications leading to death as a result of solid-organ complications. The analysis indicated that the *BrEMA*1 gene may be a suitable genetic marker for surveying *B. rossi* infections in South Africa, especially since *B. vogeli* and *B. canis* isolates were shown not to have the *BrEMA*1 gene. Early detection of virulent genotypes may alert clinicians to be especially vigilant for early signs of complications.

8.1.2. Babesia vogeli

The discovery of *Babesia vogeli* in dogs for the first time in South Africa (Chapter 2) implied that there are currently two species of *Babesia* implicated as the cause of canine babesiosis. This may explain why canine babesiosis has such varied clinical manifestations, which range from a very mild to a fatal infection. Some of these differences may be due to an excessive inflammatory response rather than due to the parasite itself. We conclude, however that the presence of both *B. rossi* and *B. vogeli* contributes partially to the varied clinical manifestations that are typical of the disease to South Africa. Thus some non-complicated cases of canine babesisois may be as a result of *B. vogeli* infections whereas severe to complicated cases may be due to *B. rossi* infections.

In general, however, Uilenberg, Franssen, Perie, and Spanjer (1989) suggested that *B. vogeli* parasites may occur in large parts of tropical and subtropical regions on all continents, coinciding with the global distribution of *R. sanguineus* ticks. Our finding confirmed this suggestions since both *H. elliptica* (regarded as a synonym of *Haemaphysalis leachi*; Apanaskevic, Horak and Camica, 2007) and *R. sanguineus* have been collected from dogs presented with babesiosis at the Onderstepoort Veterinary Academic Hospital (Horak, 1995).

8.1.3. Babesia gibsoni

Babesia gibsoni, another tick-borne pathogen causing canine babesiosis, was diagnosed in a three-month-old pit-bull pup imported into South Africa during a routine clinical

examination (Chapter 7). Diagnosis was confirmed by way of blood smear examination, PCR/RLB and sequence analysis. Prior to the blood samples being sent for PCR/RLB test it was not clear which piroplasm was parasitizing the erythrocytes. Although the dog had no obvious signs of clinical babesiosis, microscopic examination of the capillary blood smears revealed the presence of piroplasms in the erythrocytes. Prior to the atovaquone / azithromycin therapy, infected erythrocytes could still be observed on capillary blood smears on all five occasions during which the dog was examined. Treatment of canine babesiosis is effective when the disease is treated at an early stage with diminazene aceturate (Berenil RTU®) and imidocarb diproprionate (Forray-65®) singly or in combination. However, *B. gibsoni* infections will only respond to a combination drug therapy of atovaquone and azithromycin. There is a potential risk of *B. gibsoni* becoming established in South Africa, especially since the possible tick vector, *R. sanguineus*, is present in dog populations.

Although transmission experiments have not been done to demonstrate which tick species is the vector for *B. gibsoni*, both *H. leachi* and *R. sanguineus* have been incriminated in the transmission of the parasite (Kjemtrup, Kocan, Whitworth, Meinkoth, Birkenheuer, Cummings, Boudreaux, Stockham, Irizarry-Rovira and Conrad, 2000; Wozniak, Barr, Thomford, Yamane, McDonough, Moore, Naydan, Robinson and Conrad, 1997).

8.1.4. Babesia canis

Babesia canis has never been reported in South Africa and given its tick specificity is unlikely to ever be reported as an autochthonous case. An outbreak of canine babesiosis

