

**The comparative assessment of capillary and venous
Babesia rossi parasitaemias on thin blood smears and
their association with disease manifestation**

by

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Summary

This observational study of 100 dogs naturally infected with *Babesia rossi* determined whether severity of parasitaemia was associated with outcome of infection and documented the relative distribution of parasitised red blood cells (pRBC) in capillary and venous circulation. The association between increased parasitaemias and outcome with a clinically compromised circulation was also investigated. Outcome was defined as either hospitalisation with death, or hospitalisation with eventual recovery or treatment as an outpatient.

Dogs were enrolled if large babesias were found on stained thin capillary blood smears made from an ear prick. Thin venous smears were prepared from jugular or cephalic blood. Parasitaemias were manually counted and expressed as the percent pRBC.

Ten dogs died, 50 recovered after hospitalisation and 40 were treated as outpatients. Venous sampling site did not affect venous parasitaemia ($P = 0.6$). Both capillary and venous parasitaemias of dogs that died were significantly higher than those of dogs that recovered after hospitalisation ($P = 0.002$) and dogs that were treated as outpatients ($P < 0.0001$). When assessing the whole group, capillary parasitaemia (median 0.61%, range <0.05-71.6%, interquartile range (IQR) 0.22-3.75%) was significantly higher than venous parasitaemia (median 0.14%, range 0-30.6%, IQR 0.046–0.52%) with $P < 0.0001$. The 21 dogs with a clinically compromised circulation were more likely to die ($P < 0.0001$) and had significantly higher capillary (median 5.98%, range 0.09-71.6%, IQR 2.44-19.41%) and venous (median 2.81%, range <0.05-30.6%, IQR 0.17-9.03%) parasitaemias than the 79 dogs with a clinically normal circulation (capillary median parasitaemia 0.38%, range <0.05-12.87%, IQR 0.16-1.42%; venous median parasitaemia 0.096%, range 0-6.13%, IQR <0.05-0.33%; $P < 0.0001$).

This study shows that high parasitaemia is significantly associated with death in *B rossi* infected dogs. Unfortunately, there was a wide overlap in the parasitaemias of the three outcome groups

with the result that neither capillary nor venous parasitaemias appear prognostically useful. The previous clinical suspicion that capillary parasitaemias are usually higher than venous parasitaemias is confirmed. Thus capillary samples are the most appropriate diagnostic samples. Prior observations that a clinically compromised circulation is associated with death are confirmed. This association provides a rapid means of identifying patients in need of intensive monitoring and treatment. Despite the highly significant association between compromised circulation and higher parasitaemia, it is thought unlikely that parasite burden is the sole trigger for circulatory collapse.

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List of abbreviations

%	percent
<	less than
A	admitted: admitted to the hospital for treatment and survived
ANOVA	analysis of variance
<i>B</i>	<i>Babesia</i>
bpm	beats per minute
° C	degrees Celsius
circulatory score 0	clinically normal circulation
circulatory score 1	clinically collapsed circulation
cm	centimeter(s)
CRT	capillary refill time
D	dead: death despite treatment or euthanasia owing to poor prognosis
DIC	disseminated intravascular coagulation
<i>E</i>	<i>Ehrlichia</i>
e.g.	for example
EDTA	ethylenediamine tetra-acetic acid
FE	feather edge
g/l	grams per litre
H	home: treated with an antibabesial and sent home immediately
ICAM-1	intercellular adhesion molecule 1
ICC	Intra-class correlation co-efficient(s)
IQR	interquartile range
ISA	in-saline agglutination
kg	kilogram
l/l	litre per litre
ml	millilitres
mm	millimetres
n	total number
n/s	not specified
NO	Nitrous oxide
OVAH	Onderstepoort Veterinary Academic Hospital
<i>P</i>	<i>Plasmodium</i>
PCR	polymerase chain reaction
PCV	packed cell volume
PfEMP-1	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
pRBC	parasitised red blood cells
<i>R</i>	<i>Rhipicephalus</i>
RBC	red blood cell(s)
RCA	red cell area
RLB	reverse line blot
rRNA	ribosomal ribonucleic acid
s	second(s)
<i>sp</i>	<i>species</i>
syn.	synonym
TSP	total serum protein
USA	United States of America
VESA-1	Variant erythrocyte surface antigen 1

Chapter 1: Literature Review

1.1 Introduction

Canine babesiosis, a tick transmitted disease caused by piroplasms, is commonly diagnosed in South Africa (Malherbe, 1956). Approximately 12% of dogs presented to the Onderstepoort Veterinary Academic Hospital's (OVAH) Outpatients service between 1988 and 1993 were diagnosed with babesiosis and 31.4% of these were admitted for more intensive treatment (Shakespeare, 1995). This represented an average of 1253 babesiosis cases annually. A computer search of the OVAH's recent patient records revealed that babesiosis had been diagnosed in 1384 of 35285 (3.9%) dogs presented from January 2004 to November 2005. This included data from both referred and Outpatients cases. It represents an average of 755 cases annually. In a serological survey of dogs presented to animal sanctuaries in four areas of South Africa, 43.4-65.4% had detectable antibodies against babesia (Lewis *et al.*, 1996).

1.2 Canine babesias and their disease manifestations

1.2.1 Canine babesia species

Canine babesias are morphologically separated into small (1 x 3.2 µm) and large (2.4 x 5 µm) species. The most prevalent large babesiae include *Babesia canis*, *B vogeli* and *B rossi*. The parasites appear morphologically identical but vary in their geographic distribution, pathogenicity, tick vectors (Uilenberg *et al.*, 1989) and pathophysiology (Schetters *et al.*, 1997). It is suggested that they represent different species (Carret *et al.*, 1999; Depoix *et al.*, 2002). In addition, an unnamed novel large babesia most closely related to the *Babesia bigemina* of cattle was recently described in a North Carolina dog receiving chemotherapy for lymphoma (Birkenheuer *et al.*, 2004).

Initially, *Babesia gibsoni* was thought to be the only small babesia capable of infecting dogs. Recent studies have shown that small babesias isolated from dogs in California and Southeast Asia are probably separate species (Kjemtrup *et al.*, 2000; Zahler *et al.*, 2000) and the Californian

isolate has now been named *Babesia conradae* (Kjemtrup *et al.*, 2006). *Theileria annae* and *Theileria equi* are morphologically indistinguishable from small babesias. Both may infect dogs (Criado-Fornelio *et al.*, 2003) although only *T annae* causes clinical signs (Criado-Fornelio *et al.*, 2003; Guitian *et al.*, 2003). As use of more complex molecular techniques like polymerase chain reactions (PCR) becomes more widespread, it is likely that further species will be identified (Zahler *et al.*, 2000; Criado-Fornelio *et al.*, 2003).

1.2.2 Canine babesias in South Africa and their pathogenicity

B rossi and *B vogeli* (Matjila *et al.*, 2004) have been identified in South Africa. Prior to 2005, the babesia species infecting dogs presenting to the OVAH was not routinely determined. A total of 6 *B vogeli* infections were detected amongst a convenience sample from approximately 400 dogs presenting to the OVAH with clinical signs of babesiosis during 2005 (P.T. Matjila, personal communication 2006). These included the dogs sampled for this study. Thus *B rossi* appears to be the most prevalent species infecting dogs presenting to the OVAH with clinical signs of babesiosis. It is transmitted by *Haemaphysalis leachi* (Uilenberg *et al.*, 1989; Lewis *et al.*, 1996). This babesia species is highly pathogenic, resulting in diverse clinical signs and varied severe complications. Mortality is typically 10-15% in cases admitted for further treatment and greater than 50% in cases showing cerebral signs or haemoconcentration (van Zyl, 1995a; Reyers *et al.*, 1998).

B vogeli was recently isolated from an adult dog presenting to the OVAH's outpatients service with clinical signs of ehrlichiosis (Matjila *et al.*, 2004). *Ehrlichia canis* and *B vogeli* share their tick vector, *Rhipicephalus sanguineus* (Uilenberg *et al.*, 1989). *B vogeli* has also been isolated from 13 of 297 dogs in animal shelters throughout South Africa that were not showing clinical signs of babesiosis. Co-infections with *B rossi* were not detected (Matjila *et al.*, 2004).

The prevalence of *B vogeli* in South Africa is unknown at present. However, during a serological survey, which used immunofluorescent antibody titres to determine prevalence of exposure to

babesia in various South African dog populations, investigators noted two distinct immunofluorescence patterns. The less common one was noted in 11/152 dogs, which included 1/83 dogs sampled around Pretoria. Investigators speculated that these findings may indicate the presence of a different babesia strain, possibly one transmitted by *Rhipicephalus sanguineus* (Penzhorn *et al.*, 1995; Lewis *et al.*, 1996). Certainly, this tick species is abundant (Horak, 1995). *R. sanguineus* was found on 37% of dogs diagnosed with babesiosis at the OVAH (Horak, 1995) and on 66% of 344 dogs from resource poor communities in the North West Province of South Africa (Bryson *et al.*, 2000).

The pathogenicity of South African *B. vogeli* strains has not been described. This parasite is regularly isolated in the USA, on Okinawa, Japan, and in Australia. In these countries, it usually causes mild or subclinical disease in adult dogs, although some puppies may be severely affected (Sanders 1937; Farwell *et al.*, 1982; Irwin *et al.*, 1991; Taboada, 1998). In an Australian study of cases showing clinical signs suggestive of babesiosis, 26/32 affected dogs were less than 15 weeks old, with the greatest incidence in dogs less than 10 weeks old. These animals were usually presented in shock and 14/25 died. None of the adult dogs died of babesiosis (Irwin *et al.*, 1991). Although PCR was not performed in this study, it is assumed that these dogs were infected with *B. vogeli* as this is the only large babesia species known to occur in Australia (Jefferies *et al.*, 2003).

B. canis, transmitted by *Dermacentor reticularis*, occurs in Europe and Asia and is of intermediate pathogenicity (Uilenberg *et al.*, 1989). It is unlikely to occur around the OVAH owing to the absence of its tick vector. *B. gibsoni* can be transmitted by *Rhipicephalus sanguineus* and *Haemaphysalis bispinosa* (Farwell *et al.*, 1982). It has been found in Japan, southern Asia, northern Africa and the south western USA (Taboada, 1998). Although its vector is prevalent in South Africa, this parasite has only been detected in imported dogs. As capillary blood smears are performed on all dogs presented to the OVAH's outpatients service, and as parasites are morphologically distinct from the large babesia and parasitaemias relatively high (Farwell *et al.*,

1982) it seems unlikely that *B gibsoni* or any other small babesia infects dogs in the surrounding areas.

1.2.3 Clinical signs

Clinical signs of babesiosis are varied and include peracute, acute, chronic, subclinical and atypical presentations. Acute babesiosis is the most common presentation at the OVAH. It typically manifests as fever, lethargy, anaemia, thrombocytopenia and splenomegaly with or without haemoglobinuria (Abdullahi *et al.*, 1990a).

Peracute forms are usually rapidly fatal and present in hypotensive shock or a coma after less than 24 hours illness (Taboada, 1998). Cerebral babesiosis often has a peracute course, presenting as sudden death without preceding signs (Piercy, 1947; Basson *et al.*, 1965).

Chronic babesiosis has been described in greyhounds in Florida and results in a fluctuating fever, decreased appetite and marked weight loss (Sanders, 1937). Chronic infections are rarely seen at the OVAH. Subclinical infections are common amongst adult dogs infected with *B vogeli* in the USA, especially in adult greyhounds (Taboada, 1998) and have been demonstrated in France (Wlosniewski *et al.*, 1997).

