# 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.

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# 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 |

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