in the Netherlands in dogs that had never been outside the country is described in chapter 5. In the spring of 2004, pathogen DNA isolated from 18 cases (including four fatalities) was found positive for *B. canis,* whereas other haemoparasites could be excluded. According to the literature, a single dose (7.5 mg/kg) of imidocarb dipropionate (Penzhorn, Lewis, De Waal and Lopez Rebollar, 1995; Boozer and MacIntire, 2003) or two doses (7mg/kg) with a 14-day interval (Brandao, Hagiwara and Myiashiro, 2003) will sterilize the infection. In addition to the life-saving ability of the drug, sterilizing the infection in dogs in the Netherlands would render the dogs non-infective for ticks. Moreover, it would also ease matters with respect to blood-component therapy, which has become more accessible in veterinary practice over recent years. Vaccination, which sufficiently reduces clinical symptoms but does not prevent infection, would be the preferred preventive measure in an endemic area (Schetters, Kleuskens, Scholtes, Pasman and Goovaerts, 1997). A survey to identify ticks and tick-borne pathogens in dogs presented at veterinary clinics in the Netherlands was initiated after the fatal cases had occurred in 2004. The results of the survey were recently published (Nijhof, Bodaan, Postigo, Nieuwenhuijs, Opsteegh, Franssen, Jebbink and Jongejan, 2007) wherein it was shown that *Dermacentor* ticks have established themselves at several locations in the Netherlands in large numbers. *Dermacentor reticulatus* adults and nymphs were found in six localities, all with apparently suitable habitats (Nijhof et al., 2007). With *D. reticulatus* seemingly becoming established in the Netherlands, it is anticipated that *B. canis*-related canine babesiosis will be an endemic disease in the Netherlands.

8.1.5. Theileria sp.

In Chapter 6, a novel *Theileria* species that was detected in domestic dogs from Pietermaritzburg in the KwaZulu-Natal province and from the Onderstepoort Veterinary Academic Hospital is reported. Although the pathophysiology of the detected *Theileria* sp. in dogs is unknown, it is apparent from the few cases described here that haemolysis and an immune-mediated syndrome may be associated with this organism. Similar clinical findings have been reported for *Theileria annae* infected dogs (Garcia, 2006). Phylogenetic analysis showed a close similarity with one to four base pair difference with *Theileria* sp. (sable), the species that causes mortalities in sable antelopes (Nijhof, Pillay, Steyl, Prozesky, Stoltsz, Lawrence, Penzhorn and Jongejan, 2005). To our knowledge, none of the dogs from which the *Theileria s*p. was isolated, died as a result of the infection. This finding adds a *Theileria* species to the list of haemoprotozoan parasites infecting dogs in South Africa. Isolation of the parasite and subsequent tick-transmission studies will be required in order to determine the importance of this *Theileria* species.

8.1.6. Ehrlichia / Anaplasma species

Currently the two tick-transmitted *Ehrlichia* / *Anaplasma* species known to cause human disease are *E. chaffeensis* and *Anaplasma phagocytophilum*. Although we had anticipated detecting some of these zoonotic *Ehrlichia* and *Anaplasma* species, our results were negative for the known zoonotic species. The reported detection of *E. chaffeensis* in dogs and in a human being from Bloemfontein, Free State Province ((Pretorius, Venter, Ryst and Kelly, 1999), was based on serological assay and not on the detection of parasite DNA. Cross-reactivity between *Ehrlichia* infections are known to

occur (Parola, Inokuma, Camicas, Brouqui and Raoult, 2001), which suggests that molecular assays should be used to support serological evidence. On the other hand, a species closely related to *A. phagocytophilum* has been identified from 3 dog samples in South Africa (Inokuma, Oyamada, Kelly, Jacobson, Fournier, Itamoto, Okuda, and Brouqui, 2005). A more directed study considering *Ehrlichia*-infected dogs only may elucidate the existence of potentially zoonotic *Ehrlichia* species.

Our current results indicate that the abundance of tick vectors, on domestic and wild canine hosts, encourages the cyclical transmission of tick-borne pathogens in the country. Although tick vectors of *B. vogeli* and *E. canis* (*R. sanguineus*), and *B. rossi* (*H. elliptica*) have overlapping distribution and have been isolated on the same host, dual infections of blood parasites seems to be rare in South Africa. Molecular diagnostic techniques allow previously unknown species to be identified. There is no doubt that if the current momentum of research is maintained, various other important pathogens will be discovered, which will in return influence our understanding of the epidemiology, management and treatment of tick-borne pathogens of domestic dogs.