Atypical forms are usually seen as complications of acute babesiosis and include haemoconcentration, icterus and hepatopathy, immune-mediated haemolytic anaemia, acute renal failure, pancreatitis and pulmonary oedema. Rare complications include gastrointestinal disease, myalgia, upper respiratory signs, cardiac changes, necrosis of the extremities, ascites and oedema (Jacobson *et al.*, 1994).

Several authors have reported that the clinical signs of babesiosis are more severe when dogs are concurrently infected with *E canis* (Neitz, 1938; Ewing *et al.*, 1965; Van Heerden *et al.*, 1983).

1.3 Similarities between babesiosis and falciparum malaria

1.3.1 Life cycle, signalment, clinical signs and histopathology

Researchers have long remarked on the similarities between the diseases caused by virulent large babesia strains (Malherbe, 1956; Maegraith *et al.*, 1957; Clark *et al.*, 1998); *Babesia bovis* (*syn. argentina*) (Wright *et al.*, 1988; Carret *et al.*, 1999; Schetters *et al.*, 1999; Allred *et al.*, 2004), the most pathogenic babesia species infecting cattle; and *Plasmodium falciparum*, the most pathogenic of the plasmodium species infecting humans. All three haemoprotozoan parasites are transmitted by arthropod vectors. (Berendt *et al.*, 1990)

All three protozoa cause acute disease that may include severe haemolysis and haemoglobinuria, icterus, circulatory collapse and multiple organ failure (Jacobson *et al.*, 1994) and may cause cerebral signs in a small percentage of hosts (Schetters *et al.*, 1999). On post mortem capillaries, particularly cerebral capillaries, may be packed with parasitised red blood cells (pRBC) (Graham-Smith, 1905; Maegraith *et al.*, 1957; Basson *et al.*, 1965; Wright, 1972; Macpherson *et al.*, 1985; Pardini, 2000). This phenomenon has not been observed during infection with less pathogenic babesia (de Vos *et al.*, 1994) or plasmodium species (Berendt *et al.*, 1990; Schetters *et al.*, 1999). Both haemoprotozoan genera also cause similar hepatic, renal, splenic and pulmonary changes on histopathology (Maegraith *et al.*, 1957). These similarities have resulted in researchers in both fields scrutinising each other's findings in an attempt to further understand the pathogenesis of the disease in their species of interest (Aikawa *et al.*, 1985; Jacobson *et al.*, 1994; Allred, 1995; Clark *et al.*, 1998; O'Connor *et al.*, 1999; Schetters *et al.*, 1999).

1.3.2 Sludging, sequestration and cytoadhesion

In 1947, Knisely and co-workers detected RBC sludging by observing the movement of blood in living people and animals affected by a variety of diseases (Knisely *et al.*, 1947). They noted that this phenomenon did not occur in healthy subjects. They classified different types of blood sludge according to the size and uniformity of the constituent masses of agglutinated RBC. They noted

that it was possible to re-suspend RBC in some sludge whereas in others, the erythrocytes appeared to transform into a gelatinous mass. They concluded that masses large and rigid enough to resist passage through vascular bottlenecks reduced oxygen supply to the endothelium which resulted in increased vascular permeability, haemoconcentration and oedema of the surrounding tissues. In this study, 'sludging' was used to describe any situation in which RBC within vessels had lost their normal laminar flow.

In 1956 Malherbe remarked that trophozoites of South African babesia strains were more numerous in cerebral capillaries on histopathology (Malherbe, 1956). In fact, these capillaries appeared occluded by massive accumulations of pRBC and free parasites. Parasitised RBC seemed to align themselves along the endothelium much as had been observed in falciparum malaria (Malherbe, 1956; Berendt *et al.*, 1994; Cooke *et al.*, 1995) and cattle infected with *B bovis* (*syn.argentina*) (Wright, 1972; Wright, 1973; Commins *et al.*, 1988; O'Connor *et al.*, 1999; Molloy *et al.*, 2003). Electron-microscopic studies demonstrated that *P falciparum* (Berendt *et al.*, 1990; Cooke *et al.*, 2005) and *B bovis* pRBC (Potgieter *et al.*, 1979; Aikawa *et al.*, 1985; Allred *et al.*, 2004; Cooke *et al.*, 2005) as well as babesia infected red cells of dogs presented to the OVAH (Pardini, 2000) adhere to capillary endothelium. In the case of malaria, histidine related proteins (HRP) and *P falciparum* erythrocyte membrane protein 1 (PfEMP-1) were shown to be components of these knobs. Parasitised RBC were shown to bind to thrombospondin, to CD 36 on platelets, endothelial cells and dendritic cells, to chondroitin sulphate A in the placenta and to intercellular adhesion molecule 1 (ICAM-1) and E-selectin in brain endothelial cells (Sherman *et al.*, 1995; Warrell, 1997). At least 11 different receptors have been identified for *P falciparum* pRBC (Cooke *et al.*, 2005). ICAM-1 binding was positively correlated with severity of malaria symptoms and ICAM-1 expression was highest in the endothelial cells of cerebral malaria patients (Sherman *et al.*, 2003). Thus specific receptor mediated adhesion or bonding was shown to cause the preferential accumulation of pRBC in brain capillaries of patients with cerebral malaria.

During these studies, the terms sequestration and cytoadherence were introduced. Sequestration describes the accumulation and localisation of pRBC through specific interactions with blood vessel endothelial cells (Schetters *et al.*, 1999). Cytoadhesion is used to describe the receptor-mediated binding process between pRBC and host cells (Berendt *et al.*, 1990) and thus constitutes the mechanism by which sequestration occurs. Nevertheless, cytoadhesion may not always result in sequestration. *P. falciparum* infected pRBC form rosettes when the DBL1 α ligand on pRBC binds to complement receptor 1 on healthy RBC (Sherman *et al.*, 2003). Parasitised RBC adhere to each other resulting in autoagglutination (Allred, 1995). In both these cases, the resultant clumps of RBC remain suspended in the blood.

Sequestration is an invariable part of the *P. falciparum*'s lifecycle, allowing RBC containing parasites in the second half of their maturation cycle to disappear from the venous circulation (Gravenor *et al.*, 1998).

1.3.3 Sequestration in babesiosis

Electron dense elevations have been observed on cell membranes of RBC from babesia-infected dogs presented to the OVAH (Pardini, 2000). The composition and function of these protrusions has not been reported. The study of specific antigen-receptor interactions is in its infancy in babesiosis (Cortes *et al.*, 2005). Until such receptor-mediated binding of pRBC can be demonstrated in canine babesiosis, the localised accumulation of pRBC is still probably best described as sludging.

The evidence for sequestration is stronger in bovine babesiosis. Electron dense area ('stellate protrusions' (Cooke *et al.*, 2005)) have been shown to include variant erythrocyte surface antigen 1 (VESA-1) and are able to bind to thrombospondin on endothelial cells (Cooke *et al.*, 2005). *B. bovis* parasites have been shown to activate the clotting cascade resulting in fibrin synthesis which then helps agglutinate both healthy RBC and pRBC (O'Connor *et al.*, 1999; Schetters *et al.*, 1999). They also activate the kallikrein system, resulting in kinin mediated hypotension,

vasodilation and thus decreased blood flow rates through capillaries. Nitric oxide (NO) can be induced by kinin, suggesting a common pathogenesis for the development of neurological signs in babesiosis and malaria (Schetters *et al.*, 1999).

The prevalence of sequestration has not been studied in canine babesiosis. The study of its relationship to parasite maturity is hampered by the lack of easily identifiable morphological changes as the intra-erythrocytic babesia parasite matures, as well as a much shorter maturation time than *P falciparum* (17 hours as opposed to as opposed to 48 hrs) (Cooke *et al.*, 2005).

1.3.4 The importance of sequestration

As sludging or sequestration of pRBC is only observed in the most pathogenic of babesia (de Vos *et al.*, 1994) and falciparum species (Berendt *et al.*, 1990; Schetters *et al.*, 1999), the hypothesis that this phenomenon is somehow responsible for the severity of disease manifestation merits investigation. Theories have been advanced to explain the advantage sequestration represents for the parasite. They include the following: sequestration allows the parasites to avoid recognition and destruction in the spleen. It may create a micro-environment favourable to parasite replication or infection of further RBC (Berendt *et al.*, 1990; Allred, 1995). Rosetting could shield pRBC from recognition by the host immune system (Allred, 1995; Sherman *et al.*, 2003). The prevalence of sequestration and its association with relative parasite distribution and outcome have not been studied in canine babesiosis.

It has long been postulated that some atypical presentations of canine babesiosis may be the result of a concentration of parasites in the clinically most severely affected organs. There is some evidence to support this theory in the cases of pulmonary oedema (Shortt, 1973) and cerebral babesiosis (Purchase, 1947; Malherbe *et al.*, 1951; Malherbe, 1956; Basson *et al.*, 1965). Although localisation of pRBC in renal, muscular, splenic, intestinal and / or lymph node capillaries have occasionally been observed in post mortem studies (Basson *et al.*, 1965a; Irwin *et al.*, 1991), their causal relation to complicated clinical forms is less certain (Maegraith *et al.*,

1957). Similarly, several histopathological studies have noted ocular capillaries distended with pRBC, yet ocular signs are rarely reported clinical findings in babesiosis (Malherbe *et al.*, 1951; Basson *et al.*, 1965). Other atypical presentations of babesiosis like icterus and hepatopathy (Gilles *et al.*, 1953; Jacobson *et al.*, 1994), immune mediated haemolysis and haemoconcentration cannot be explained by a localised increased parasitaemia within capillaries. Some workers postulated that atypical presentations develop as a result of the inflammatory response mounted by individual dogs in response to the infection, and are not directly triggered by the parasite (Jacobson *et al.*, 1994; Reyers *et al.*, 1998).

It has not been proven that canine cerebral babesiosis is related to parasite sequestration i.e. to receptor-mediated localisation of pRBC in cerebral capillaries. A post mortem study of 32 dogs diagnosed with babesiosis that showed neurological signs and / or brain lesions on histopathology, revealed no correlation between the density of pRBC in cerebral capillaries and presence or absence of neurological signs (Pardini, 2000). A weakness in this study was that the time between treatment and post mortem was not reported. This happened because the study was designed to document pathological changes and not clinical signs. It has been shown that venous parasitaemia of virulent South African large babesia strains is low or undetectable within 24 hours of treatment with diminazene (Jacobson *et al.*, 1996). This means that sequestered parasites may have been killed prior to post mortem examination in some dogs. In bovine babesiosis, one study found no correlation between sequestration and cerebral signs (Wright, 1972) and in vitro cytoadherence appeared not to correlate with strain virulence (Molloy *et al.*, 2003).

1.3.5 Sequestration in subcutaneous capillaries as a surrogate marker for cerebral sequestration

It is an accepted phenomenon in falciparum malaria infections that pRBC are more likely to be found on capillary blood smears made from a finger prick than on venous smears (Moody, 2002). In addition, Chinese clinicians reported that intradermal blood smears were more sensitive at

detecting *P falciparum* pRBC than the usual capillary smears made from a finger prick (Li *et al.*, 1983). Later investigators confirmed this observation and showed that it was true for falciparum but not vivax malaria (Singh *et al.*, 2003).

The study of the pathogenesis of cerebral malaria and babesiosis has been hampered by the inaccessibility of the affected tissue. Various groups have since investigated dermal sequestration as a more accessible surrogate for the detection or study of cerebral sequestration in falciparum malaria. Electronmicroscopic studies showed that pRBC in dermal capillaries developed electron dense knobs in people (Macpherson *et al.*, 1985) and in a rhesus monkey (Nakano *et al.*, 1996). One study found an average of 25% more pRBC in skin biopsies than in venous blood but noted that quantitatively, sequestration was least apparent in the skin of all the tissues examined (Macpherson *et al.*, 1985). A later study found that compared with patients with uncomplicated severe malaria, patients with cerebral malaria had 15 times more pRBC in dermal capillaries (Wilairatana *et al.*, 2000). A single case of cerebral malaria in which pRBC could be detected on skin biopsy but not on blood smear has been reported (Nakazawa *et al.*, 1995). Further investigations are needed before the diagnostic utility of skin biopsies for falciparum malaria can be determined.

There is sufficient evidence to conclude that the distribution of different plasmodium species within their hosts varies and that the comparison of capillary and venous smears from a patient may detect this differential distribution. Thus parasite distribution characteristics may aid species identification and prognostication.

1.4 The influence of circulatory status on capillary parasitaemia

The association between parasitaemia and circulatory compromise has not been studied in canine babesiosis. Circulatory collapse has been offered as a common cause of death (Maegraith *et al.*, 1957) and appeared to be associated with a poor prognosis in this disease (Malherbe *et al.*, 1976; Abdullahi *et al.*, 1990). Clinical collapse has been associated with hypotension (Jacobson

et al., 2000), hyperlactaemia (Malherbe *et al.*, 1976; Leisewitz *et al.*, 2001; Jacobson *et al.*, 2005), hypoglycaemia (Keller *et al.*, 2004) and acid-base disturbances (Leisewitz *et al.*, 2001). Thus circulatory compromise appears to be associated with severely affected individuals.

1.5 Parasitaemia

1.5.1 Parasite detection

Traditionally, capillary blood smears collected from an ear prick have been used to diagnose canine babesiosis (Paine, 1934; Neitz, 1938; Ewing, 1965; Stewart, 1983; Irwin *et al.*, 1991; Lewis *et al.*, 1995; Penzhorn *et al.*, 1995; Brandao *et al.*, 2003). Canine large babesias are usually easy to detect on capillary smears in acute infections. In chronic or subclinical infections, subinoculation into splenectomised dogs may be necessary (Penzhorn *et al.*, 1995). American workers found bone marrow aspirates useful in detecting chronic infections (Ewing, 1966) while Australians found that smears prepared from the red cells just below the buffy coat appeared to increase sensitivity of detecting parasitaemia (Irwin *et al.*, 1991). In addition, flow cytometry has been used to detect and quantify low parasitaemias in chronic *B vogeli* infections (Bicalho *et al.*, 2004). Lastly, PCR is used in some diagnostic laboratories (Birkenheuer *et al.*, 2003).

Although many workers have remarked that canine babesiosis is best diagnosed on capillary smears (Paine, 1934; Malherbe *et al.*, 1951; Malherbe, 1956; Maegraith *et al.*, 1957; Vaughan-Scott, 2001), the relative distribution of pRBC in *B rossi* infections has not been formally determined by any means. In *B bovis* infections, capillary parasitaemia is significantly greater than venous parasitaemia (Callow *et al.*, 1974). The converse has been shown for a North American large babesia assumed to be *B vogeli* (Ewing, 1966; Birkenheuer *et al.*, 2005). Seventy three percent of dogs showed a higher venous parasitaemia and in 7/72 cases trophozoites were only detected on venous blood smears (Ewing, 1966). A meeting report described capillary parasitaemias to be 10 times higher than venous parasitaemias in 119 different French large canine babesia isolates (Mendis *et al.*, 1995). As some of these isolates were capable of causing severe acute infections (Mendis *et al.*, 1995), some if not all of the isolates were probably *B canis*.

It is possible that there are species specific variations in the sensitivity of different sampling sites for the detection of large babesias, as has been shown amongst *Plasmodium* species (refer 1.3.5). Capillary parasitaemias should exceed venous parasitaemias if sequestration is prevalent during infection with *B. rossi*.

Where blood smears made from capillary blood appear to be a relatively sensitive diagnostic tool for babesiosis, several investigators have noted that estimations of parasitaemia from these samples are poorly repeatable even with smears taken within minutes of each other (Maegraith *et al.*, 1957; Callow *et al.*, 1974). Ratios between capillary and venous parasitaemias varied between 1.5:1 and 16:1 in one calf (Callow *et al.*, 1974). In falciparum malaria, some of the variability in capillary parasitaemia is thought to be due to sequestration of mature parasites (Gravenor *et al.*, 1998) and mathematical models have been designed to overcome this (Gravenor *et al.*, 1998). The repeatability of parasitaemia estimations from capillary and venous samples has not been investigated to our knowledge.

Despite widespread use of smears, there is limited information on the association between parasitaemia and outcome in *B. rossi* infections. Early workers' impressions (Purchase, 1947; Maegraith *et al.*, 1957) and a preliminary study (Reyers *et al.*, 1995) could show no association between the extent of virulent large babesia parasitaemia and outcome. However parasitaemia was associated with the severity of clinical signs in experimental *B. rossi* infections (Schetters *et al.*, 1997).

1.5.2 Parasitaemia

As a generalisation, parasitaemia seems to be markedly lower during *B. vogeli* infections compared to those caused by *B. rossi* (Table I). Although the large babesia strain was not identified by PCR in any of the South African studies listed below, the preliminary data on the prevalence of *B. vogeli* amongst dogs presented to the OVAH with signs consistent with babesiosis (1.5%, refer 1.2.2), suggests that the vast majority were in fact infected with *B. rossi*.

Table I: Summary of literature on parasitaemias of large canine babesia species

Study	Likely babesia species	co-infected with E canis	Collection site	site on smear counted	Highest parasitaemia	infection	total no of infected animals
Nuttall	<i>B canis</i>		n/s	n/s	3-4%	natural	1
Schetters 1994	<i>B canis</i>		venous	n/s	Mean parasitaemia <0.1%	experimental	25
Schetters 1996	<i>B canis</i>		peripheral	n/s	Mean parasitaemia <0.1%	experimental	20
Schetters 1997 ^a	<i>B canis</i>		peripheral	n/s	0.10%	experimental	5
du Plessis 1990	<i>B rossi</i>	37	capillary	FE	mixed infections had 5-30% pRBC	natural	47
Jacobson 2002 ^b	<i>B rossi</i>		venous	RCA	<2.43	natural	30
Jacobson 2005 ^b	<i>B rossi</i>		venous	n/s	<2.43%	natural	20
Lewis 1995	<i>B rossi</i>		peripheral	n/s	10.40%	experimental	4/6 reported
Schetters 1997 ^a	<i>B rossi</i>		peripheral	n/s	4%	experimental	5
Stewart 1983	<i>B rossi</i>		capillary	n/s	10%	experimental	14
Van Heerden 1983	<i>B rossi</i>	3	capillary	Borders and FE	--	experimental	6
Vaughan-Scott ^b	<i>B rossi</i>		venous	n/s	<2.43%	natural	31
Bicalho	<i>B vogeli</i>		venous	n/s	<0.25%, then 0-0.01% for 16 weeks	natural	162
Ewing 1965	<i>B vogeli</i>	21	capillary	n/s	<0.5%	experimental	23
Ewing 1966 ^c	<i>B vogeli</i>		cephalic vein	n/s	<0.7% of 100 000 RBC	experimental	6
Ewing 1966 ^c	<i>B vogeli</i>		capillary	n/s	<0.27%	experimental	6
Ewing and Buckner 1965	<i>B vogeli</i>	8	n/s	n/s	0.3%	experimental	10
Irwin 1991	<i>B vogeli</i> likely		venous	n/s	<2%	natural	32
Piercy 1947	n/s		n/s	n/s	22%	natural	1
Graham-Smith 1905	n/s		peripheral	n/s	6%	experimental	11

n/s: not specified; FE: feather edge; RCA: red cell area

peripheral: Some authors use this term to describe jugular or cephalic samples (Schetters *et al.*, 1994; Bicalho *et al.*, 2004). Others use it to describe smears made from an ear prick (Van Heerden *et al.*, 1983). When articles described smear technique in sufficient detail, 'venous' has been used to indicate samples drawn from the jugular or cephalic vein while 'capillary' indicates that the smear was made from an ear prick. In cases where the origin of the smear is not clear, 'peripheral' has been retained.

^{a,c}: same paper

^b: same group of dogs

Thus, Lewis and others found maximum capillary parasitaemias of 3.73-10.4% (Lewis *et al.*, 1995) whereas pre-treatment venous parasitaemias of clinical cases at the OVAH varied between 0.03% - 3.78% (Jacobson *et al.*, 1996) and 0 to 2.4% (Vaughan-Scott, 2001). In contrast, most reported *B vogeli* parasitaemias are below 0.5% (Ewing, 1965; Farwell *et al.*, 1982) although rare instances of parasitaemias around 2% have been reported (Irwin *et al.*, 1991). The typically lower *B vogeli* parasitaemias supported the need to exclude this pathogen from our study group.

Babesia vogeli parasitaemia is known to vary over time. Twelve to sixteen days after experimental infection with this pathogen, pRBC increased markedly for 4-6 days then decreased to less than 1 in 100 000 pRBC (Ewing, 1965). In a recent study validating the use of flow cytometry for detection of *B vogeli*, pRBC reached 0.20-0.25% one week after infection but decreased to less than 0.02% by the middle of the second week. Parasitised RBC were only intermittently detectable on blood smear at very low levels for 15 weeks after that. For a further 10 weeks, parasitaemia was not detected on blood smear yet pRBC could be identified by flow cytometry throughout the study (Bicalho *et al.*, 2004). We have not found similar data for *B rossi* parasitaemias following natural infection, most probably because untreated dogs usually succumb to the disease. A paper describing the development of a preliminary vaccine against the Thomas strain of *B rossi* describes a final parasitaemia of less than 0.01% 6 weeks after challenge with a homologous strain in three of six dogs (Lewis *et al.*, 1995). Co-infection with *Ehrlichia canis* has been shown to result in a higher babesia parasitemia (Ewing *et al.*, 1965; Van Heerden *et al.*, 1983).

1.5.3 Quantifying parasitaemia

No standard method for scoring parasitaemias has been described for babesiosis or malaria. Methods used can be broadly separated into quantitative, semi-quantitative and qualitative methods (Jacobson, 1994).

Qualitative techniques assign a subjective score to the parasitaemia e.g. one group assigned a score of 2 if very few parasites were found, a score of 5 if one pRBC per 330-5000 erythrocytes was seen, a score of 10 if the parasitaemia was estimated at 0.33-2% pRBC and a score of 15 if more than 2% RBC appeared infected (Callow *et al.*, 1974). Jacobson counted pRBC in 50 oil immersion (x1000 magnification) fields along both edges of the smear starting at end of the feather edge. Results were expressed in total numbers of pRBC per 100 oil immersion fields. Dogs were divided into high, medium and low parasitaemia groups on the basis of these results (Jacobson, 1994). Investigators resorted to these techniques because they were concerned about the poor repeatability of absolute percentage parasitaemias (Callow *et al.*, 1974; Van Heerden *et al.*, 1983; Jacobson, 1994; Kettner *et al.*, 2003; Singh *et al.*, 2003). It is likely that the above qualitative approach would not have been sensitive enough to detect small differences in capillary and venous parasitaemia.