Travel within and movement of animals into South Africa has increased the possibility of new tick-borne pathogens being introduced to non-endemic areas. The risk of *B. gibsoni* becoming established in South Africa is limited, due to the requirements of pre-import testing of all dogs from countries that are not free of *B. gibsoni*. It is also crucial that blood testing should always include serological and molecular testing. Without vigilant surveillance and stringent import control, the presence of potential tick vectors increases the risk of this pathogen becoming established in South Africa.

8.1.7. Multiple infections

Dual infections of *B. rossi* and *E. canis* were also detected in all our sampled areas except in Free State and Eastern Cape provinces. These could indicate that *H. elliptica* and *R. sanguineus* have overlapping distributions and also feed on the same hosts in those areas.

Therefore there exists a wide variety of tick-borne pathogen in dog populations, in so far as we can generalize:

- *Babesia rossi* is probably the most prevalent tick-born pathogen of dogs in South Africa.
- *Babesia vogeli* occurs in South Africa, but it is not as prevalent as *B. rossi*.
- There is a novel *Theileria* species infecting dogs, which may be linked to anaemia (possibly haemolytic) and a possible immune-mediated syndrome.
- *Ehrlichia canis* is present in dog populations and it is usually found in dual infections with *B. rossi*, *B. vogeli* or the novel *Theileria* sp. (dog).
- Mixed infections of *B. rossi* and *B. vogeli* and of *B. rossi, B. vogeli* and *E. canis* occurring simultaneously in dogs are rare.

We are also able to conclude the following:

- Although the occurrence of *B. vogeli* has been confirmed, its clinical importance in South Africa is regarded as less than that of *B. rossi*.
- There are several *B. rossi* genotypes, based on the *BrEMA*1 gene.
- There may be a correlation between *B. rossi* genotypes and canine babesiosis phenotypes.

- *BrEMA1* gene might be a good genetic marker for surveying *B. rossi* infections in South Africa.
- Virulent *B. rossi* genotypes could possibly cause differing host responses to infection.
- *Babesia vogeli* and *B. canis* parasites do not have the gene *BrEMA*1.

There is evidence to suggest that tick-borne pathogens have been introduced in previously non-endemic areas based on the following:

- *Babesia canis* is becoming endemic in pockets of dog populations in the Netherlands.
- There are localized populations of *Dermacentor reticulatus* in the Netherlands.
- A previously unknown *Theileria* sp. occurs in dogs in South Africa.
- *Babesia gibsoni* infections can be misdiagnosed if diagnosis is based only on smear examination without the use of molecular diagnostics.
- Diminazene aceturate (Berenil RTU®) and imidocarb diproprionate (Forray-65®) are only effective against the large *Babesia* of dog infections.
- A 10-day course of combination drug therapy of atovaquone and azithromycin is effective against *B. gibsoni* infections.

8.2. Conclusion

In this thesis we were able to identify the most prevalent tick-borne pathogens infecting domestic dogs in the areas studied. Species of organisms responsible for canine babesiosis and ehrlichiosis were isolated and characterized. Although canine babesiosis

is an endemic disease in South Africa, it has only been associated with *B. rossi*. We discovered that two species of *Babesia* were responsible for canine babesiosis in South Africa. Canine babesiosis associated with *B. canis* infections was found to be the only cause of out-breaks in the Netherlands. *Babesia vogeli* was identified for the first time in South Africa, but clinical canine babesiosis is mostly associated with *B. rossi* infections. Characterization of the *B. rossi (BrEMA*1) revealed several genotypes. These genotypes seem to differ in virulence. It was found that only genotypes 11, 19 and 28/29 were associated with solid-organ complications. Although genotype19 induced around twice as many solid-organ complications and death than genotype 28/29, genotype 28/29 showed the same severity in causing complicated disease and subsequent death.

The only *Theileria* species known to cause clinical disease in dogs is *Theileria annae*, which is endemic only in Spain. We detected for the first time a novel *Theileria* species of dogs in South Africa. The *Theileria* species described seems to be associated with haemolysis and an immune-mediated syndrome in infected dogs. This species was also found concurrently with *E. canis* infections. Although the tick vector of this species is unknown, it is suspected that *R. sanguineus* may play a role in the transmission of the *Theileria* species.