Semi-quantitative methods can be divided into volumetric and non-volumetric ones. Volumetric methods determine the number of pRBC in a known volume of blood. Previous workers experimented with two of these techniques, but found practical problems with them that made them unsuitable for work with babesiosis (Jacobson, 1994). Non-volumetric methods compare the number of pRBC with total numbers of RBC or white blood cells. These are the most commonly used techniques in babesia research (Ewing, 1966; Stewart, 1983; Lewis *et al.*, 1995; Vaughan-Scott, 2001). Most researches initially determined the average number of RBC per field for each animal and then counted pRBC in either a fixed number of fields (Stewart, 1983; Lewis *et al.*, 1995) or a fixed number of RBC (Ewing, 1966; Vaughan-Scott, 2001). Pardini calculated the percentage pRBC by counting both the total number of RBC and the number of pRBC in 4 fields (Pardini, 2000). Only two of the 20 papers listed in 1.5.2 describe which area of the smear was scored (du Plessis *et al.*, 1990; Jacobson *et al.*, 2002).

In malaria diagnosis and research, several semi-quantitative non-volumetric methods are used. pRBC per 10 000 RBC (Moody, 2002) or 1000 RBC (Gravenor *et al.*, 1998) are usually counted and then expressed as a percentage.

Flow cytometry has been used to detect babesia and malaria parasitaemias (Saito-Ito *et al.*, 2001; Bicalho *et al.*, 2004). No papers describing the accuracy or repeatability of this technique were found. Its use was investigated for this project but deemed impractical as there is no flow cytometer at the veterinary faculty. Samples need to be stained within 24 hours of collection and processed within half an hour of staining^a, precluding the use of an off-site machine.

Real time quantitative PCR (Hermsen *et al.*, 2001) and real time nuclei acid sequence based amplification (Schneider *et al.*, 2005) have been used in malaria research. Both techniques are approximately one thousand times more sensitive than blood smears in detecting parasites and are accurate, but their use in studies on babesiosis has not been described.

1.6 Conclusion

In summary, the above discussion shows that there are two large babesia species that infect dogs in South Africa. The two species probably differ in the severity of their disease manifestations. Co-infection with *E canis* appears to both increase parasitaemia and worsen clinical signs. Thus it was important to know which babesia species was present and to concentrate on a uniform population of dogs infected solely with *B rossi*.

The discussion details the similarities between canine babesiosis and malaria, particularly those between infections with virulent South African large babesias and falciparum malaria. Both cause the most severe form of disease in their host, are associated with the highest mortalities and with cerebral complications. In malaria, it is thought that parasite sequestration in various capillary beds is responsible for some of this increased pathogenicity. In particular, cerebral complications

^a OA Martins-Filho, personal communication 2005

are thought to be the result of sequestration in brain capillaries. Nevertheless, sequestration in more accessible (e.g. dermal) capillaries also occurs. Preliminary evidence of sequestration in *B. rossii* infections is presented. It is possible that the higher ear capillary parasitaemias observed (but not documented) by previous investigators in infections with South African strains assumed to be *B. rossii* is the result of dermal parasite sequestration.

The terms sequestration, cytoadhesion and sludging are defined. Sludging is associated with circulatory compromise. Evidence that associates a collapsed circulatory state with a poorer prognosis is presented. The mechanisms by which a collapsed circulation could result in higher capillary parasitaemias independent of sequestration are discussed.

Lastly, means of detecting large babesias and quantifying parasitaemia are reviewed. Blood smears are most commonly used to detect infection as they are cheap and quick to assess, although flow cytometry and PCR are more sensitive. In addition, available information on capillary and venous parasitaemias is presented.

Chapter 2: Objectives

2.1 Problem statement

B. rossii infections are common in South Africa. Capillary smears are the most common means of diagnosis. This is based partially on convenience and partially on the accepted wisdom that capillary parasitaemias exceed venous ones. No studies prove this. Indeed a study of dogs infected with the related *B. vogeli* shows that venous parasitaemias are higher. Thus this study documented the relative distribution of pRBC in capillary and venous circulation of dogs naturally infected solely with *B. rossii*.

No prior study has been designed to explore the association between parasitaemia and outcome. Information on this aspect could add to our ability to prognosticate in clinical cases. Outcome was defined broadly according to survival and the intensity of required therapy as the wide diversity of clinical signs shown by patients presented to the OVAH precluded a simple clinical score.

It has been postulated that sequestration of *B. rossii* infected RBC in capillaries is responsible for severe clinical signs and a poor prognosis. This study sought clinical evidence to support this hypothesis. An association between capillary (rather than venous) parasitaemia and outcome would provide clinical evidence to support the theory that sequestration may be an important part of *B. rossii*'s pathomechanism, as it is in *P. falciparum* infections.

Prior investigators had remarked, but not documented, that parasitaemias determined on capillary smears were poorly repeatable. Thus repeatability of the slide scoring, smear making and sampling technique were determined to be able to identify which individual dogs had a capillary parasitaemia that exceeded venous parasitaemia by an amount that was greater than the error introduced by sampling, smear making or slide scoring. If a means of identifying individual dogs with a higher capillary parasitaemia could be found, dogs with potentially sequestered pRBC could be identified. Their total parasitaemias, outcome and circulatory status could then be assessed separately.

Circulatory status was assessed as circulatory failure was seen as an alternative explanation for high capillary parasitaemias. Sequestration could also decrease microcirculatory perfusion, but is unlikely to influence global circulatory parameters such as the ones assessed. Circulatory status was correlated with outcome and parasitaemia.

Lastly, and as a by-product of all of the above, capillary and venous parasitaemias were documented for 100 dogs prior to treatment. This group is far larger than any reported in the literature.

2.2 Research questions

1. Are *Babesia rossi* infected red blood cells more numerous in capillary (i.e. ear prick) smears than in venous blood smears taken at the same time?
2. Does parasite density in either capillary or venous smears correlate with outcome of infection?
3. Does the ratio of capillary to venous pRBC correlate with outcome in canine babesiosis?
4. Does a clinically compromised circulation correlate with parasitaemia or outcome?

2.3 Hypotheses

1. RBC parasitised by *Babesia rossi* are more prevalent in capillary than in venous smears.
- 2a. Higher capillary parasitaemias are correlated with poorer outcomes.
- 2b. Higher venous parasitaemias are correlated with poorer outcomes.
3. An increased ratio between capillary and venous parasitaemia is correlated with a poorer outcome.
- 4a. A clinically compromised circulation is correlated with poorer outcomes.
- 4b. A clinically compromised circulation is associated with higher capillary and venous parasitaemias.

2.4 Benefits

- Information on the relative sensitivity of ear prick smears and venous smears in detecting parasitaemia will allow general practitioners to choose the most appropriate diagnostic test to detect canine babesiosis.
- If increased parasitaemia correlates with more severe disease, information derived from this study may increase prognostic accuracy in canine babesiosis.
- Information on the repeatability of capillary and venous parasitaemias would result in a more accurate appreciation of the limitations of these techniques.
- The following findings would be consistent with the theory that sequestration plays an important role in the pathophysiology of severe *B. rossii* infections:
 1. capillary parasitaemias being higher than venous ones (the converse would make it highly unlikely)
 2. an association between capillary but not venous parasitaemia and outcome or circulatory collapse
 3. poorly repeatable capillary parasitaemiasIf these are detected, molecular and ultrastructural investigations of the mechanisms causing the accumulation of pRBC in the capillaries of *B. rossii* infected dogs would then be indicated.
- If circulatory status could be shown to correlate with outcome, this would provide a rapid means of identifying more severely affected patients.
- If circulatory status could be shown to correlate with parasitaemia, this would provide a means of identifying patients likely to harbour higher parasite burdens.
- Basic information on severity of parasitaemia would be added to the literature.
- The research conducted fulfils part of the requirements of the principal investigator's MMedVet (Med) degree.

Chapter 3: Materials and Methods

3.1 Study population

One hundred and seventeen naturally infected dogs that had large babesias identified on a stained thin capillary blood smear were enrolled in this observational study. Dogs were not sampled if their condition was complicated by any of the following: treatment for babesiosis in the preceding three weeks, prior splenectomy, suspected or confirmed severe concurrent disease, or detection of *Ehrlichia canis* morula on capillary smear. Dogs were excluded after sampling if their slides were damaged, there was inadequate follow-up, elective euthanasia was performed, severe concurrent disease became evident or *B vogeli* or *Ehrlichia canis* were detected by PCR and reverse line blot (RLB) (Matjila *et al.*, 2004). The study was approved by the University of Pretoria's Animal Use and Care Committee.

3.2 Clinical examination to determine circulatory score

The primary investigator examined all the dogs and recorded rectal temperature, heart rate, pulse quality, capillary refill time (CRT), habitus and hydration status. A circulatory score was assigned based on clinical information (Table IIIa and b). The owner was questioned about previous episodes of babesiosis in all cases and about prior surgery if the spleen was not clearly palpable.

Shock has been defined as “acute circulatory failure with inadequate or inappropriately distributed tissue perfusion resulting in generalised cellular hypoxia” (Graham *et al.*, 2005). The clinical criteria used to define the circulatory score were selected by consulting a standard text on shock (Day, 2000) and omitting parameters that would usually be abnormal in babesia cases as a consequence of anaemia (Table IIIc). Clinically detectable dehydration was added to the parameters as this would not be the direct result of babesiosis and could contribute towards circulatory failure. Dehydration was recorded if the eyes were sunken, skin fold turgidity was decreased and/or the oral mucosae were dry. Blood glucose was determined and hypoglycaemia treated before habitus was assessed. Mental depression, stupor and coma were included under the criterion ‘collapse, unable to stand’ as the latter was more easily assessed objectively (Table

IIIa). Bradycardia would be very unusual in babesiosis. It has been defined as a heart rate below 100 beats per minute (bpm) for small dogs and below 60 for large breeds (Miller *et al.*, 1999). In a normovolaemic anaemia model in conscious beagles weighing 9.5-15.2 kg, heart rate increased by a mean of 25 bpm as the haematocrit fell from a mean of 0.47 l/l to 0.13-0.17l/l (Spotswood *et al.*, 2005). This information was used to define bradycardia for the study population (Table IIIb).

Table III a: Circulatory score

Score	Description
1	<p>Presence of 2 or more of the following:</p> <ul style="list-style-type: none"> • Hypothermia < 37.5 ° C • Bradycardia • Pulse weak • CRT < 1 sec or > 2 sec • Collapse i.e. unable to stand • Clinically detectable dehydration
0	Presence of 1 or none of the above

Table III b: Criteria for diagnosis of bradycardia (Miller *et al.*, 1999; Spotswood *et al.*, 2005)

	<10kg	>10-25kg	>25 kg
PCV < 15%	<120 bpm	<100 bpm	<80 bpm
PCV 15 to <25%	<110 bpm	<90 bpm	<70 bpm
PCV 25% or more	<100 bpm	<80 bpm	<60 bpm

Table III c: Selection of criteria included in the circulatory score

	sign of shock (Day, 2000)	sign expected as result of anaemia	included in circulatory score
Compensatory stage of shock	CRT < 1s	No	included
	tachycardia	Yes	no
	injected mucosae	difficult to show when anaemic	no
Early decompensatory stage of shock	CRT > 2s	No	included
	tachycardia	yes	no
	pale mucosae	Yes	no
	Mental depression	No	included, after hypoglycaemia ruled out (Keller <i>et al.</i> , 2004)
	hypothermia	No	included
Terminal shock	bradycardia	No	included
	pale mucosae	Yes	no
	cyanotic mucosae	no: need haemoglobin to become cyanotic	no
	hypothermia	No	included
	weak pulses	No	included
	stupor / coma	No	included, after hypoglycaemia ruled out (Keller <i>et al.</i> , 2004)

3.3 Sampling

Samples were collected prior to treatment. A thin capillary blood smear was made from the first drop of blood that formed after the clipped inner surface of an ear was pricked with a 21 gauge needle 5-15mm away from the ear margin and away from the marginal ear vein. Occasionally it was necessary to squeeze the ear to encourage a sufficiently large droplet to form. The blood was transferred to a glass slide with a heparinised microhaematocrit tube. This was necessary to prevent ear debris affecting the integrity of the feather edge. A spreader slide with a leading edge that was narrower than a normal slide was used to make a thin smear.

Venous blood (1/2 to 4 ml) was collected into EDTA within 10 minutes of making the capillary smear and a thin smear was made from this blood. Jugular samples were usually collected. Cephalic samples were collected if the jugular vein was not accessible for some reason, if the

dog was fractious or if a cephalic catheter needed to be placed for therapeutic reasons. In the latter case, a sample was withdrawn immediately after the catheter was placed. The remaining EDTA blood was stored at 4°C until it was submitted for PCR and RLB.

All smears originated at the frosted edge of the slide. Quality was judged unacceptable if the smear was less than 1 cm long or if more than 2 streaks extending more than 5 mm from the feather edge were present. If smear quality was unacceptable, a further smear was made. For the first 40 dogs, two capillary smears were made from opposite ears within 5 minutes (and usually within a minute) of each other. Usually, the second smear was made from the opposite ear. Two smears were made from the single venous sample of these same dogs within 5 minutes (and usually within a minute) of each other. The primary investigator made all the smears.

3.4 PCR and RLB

The PCR and RLB were performed as previously described (Matjila *et al.*, 2004). PCR was conducted with a set of primers that amplified a 460 - 540 base pair fragment of the 18S SSU rRNA spanning the V4 region, a region conserved for *Babesia* and *Theileria*. The *Ehrlichia* PCR amplified the V1 hypervariable region of the 16S SSU rRNA (Schouls *et al.*, 1999; Bekker *et al.*, 2002). The membrane used for RLB included probes for *B vogeli*, *B rossi*, *B canis* and *Ehrlichia canis*.

3.5 Groups of dogs

Animals were grouped according to the following outcomes: treated with an antibabesial during the consultation and sent home immediately (H), admitted for treatment and survived until discharge (A), and death despite treatment or euthanasia owing to poor prognosis (D). Owners of dogs in the H group were contacted on completion of the study to confirm that their pet had recovered completely. Dogs that were represented to the OVAH with complications of their babesiosis after being treated as an outpatient were transferred to group A.

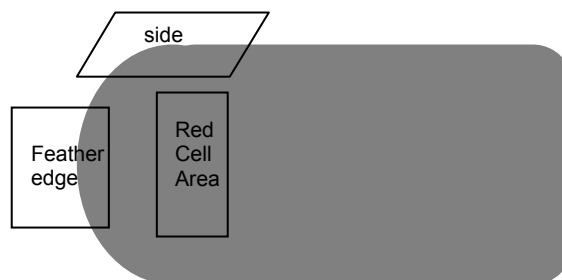
The duty clinician decided whether the dog should be admitted to the hospital or treated as an outpatient. All diagnostic and therapeutic decisions were made by the attending clinician, not the primary investigator. Because this was a clinical study of client owned dogs, therapy was not standardised. Nevertheless babesia cases are treated in a similar manner by all clinicians in the OVAH.

3.6 Analysis of parasitaemia

Blood smears were stained with Kryo-quick (Kyron Laboratories, Benrose, South Africa), a Romanowski stain, and scored at 1000× magnification with the aid of a digital image analysis program (Optimas 6 for Win 95/NT 4.0, Media Cybernetics, distributed by Carl Zeiss Ltd, Randburg, South Africa). A digital photograph of a section of the smear was transferred to a computer screen and magnified. The computer program allowed markers to be placed over counted red blood cells (RBC), which ensured accurate counting.

A semi-quantitative non-volumetric method (Jacobson, 1994) that was a combination of those used by Vaughan Scott (Vaughan-Scott, 2001) and Pardini (Pardini, 2000) was used to quantify parasitaemias. Free parasites were ignored. Unparasitized RBC and pRBC were counted separately in each field.

Figure 3: Areas of the blood smear



Approximately 650 RBC were examined in the red cell area and the same number along the feather edge and along the sides of the smears. Thus at least 1950 RBC were examined per smear. Complete oil immersion fields were scored, as scoring only proportions of fields could artificially increase parasitaemias. Identification markings on the slides were covered to blind analysis. The primary investigator scored all the slides.

Results were added and expressed as a percent pRBC. A score of < 0.05% was given if no pRBC were detected in the designated areas but were observed somewhere else on the smear. A score of 0% was assigned if no pRBC were detected on the smear after 15 minutes of scanning the slide without counting.

3.7 Repeatability of methodology

Twenty one complete sets consisting of 2 capillary and 2 venous smears were selected to include a range of parasitaemias. The repeatability of the slide scoring method was assessed by scoring all 84 smears twice. The repeatability of the smearing technique was assessed by scoring two venous smears for the same 21 dogs. The repeatability of the sampling method was quantified by scoring two capillary smears for 21 dogs. The difference between the two capillary smears from the same dog would be the result of variation of the distribution of parasites in the capillary capillaries of the individual over time or space as well as the variation induced by the smearing and slide scoring techniques. Pairs of capillary smears were analysed for a further 13 dogs, resulting in a total of 34.

3.8 Data analysis

Scores of <0.05% were converted to 0.04% for the statistical analysis. The score of 0% was converted to 0.001% when the logs of capillary and venous parasitaemias were calculated and compared.

Multiple regression analysis was used to compare parasitaemias from cephalic and jugular samples, adjusting for outcome group. This was performed to demonstrate that venous blood sampling site was not responsible for any bias in the results. The significance level was set at $\alpha = 0.05$.

Intra-class correlation coefficients (ICC) were calculated to describe the repeatability of scores for slides that were scored twice as well as for repeated venous and capillary smears within the same animal (Lachin, 2004). Dogs were divided into two groups according to their circulatory score. The Mann-Whitney U test was used to determine whether circulatory score was associated with the absolute difference between pairs of capillary slides. In addition, ICCs were calculated for the pairs of capillary smears for two groups.

Medians, ranges and interquartile ranges (IQR) were used to describe the capillary and venous parasitaemias as these were not normally distributed and log-transformation did not achieve a near-normal distribution. Parasitaemias were log-transformed before the correlation co-efficient was calculated. The Wilcoxon signed-rank test was used to compare capillary and venous parasitaemias for all the dogs together as well as within each of the three outcome groups. Kruskal-Wallis one-way ANOVA on ranks was used to determine whether capillary and venous parasitaemia differed between outcome groups. The Fisher exact test was used to confirm reports by previous investigators that dogs with a collapsed circulation were significantly more likely to die. The Wilcoxon rank-sum test was performed to compare the capillary and venous parasitaemias for dogs with and without circulatory compromise.

Statistical analyses were performed using NCSS 2004 (Kaysville, UT, USA) unless otherwise stated. Stata version 8.2 (StataCorp, College Station, Texas, USA) was used to calculate ICCs. SigmaPlot version 4.01 (SPSS Inc, Chicago, Illinois, USA) was used to calculate the correlation coefficient.

Chapter 4: Results - Description of study population

4.1 Total number of dogs and reasons for exclusion

Of the 117 dogs sampled, 17 dogs were excluded for the following reasons: 8 dogs were infected with *Ehrlichia*. One dog had concurrent pneumonia. One had demodecosis and its babesia parasitaemia, anaemia and splenomegaly required three months of repeated babesiocidal treatments before they finally resolved. Two dogs were euthanased electively. One dog died unexpectedly two days after diagnosis, having made an uneventful recovery from its babesiosis. Another died a week after being treated as an outpatient. The owner had observed this dog's condition deteriorate but felt he could not afford further treatment. Neither of the last two dogs was available for a post mortem examination. One dog treated as an outpatient (group H) was excluded because no follow up information could be obtained. Two dogs were excluded because their slides were damaged. This left 100 dogs in the study group.

4.2 Circulatory score

There were 21 dogs with a clinically compromised circulation and 79 dogs with normal circulation. A CRT below 1 second was the most prevalent circulatory abnormality (n=75). This was followed by collapse (n=17), hypothermia (n=6), dehydration (n=4), bradycardia (n=1) and weak pulse (n=1). The CRT was not determined in 4 dogs as they were muzzled. The temperature was not recorded for 1 dog. These 5 dogs had no other abnormal criterion, thus the missing information could not have changed their classification (Table IIIa).

4.3 Venous sampling sites

Venous samples were collected from the jugular in 77 dogs and the cephalic vein in 22 dogs. The collection site was not recorded for 1 dog. Venous sampling site did not affect the magnitude of the venous parasitaemia ($P = 0.6$).

4.4 PCR and RLB

Three of the 8 dogs infected with *Ehrlichia sp* were co-infected with *B rossii* and 5 with *B vogeli*. No dog was infected with more than one *Babesia sp*. No dog was infected with *B vogeli* only.

4.5 Groups of dogs

Of the 100 dogs included for further analysis, 60 dogs were admitted for further treatment and 40 discharged immediately after the consultation (group H). Ten of the admitted dogs died (group D), and 50 recovered (group A). Thus 16.7% of admitted dogs died.

4.6 Discussion

If this study were to be repeated, damaged slides could be minimised by staining all smears immediately after they are made and by placing a permanent cover-slip over them.

The circulatory score selected could be criticised for not using objective measures of tissue perfusion or cellular hypoxia. Perfusion may be globally inadequate owing to myocardial dysfunction, real or relative hypovolaemia (Hinds *et al.*, 1999). Direct arterial blood pressure, central venous pressure and pulmonary artery occlusion pressure have been used to monitor cardiovascular status objectively (Hinds *et al.*, 1999). A single reading using any of these techniques gives very limited information (Hinds *et al.*, 1999). Blood pressure in particular may be normal, increased or decreased in shock (Graham *et al.*, 2005). All the techniques are invasive, time consuming and some require expensive equipment. Hyperlactaemia has been used as a marker of tissue hypoxia, but is not a specific finding as lactate may also be elevated by hypermetabolism and organ disease (Jacobson *et al.*, 2005). Organ damage is known (Maegraith *et al.*, 1957; Welzl *et al.*, 2001) and hypermetabolism is expected to occur (Jacobson *et al.*, 2005) in some cases of canine babesiosis. Previous work in canine babesiosis showed that lactate concentrations on admission overlapped markedly between survivors and animals that died. The change in blood lactate in the first 24 hours of treatment was a much more accurate prognostic

indicator (Nel *et al.*, 2004). Thus there was no objective way to diagnose shock that did not require repeated readings.