8.2.1. Control measures

Soluble parasite antigens (SPA) derived from serum of *Babesia*-infected animals or supernatants of *in vitro* culture of *Babesia* parasites have been shown to confer protection against challenge infection when used as a vaccine (Schetters and Montenegro-James,

1995). Initial attempts to produce such a vaccine against *Babesia rossi* infection using SPA from *B. rossi* culture supernatants were not or were only partially successful. Recent reports have shown that a vaccine containing a mixture of SPA obtained from *in vitro* cultures of *B. rossi* and *B. canis* induces protection in dogs against heterologous challenge infection with *B. canis* (Schetters, Kleuskens, Scholtes, Gorenflot, Moubri and Vermeulen, 2001) and heterologous challenge infection with *B. rossi* (Schetters, Strydom, Crafford, Kleuskens, Van de Crommert, and Vermeulen, 2007). Although this improved vaccine (Nobivac Piro, Intervet) (Schetters, Kleuskens, Carcy, Gorenflot and Vermeulen, 2007) is available commercially in Europe, it is not yet available in South Africa. Chemotherapy is still the method of choice for treatment and control of *Babesia* infections in dogs in South Africa. Treatment of canine babesiosis is effective when the disease is treated at an early stage with diminazene aceturate (Berenil RTU®) and/or imidocarb diproprionate (Forray-65®). However, *B. gibsoni* infections will only respond to a combination drug therapy of atovaquone and azithromycin. Since *B. gibsoni* is not endemic in South Africa, this treatment regimen is often not used. Dogs infected with *Ehrlichia* often recover from infection after treatment with tetracyclines. Alternatively, prophylactic use of impregnated dog-collars seems to be effective in preventing tick-infestations and ultimately the transmission of tick-borne pathogens (Last, Hill, Matjila and Reme, 2007). The implications are that if clinical diagnosis is not accompanied by molecular confirmation, then chances of the wrong treatment being administered are high. Also, abundance of different tick species in endemic areas further validates the use of molecular techniques as a tool in ensuring that the correct treatment is used for the specific pathogen infection.

8.2.2. Scope for future research

The indication of a possible correlation of *B. rossi* genotypes to disease phenotype was one of the interesting findings of this thesis. The significant suggestion of this work is that different parasite genotypes may cause differing host responses to infection (i.e. there could be a relationship between parasite genotypes and disease pathogenesis). Changing disease outcome through treatment depends heavily on understanding of disease pathogenesis. We have identified *BrEMA*1 as a valuable genetic marker for the diagnosis of virulent genotypes. The currently available test to date is however, a double PCR (18S rDNA PCR first, followed by *BrEMA*1 PCR). Thus, a real-time PCR-based test will have the advantages of being more sensitive, rapid and less time-consuming than our current double PCR test.

Furthermore, the *BrEMA*1 is located on the cytoplasmic side of red blood cell-membrane under an insoluble phosphoprotein. A soluble antigen found in the supernatant of *B. rossi in vitro* culture is also present, which is immunogenic to the hyperimmune serum from *B. rossi* infected dogs. Moreover, like many repetitive regions of parasitic antigens, the recombinant repetitive region of *BrEMA*1 is highly immunogenic. Currently, there is no ELISA test available to evaluate the incidence of babesiosis in South Africa at the immunological level. The recombinant *BrEMA*1 is a valuable antigen for the development of such test.

The relationship between virulence and sequestration (by molecular and cellular analysis of the surface of the infected erythrocytes and by identifying parasite proteins present on

the membrane of the infected erythrocyte by electron microscopic analysis of the surface modifications) needs to be investigated. To date, available data concerning the function of adhesive antigens, located on the extracellular side of infected red blood cell-membrane, in the pathogenicity of apicomplexan haemoparasites is mostly derived from the human malaria parasite *P. falciparum* and, to a lesser extent, from the bovine babesiosis parasite *B. bovis.* Adhesive surface antigens from the extracellular side of the sequestering canine *Babesia* species *B. rossi* are unknown. This antigen needs to be characterized, not only for a better understanding of mechanisms governing the pathogenicity of *B. rossi* in dogs, but also of the genus *Babesia* in general.