It has been shown that resistance to blood flow decreases and flow rates increase in a variety of vascular beds in anaemic dogs (Vatner *et al.*, 1972). This is the likely explanation of the high prevalence of CRTs of less than 1 second (75 dogs) amongst dogs in this study. As there is a plausible alternative explanation for this observation in this study population that is unrelated to the development of shock, this criterion could probably be safely ignored in similar studies in the future.

The confusion regarding where 'peripheral' smears originate from was detailed in literature review (refer 1.5.2). The results presented here revealed no significant difference in parasitaemias obtained from cephalic and jugular venous samples. The results also demonstrate a clear difference between parasitaemias from ear prick (capillary) and venous samples. Future use of the terms 'capillary' and 'venous' may aid clarity.

PCR and RLB were used to identify the large babesia species that had been seen on capillary smears as *B vogeli* causes less severe clinical signs (refer 1.2.2), results in a lower parasitaemia (refer 1.5.2) and may distribute differently within its host (refer 1.5.1) than *B rossi*. As all samples submitted for PCR and RLB were known to be babesia positive, the data presented here cannot be used to determine the sensitivity of this test.

PCR and RLB were also used to exclude concurrent ehrlichiosis as this has been associated with higher babesia parasitaemias (refer 1.5.2) and more severe clinical signs (refer 1.3.2). Confirming canine ehrlichiosis is difficult (Van Heerden *et al.*, 1983). Morula are a highly specific finding on capillary smears but are rarely seen (Mylonakis *et al.*, 2003). Clinical, hematological and biochemical criteria (epistaxis, lymphadenopathy, petechiae, anaemia, neutropenia, thrombocytopenia, hyperglobulinemia) are unreliable (van Heerden, 1982) and all with the

exception of epistaxis are noted in babesiosis cases. Antibody detection by immunofluorescent assay (Waner *et al.*, 2001), PCR (McBride *et al.*, 1996) and tissue culture (Iqbal *et al.*, 1994) have all been considered to be the test of choice by various authors. PCR with RLB was the most sensitive and specific of all the diagnostic options available to us to exclude infection. The sensitivity of the test used for detecting *Ehrlichia canis* has not been determined. Based on information from 6 chronically infected dogs, spleen aspirates may be more likely to yield organisms than blood sampled (Harrus *et al.*, 1998). Collection of splenic aspirates was not an option in client owned and usually markedly thrombocytopenic dogs.

Animals were not grouped according to clinical signs as clinical signs are too varied to allow a simple clinical score to be derived (refer 1.2.3). The system is imperfect in that an animal that receives a blood transfusion and is discharged the next day will be in the same category as one that develops pulmonary and renal complications but survives. Alternative outcomes like cost of treatment and length of hospital stay are influenced significantly by factors not associated with the severity of the disease e.g. the fee structure for referred cases is higher, duration and extent of treatment will be affected by financial constraints of the owner, occasionally medication and blood will be donated to poor owners, animals may be hospitalised for several days after they are clinically ready to be discharged owing to transport or other client-related issues. Some of these factors will also influence survival as well as the decision to admit the case, but to a lesser extent.

Ten of the 60 dogs (16.7%) admitted for further treatment died. This mortality rate is similar to previously reported ones of 12-15% (van Zyl, 1995a; Nel *et al.*, 2004; Reyers *et al.*, 1998). The percentage of admitted cases appears to have increased gradually from 31% annually between 1988 and 1993 (Shakespeare, 1995), to 111/250 (44%) in 2002 (Keller *et al.*, 2004) and 60/100 (60%) in the group of dogs presented here. The difference between successive groups is significant ($P = 0.00002$ and $P = 0.011$ respectively). A possible explanation for this increase could be that private veterinarians in surrounding practices have taken over the treatment of simple babesiosis cases, resulting in the decreased number (refer 1.1) but increased severity of

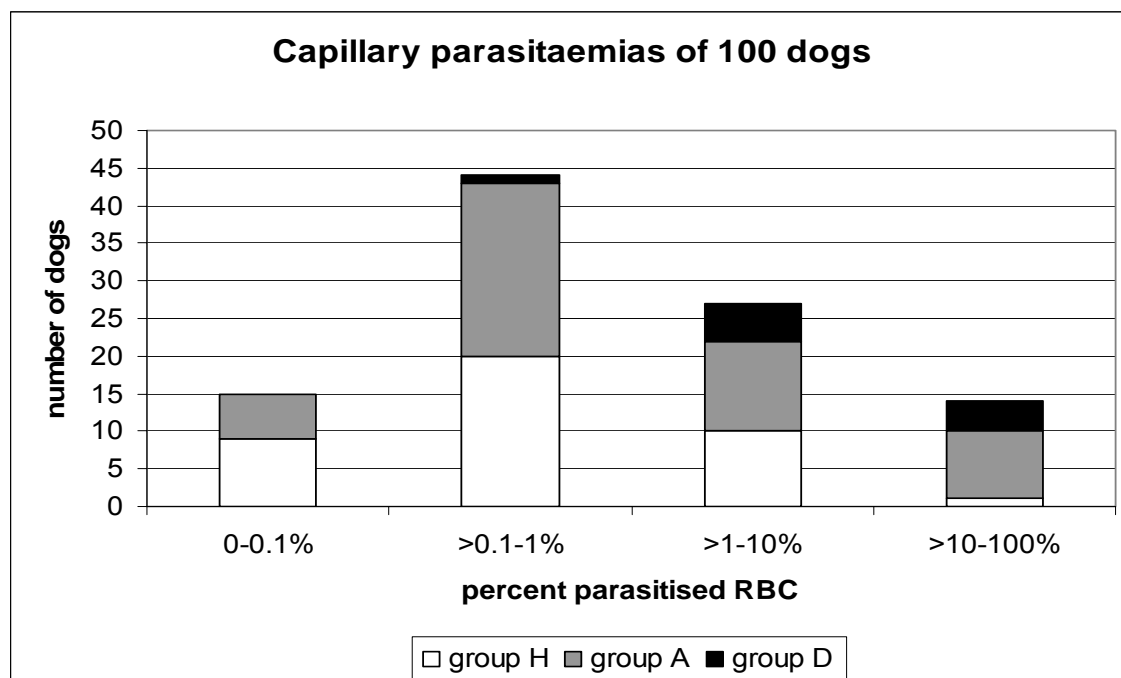
cases presented to the OVAH. Alternatively, veterinarians employed by the OVAH may have become more cautious about treating babesiosis cases as outpatients owing to an increased awareness of potential complications. Lastly, it is possible that a significantly greater percentage of clients presenting their dogs between 1988 and 1993 may have refused hospitalisation owing to financial constraints.

Chapter 5: Results – Methodology

5.1 Range of parasitaemias

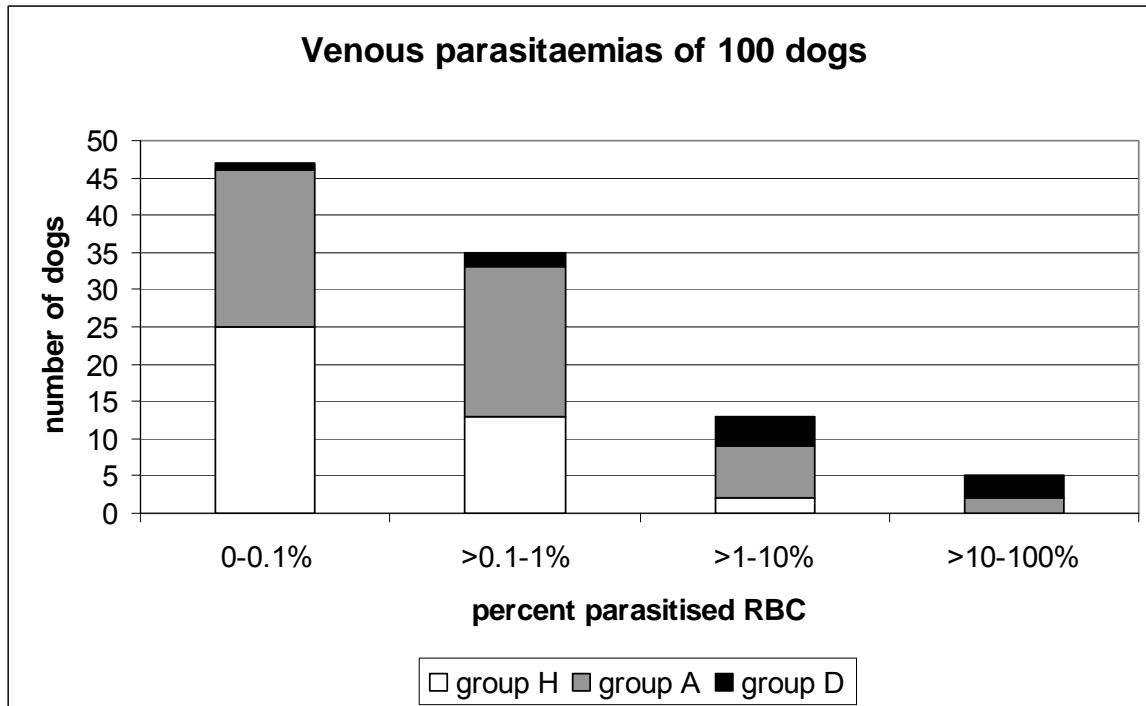
All capillary smears contained parasites. One venous smear was negative for parasites after 15 minutes of continuous searching without scoring fields. Fourteen dogs had capillary parasitaemias above 10% (range 10.2-71.6%) and 5 dogs had venous parasitaemias above 10% (range 11.56-30.6%).

Figure 5a: Capillary parasitaemias of the whole group



Groups H: 40 dogs treated as outpatients. Group A: 50 dogs that were hospitalised and recovered. Group D: 10 dogs that died of babesiosis.

Figure: 5b Venous parasitaemias of the whole group



Groups H: 40 dogs treated as outpatients. Group A: 50 dogs that were hospitalised and recovered. Group D: 10 dogs that died of babesiosis.

5.2 Repeatability of analysis of the same slide

Forty-two capillary slides from 21 dogs and 42 venous slides from the same 21 dogs were scored twice. Repeated scoring of capillary and venous slides showed ICCs (95% confidence intervals) of 0.988 (0.971 to 0.995) and 0.994 (0.986 to 0.998) respectively. The largest absolute difference in repeat scores for a capillary smear was for a slide that had a 18.89% parasitaemia on the first and a 24.41% parasitaemia on the second scoring – a difference of 5.52%. The largest percentage difference in repeat scores for a capillary was for a slide that was scored at 0.32% the first time and 0.7% the second time. Thus the second score was 2.19 times higher than the first score – or 219% of the first score. The largest absolute difference in repeat scores for a venous smear was for a slide that had a score of 4% on the first scoring and 6.64% on the second scoring – a difference of 2.65%. The largest percentage difference in scores for a venous slide

was for one that had a score of 0.69% the first time and 0.24% the second time. Thus the first score was 2.88 times higher than the second – or 288% of the second score.

5.3 Repeatability of parasitaemia in duplicate venous smears from the same dog

Comparison of the scores of two venous smears made from the same sample from the same dog revealed an ICC of 0.996 (0.991-0.999). Absolute differences in venous scores from two slides made from the same venous sample were under 1% in 19 of the 21 dogs. The dog with the largest difference between its two venous slides had parasitaemias of 27.4 and 29.7%.

5.4 Repeatability of parasitaemia in duplicate capillary smears from the same dog

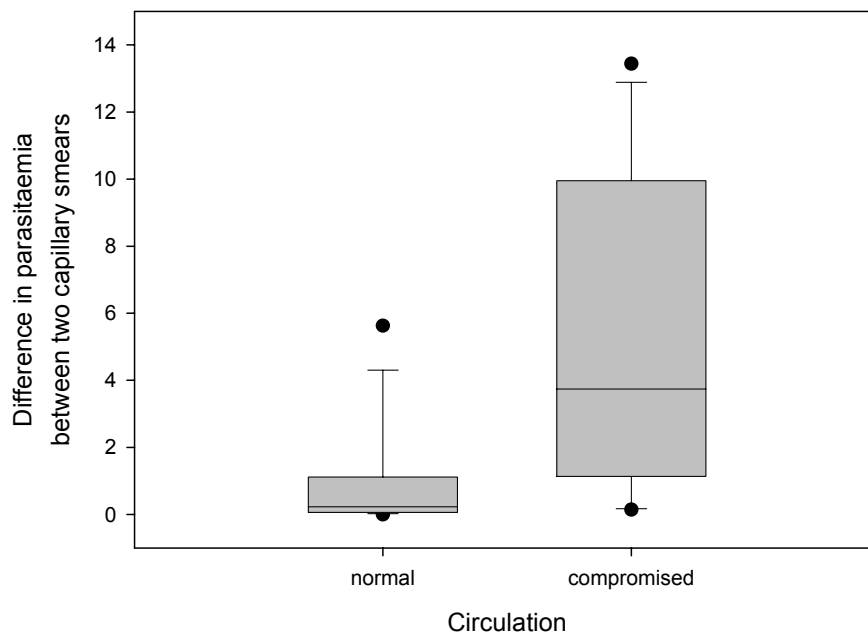
Pairs of capillary smears from the same dog demonstrated a greater variability with a lower ICC of 0.961 (0.924 to 0.98). Pairs of capillary slides showing the greatest variability in their score were a pair with 25.8 and 12.4% pRBC and a pair with 18.1 and 5.46% pRBC.

5.5 The influence of circulatory status on repeatability of capillary parasitaemias

Amongst the 34 pairs of capillary smears analysed, there were 18 from dogs with a clinically normal circulation (score 0) and 16 from dogs with a clinically collapsed circulation (score 1). The repeatability of the parasitaemia scores were examined in two ways. When the absolute difference between the two parasitaemia scores in pairs of capillary smears was compared, it was significantly greater in dogs with a circulatory score of 1 than dogs with a circulatory score of 0 ($P = 0.002$) (see Figure 5c).

When the ICCs of the same groups were compared there was no clear difference: the ICC for the dogs with a clinically normal circulation was 0.804 (range 0.566 – 0.918), whereas that for the dogs with a clinically compromised circulation was 0.953 (range 0.873 – 0.982).

Figure 5c: Association between circulatory status and absolute difference in percentage parasitaemias of pairs of capillary smears from the same dog



Horizontal lines represent the median, boxes the interquartile range, whiskers the 10th and 90th percentiles, and circles the outliers

5.6 Discussion

Despite widespread use of blood smears to determine parasitaemia in babesia research, no standard sampling site or methodology is described. In addition, only 3 of 20 papers discussing babesia parasitaemia described which area of the smear was scored (van Heerden *et al.*, 1983; Jacobson *et al.*, 1996; Jacobson *et al.*, 2002). We used a semi-quantitative non-volumetric method as the best compromise between accuracy and practicality. At least 650 RBC were examined in the red cell area and the same number along the feather edge and along the sides of the smears (see figure 3a). Thus two thirds of the RBC examined were from areas on the smear where pRBC are known to accumulate (Irwin *et al.*, 1991; Pardini, 2000). Total RBC density appeared to be highest along the sides of the smear and lowest along the feather edge.

The reason the above scoring method was chosen was that it took the uneven distribution of RBC and pRBC on the slide into account and tried to standardize the analysis with this in mind. It was theorised that results would be more repeatable, even if parasitaemias were higher than in other studies. Any magnification of parasitaemia was expected to affect all smears to a similar extent. Thus detection of a difference between the groups should not have been affected negatively. If anything, it should have been enhanced as higher absolute parasitaemias could exaggerate the differences between groups. If a fixed number of fields had been examined instead, anaemic samples would have had significantly fewer RBC examined, resulting in less repeatable results. This was important as dogs' PCVs ranged from 6 to 54% in this study.

Parasitaemias recorded in this group of dogs are higher than those reported previously for dogs thought to be infected with *B. rossii*. Maximum parasitaemias of 10% were recorded in capillary or venous samples from experimentally infected dogs (Stewart, 1983; Lewis *et al.*, 1995; Schetters *et al.*, 1997) and maximal venous parasitaemias of around 2.43% in a group of 31 dogs naturally infected with virulent South African babesias (Vaughan-Scott, 2001; Jacobson *et al.*, 2002; Jacobson *et al.*, 2005). The scoring method chosen may be partially responsible for the fact that parasitaemias recorded in this study were higher than those previously reported (refer Table I in 1.5.2). Until the effect of methodology on parasitaemias has been determined, parasitaemias should not be directly compared between studies. Treatment of experimentally infected dogs before higher parasitaemias developed could have contributed to the observed discrepancy (Stewart, 1983; Lewis *et al.*, 1995; Schetters *et al.*, 1997). Lastly, low prevalence of high parasitaemias and the small numbers of dogs previously reported on may also have played a role as prior reports of capillary, peripheral and venous *B. rossii* parasitaemias are based on 24, 9 and 31 dogs respectively.

Although two sets of slides were prepared from 40 dogs, some of these were not analysed. In a few cases, slides were damaged. The main reason analysis was terminated early was that slides took an average of 30 minutes to score.

The ICC may be approximated as follows:

$$\frac{\text{Mean square}_{\text{(variation between subjects)}} - \text{mean square}_{\text{(variation within subjects)}}}{\text{Mean square}_{\text{(variation between subjects)}} + \text{mean square}_{\text{(variation within subjects)}}}$$

Thus the ICC is a measure of the variation in parasitaemia that is explained by differences between slides rather than differences in repeat examinations of the same slide. It increases when the range of differences in the parasitaemias of a group decreases relative to the range of absolute parasitaemias in the group.

The 95% confidence intervals of the ICC for the repeat scoring of individual capillary and venous slides overlapped widely, suggesting that there was no clear difference between the repeatability of slide scoring for these two groups. This is not surprising as the variability in both analyses would be expected to be predominantly operator-induced. It is likely that the confidence interval for the capillary smears is wider because the parasitaemias in this groups are also higher, resulting in a higher absolute difference between scores.

Parasitaemias on repeated venous smears show more consistent results with an ICC of 0.996 (0.991-0.999) than repeated capillary smears (0.961 (0.924-0.98)). The fact that the 95% confidence intervals do not overlap at all suggests that this is a real difference in the repeatability of capillary and venous parasitaemias. The pairs of venous slides were prepared from the same sample. Thus the difference in the parasitaemias of pairs of venous smears would result from error inherent in the manual scoring technique as well as non-uniform distribution of parasites through the sample or smear. The pairs of capillary smears were prepared from separate ear pricks on different ears. The difference between pairs of capillary smears would also be affected by the factors mentioned above. The decreased repeatability of capillary smears suggests the presence of an additional factor. The most likely cause for the additional variability is that parasite distribution within ear capillaries is either uneven or varies from minute to minute. Prior

histopathological studies of changes in cerebral capillaries of fatal cases of canine babesiosis demonstrated uneven vascular filling in 19/54 cases (Pardini, 2000) and localised accumulation of pRBC in cerebral capillaries was noted in several case reports (Purchase, 1947; Basson *et al.*, 1965). Our observation is also consistent with previous observations of *B. rossi* (Maegraith *et al.*, 1957) and *Babesia bovis* (syn. *argentina*) infections (Callow *et al.*, 1974) although these investigators did not quantify the variability they observed. It means that venous smears would be more appropriate in a research setting when accuracy is more important, for example while monitoring parasitaemia in an individual over time. The pathomechanism behind these observations is not clear although similar observations in falciparum malaria make it tempting to infer that they are the result of active sequestration of pRBC. Much work is still needed to prove this.

The effect of circulatory status on the repeatability of capillary parasitaemia is not clear cut. When the absolute difference between the parasitaemias of pairs of capillary smears of dogs with a clinically normal or compromised circulation are compared, there is a significantly greater variability in the parasitaemia of dogs with a collapsed circulation. However, the absolute parasitaemia in this group is also higher and probably explains a lot of this difference. When the ICCs for the two groups are compared, the 95% confidence intervals overlap, so there is no clear difference between the groups. It appears strange that the ICC for the dogs with a clinically normal circulation (0.804) is a lot lower than that of the dogs with a clinically compromised circulation (0.953). This can be explained by referring back to the calculation for the ICC and then noting that the range of differences in the two capillary parasitaemias of dogs with and without circulatory compromise is similar (0-7.8% and 0.1-12.64% respectively) while the range of parasitaemias is markedly higher for the dogs showing circulatory compromise (0.04-73.9%) than for the dogs with a clinically normal circulation (0.04-19.6).

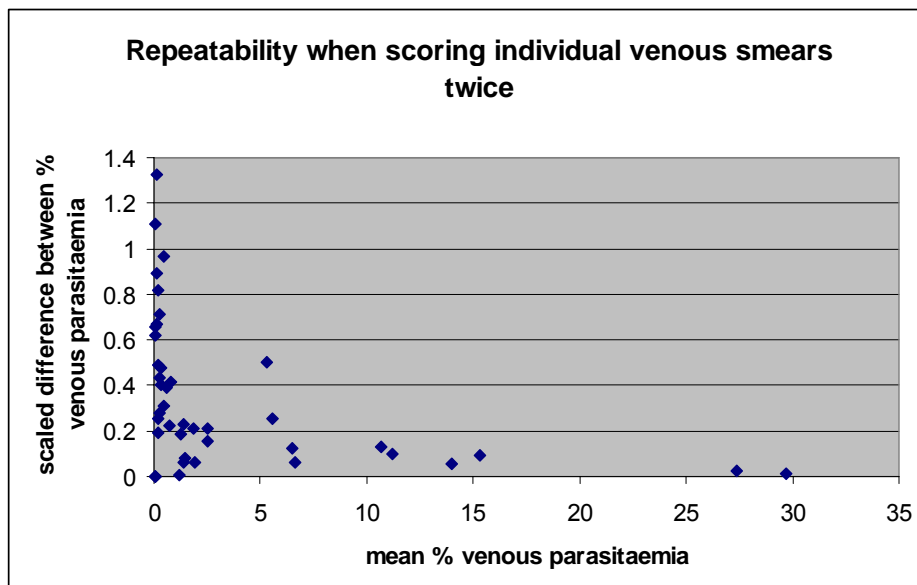
Only one prior paper was found that looked at sources of variability when parasitaemia was determined by microscopy (Prudhomme O'Meara *et al.*, 2005). The paper was published after the

design of and sample collection for this study had been completed, thus it was not included in the literature review. Variability was determined when the same venous smear was scored twice by the same person, the same smear was scored by 2 people or when two smears made from a single venous sample per malaria-infected patient were scored by the same person. Variability was expressed as the scaled difference:

$$\frac{\text{parasitaemia A} - \text{parasitaemia B}}{(\text{parasitaemia A} + \text{parasitaemia B}) / 2}$$

It was shown that variability decreased as absolute parasitaemia increased, irrespective of what scores were being compared. Repeatability was, in part, operator-dependent.