Preliminary evidence presented in this thesis indicated the presence of a *Theileria* species which may be linked to a haemolytic and immune-mediated syndrome. Although the parasite was detected from a number of samples, only three samples were from dogs showing clinical signs. The clinical significance of this *Theileria* species needs to be investigated. All the *Theileria*-positive dogs had a common feature in that they each had thrombocytopenia in the absence of *Ehrlichia* infection. Dogs that are presented with an unexplained thrombocytopenia should be selected and sampled. Attempts should be made to isolate the parasite and adapt it to *in vitro* cultures. Experimental tick-transmission trials should be undertaken, to establish the tick vector. Additionally, the life cycle of this *Theileria* species could be investigated by conducting experimental infections in dogs.

Although there is no evidence to link *B. vogeli* to severe clinical disease in South Africa, it has been linked to significant clinical disease in the USA and Australia, areas from which *B. rossi* infections are known not to occur. The hypothesis is that in the absence of *B. rossi*, *B. vogeli* can cause a significant clinical disease. There is a need to compare the different *B. vogeli* isolates from different regions, especially those areas free of *B. rossi*. This will provide information on genetic diversity. Correlations could also be made between virulent genotypes and clinical manifestations.

Finally, the work done in this thesis has shown that populations of dogs that live in tick-endemic areas are exposed to single or multiple tick-borne pathogens. These pathogens continue to cause morbidity and mortality in susceptible dogs. Correct diagnosis (supported by molecular diagnostic tools) followed by appropriate treatment offers a better understanding and management of these tick-borne pathogens. Preventative measures either by vaccination or transmission-blocking acaricidal treatments should be fully evaluated and applied to prevent these tick-borne pathogens from adversely affecting the canine population in South Africa and elsewhere.

8.3. References

Apanaskevich, D.A., Horak, I.G., Camica J.-L., 2007. Redescription of *Haemaphysalis* (*Rhipistoma*) *elliptica* (Koch, 1844), an old taxon of the *Haemaphysalis* (*Rhipistoma*) *leachi* group from East and Southern Africa, and of *Haemaphysalis* (*Rhipistoma*) *leachi* (Audouin, 1826) (Ixodida, Ixodidae). Onderstepoort Journal of Veterinary Research 74, 181-208.

Boozer, A.L., MacIntire, D.K., 2003. Canine babesiosis. Veterinary Clinics of the North American Small Animal Practice 33, 885-904.

Brandao, L.P., Hagiwara, M.K., Myiashiro, S.I., 2003. Humoral immunity and re-infection resistance in dogs experimentally inoculated with *Babesia canis* and either treated or untreated with imidocarb dipropionate. Veterinary Parasitology 114, 253-265.

Garcia, A.T.C., 2006. Piroplasma-infection in dogs in northern Spain. Veterinary Parasitology 138, 97-102.

Horak, I.G., 1995. Ixodid ticks collected at the Faculty of Veterinary Science, Onderstepoort, from dogs diagnosed with *Babesia canis* infection. Journal of the South African Veterinary Association 66, 170-171.

Inokuma, H., Oyamada, M., Kelly, P.J., Jacobson, L.A., Fournier, P.E., Itamoto, K., Okuda, M., Brouqui, P., 2005. Molecular detection of a new *Anaplasma* species closely related to *Anaplasma phagocytophilum* in canine blood from South Africa. Journal of Clinical Microbiology 43, 2934-2937.

Kjemtrup, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad, P.A.,

2000. There are at least three genetically distinct small piroplasms from dogs. International Journal of Parasitology 30, 1501-1505.

Last, R.D., Hill, J.M., Matjila, P.T., Reme, C.A., 2007. A field trial evaluation of the prophylactic efficacy of amitraz-impregnated collars against canine babesiosis (*Babesia canis rossi*) in South Africa. Journal of the South African Veterinary Association 78, 63-65.

Nijhof, A.M., Bodaan, C., Postigo, M., Nieuwenhuijs, H., Opsteegh, M., Franssen, L., Jebbink, F., Jongejan, F., 2007. Ticks and associated pathogens collected from domestic animals in the Netherlands. Vector-Borne and Zoonotic Diseases 7, 585-596.

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. J. Clin. Microbiol. 43, 5907-5911.

Parola, P., Inokuma, H., Camicas, J.L., Brouqui, P., Raoult, D., 2001. Detection and identification of spotted fever group Rickettsiae and Ehrlichiae in African ticks. Emerging Infectious Diseases 7, 1014-1017.

Penzhorn, B.L., Lewis, B.D., De Waal, D.T., Lopez Rebollar, L.M., 1995. Sterilisation of *Babesia canis* infections by imidocarb alone or in combination with diminazene. Journal of the South African Veterinary Association 66, 157-159.

Pretorius, A.M., Venter, T.P., Ryst, E.V.D., Kelly, P.J., 1999. Possible human ehrlichiosis in the Free State. South African Medical Journal 89, 961-961.

Schetters, T.P., Kleuskens, J., Carcy, B., Gorenflot, A., Vermeulen, A., 2007. Vaccination against large *Babesia* species from dogs. Parassitologia 49, 13-19.

Schetters, T.P., Kleuskens, J.A., Scholtes, N.C., Gorenflot, A., Moubri, K., Vermeulen, A.N., 2001. Vaccination of dogs against heterologous *Babesia canis* infection using antigens from culture supernatants. Veterinary Parasitology 100, 75-86.

Schetters, T.P.M., Kleuskens, J.A.G.M., Scholtes, N.C., Pasman, J.W., Goovaerts, D., 1997. Vaccination of dogs against *Babesia canis* infection. Veterinary Parasitology 73, 35-41.

Schetters, T.P., Montenegro-James, S., 1995. Vaccines against babesiosis using soluble parasite antigens. Parasitology Today 11, 456-462.

Schetters, T., Strydom, T., Crafford, D., Kleuskens, J.A.G.M., Van de Crommert, J., Vermeulen, A.N., 2007. Immunity against *Babesia rossi* infection in dogs vaccinated with antigens from culture supernatants. Veterinary Parasitology 144, 10-19.

Uilenberg, G., Franssen, F.F., Perie, N.M., Spanjer, A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Veterinary Quarterly 11, 33-40.

Wozniak, E.J., Barr, B.C., Thomford, J.W., Yamane, I., McDonough, S.P., Moore, P.F., Naydan, D., Robinson, T.W., Conrad, P.A., 1997. Clinical, anatomic, and immunopathologic characterization of *Babesia gibsoni* infection in the domestic dog (*Canis familiaris*). Journal of Parasitology 83, 692-699.

Scientific publications connected with this thesis

Matjila, P.T., Penzhorn, B.L., Bekker, C.P., Nijhof, A.M., Jongejan, F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Veterinary Parasitology 122, 119-125.

Matjila, T.P., Nijhof, A.M., Taoufik, A., Houwers, D., Teske, E., Penzhorn, B.L., De Lange, T.D., Jongejan, F., 2005. Autochthonous canine babesiosis in The Netherlands. Veterinary Parasitology 131, 23-29.

Matjila, P.T., Penzhorn, B.L., Leisewitz, A.L., Bhoora, R., Barker, R., 2007. Molecular characterization of *Babesia gibsoni* infection from a pit-bull terrier pup recently imported into South Africa. Journal of the South African Veterinary Association 78, 2-5.

Matjila, P.T., Leisewitz A.L., Jongejan, F., Penzhorn, B.L., 2008. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. Veterinary Parasitology 155, 152-157.

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.

Matjila, P.T., Carcy, B., Leisewitz A.L., Schetters, T., Jongejan, F., Penzhorn, B.L., 2008. Correlation between *Babesia rossi* genotypes and clinical canine babesiosis (Submitted) Journal of Clinical Microbiology.