Figure 5d: Repeatability of slide scoring expressed at a scaled difference (Prudhomme O'Meara *et al.*, 2005)



When the repeatability of parasitaemia scores was expressed as a scaled difference for babesia infected dogs in this study, a similar decrease in variability was noted as parasitaemia increased. Figure 5d illustrates this. This is not surprising as parasites are more likely to be predictably and uniformly distributed at higher parasitaemias. In addition, the proportion of the error that is operator induced is likely to decrease rather than increase as parasitaemia increases. Lastly, mathematics dictates that when the difference between the two scores is expressed as a proportion, this will of necessity decrease as the percentage pRBC increases. Thus scaled differences could not be used to overcome the problems associated with ICCs.

Operator-dependent variability was not quantified in this study. As a single operator scored all the slides, the difference between two slides should not have been affected.

Chapter 6: Results - correlations between parasitaemias, circulatory status and outcome

6.1 Capillary compared with venous parasitaemia

Capillary parasitaemias of the group of 100 dogs (median 0.61%, range <0.05-71.6%, IQR 0.22-3.75%) were significantly higher than venous parasitaemia (median 0.14%, range 0-30.6%, IQR 0.046-0.52%) with $P < 0.0001$. Within each individual outcome group, this difference was also significant: Amongst the dogs that died, capillary parasitaemia (median 5.89%, range 1.21-71.6%) was significantly higher than venous parasitaemia (median 4.91%, range 0.1-30.6%) ($P = 0.025$). This was also true for the dogs surviving after hospitalisation (capillary parasitaemia median 0.56%, range <0.05-62.5%; venous parasitaemia median 0.17%, range <0.05-28.6%; $P < 0.0001$) and the dogs treated as outpatients (capillary parasitaemia median 0.37%, range <0.05-11.7%; venous parasitaemia median 0.09%, range 0-1.56%; $P < 0.0001$).

When the logs of capillary and venous parasitaemias were compared, the correlation co-efficient (r) was 0.797. Thus 63.5% of the variance in the capillary parasitaemia is explained by the relationship between capillary and venous parasitaemias (r^2). The correlation is graphically represented in Figure 6a. The venous parasitaemias cluster around -1.4. This is the log of 0.04, the value assigned to dogs with parasitaemias below 0.05% (see 3.8). The results of the single dog with a venous score of 0% were omitted for this analysis.

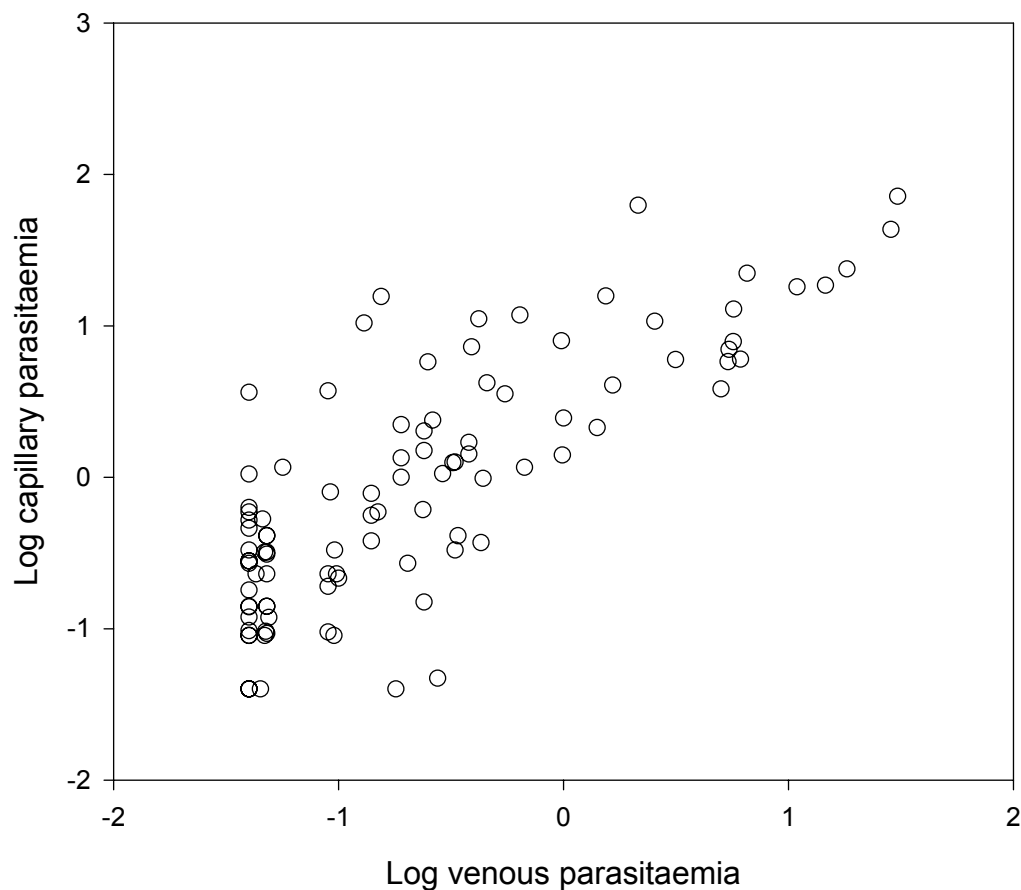


Figure 6a: Correlation between the logs of capillary and venous parasitaemias

Six of the 100 dogs had higher venous parasitaemias. One dog had both capillary and venous parasitaemias of 0.33%. Nineteen dogs had both capillary and venous parasitaemias below 0.15%, making it difficult to compare relative parasite density in the two sampling sites. Table VI illustrates that parasitaemias of 0.15% would mean that 3 pRBC were observed on the smear. Owing to the non-uniform distribution of parasites on a smear, scoring the other side and other edge of the feather edge could easily have resulted in just one parasite being observed. Thus the manual slide scoring technique used is not sensitive enough to offer meaningful comparisons of very low parasitaemias.

Table VI: Correlation between percentage parasitaemia and number of pRBC seen on a smear

Total pRBC seen	Parasitaemia if 2000 RBC examined
1	0.05%
2	0.1%
3	0.15%
4	0.2%
5	0.25%
10	0.5%
100	5%
1000	50%

The difference between capillary and venous parasitaemias in individual dogs varied. The largest absolute differences were capillary and venous parasitaemias of 62.5 and 2.51%, and 71.6 and 30.6% in two dogs. One dog had a capillary parasitaemia of 5.81% and a venous one of 5.4%, thus even dogs with relatively high parasitaemias could have small differences in parasitaemias of the two sample sites. It is difficult to analyse this data further in the absence of information on the reliability of individual measurements and of an objective measure of whole body parasitaemia.

6.2 Parasitaemia and outcome

Both capillary and venous parasitaemias of the dogs that died (group D) were significantly higher than those of the dogs that were treated as outpatients (group H) ($P < 0.0001$ for both sampling sites) and also significantly higher than those of the dogs that were admitted for treatment and survived (group A) ($P = 0.002$ for both sampling sites). There was no significant difference in the capillary and venous parasitaemias of groups A and H ($P = 0.1$ and 0.2 respectively) (Figures 6b and 6c).

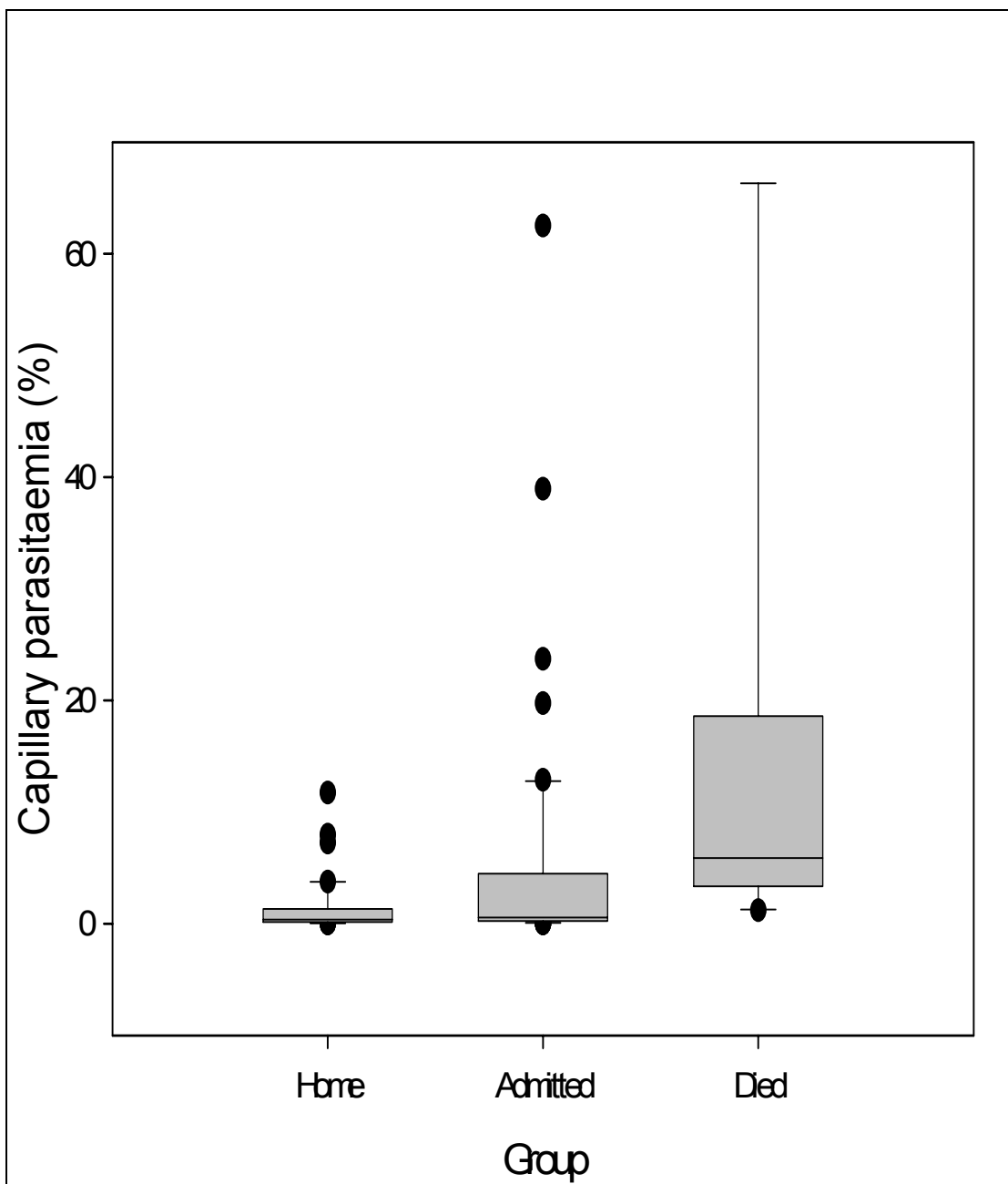


Figure 6b: Capillary parasitaemias of the three outcome groups. Horizontal lines represent the median, boxes the interquartile range, whiskers the 10th and 90th percentiles, and circles the outside values. The difference was significant when the 10 dead dogs were compared with the 40 dogs that went home ($P = 0.0001$) and the 50 admitted dogs ($P = 0.002$). The difference between 'home' and the 'admitted' groups was not significant ($P = 0.1$).

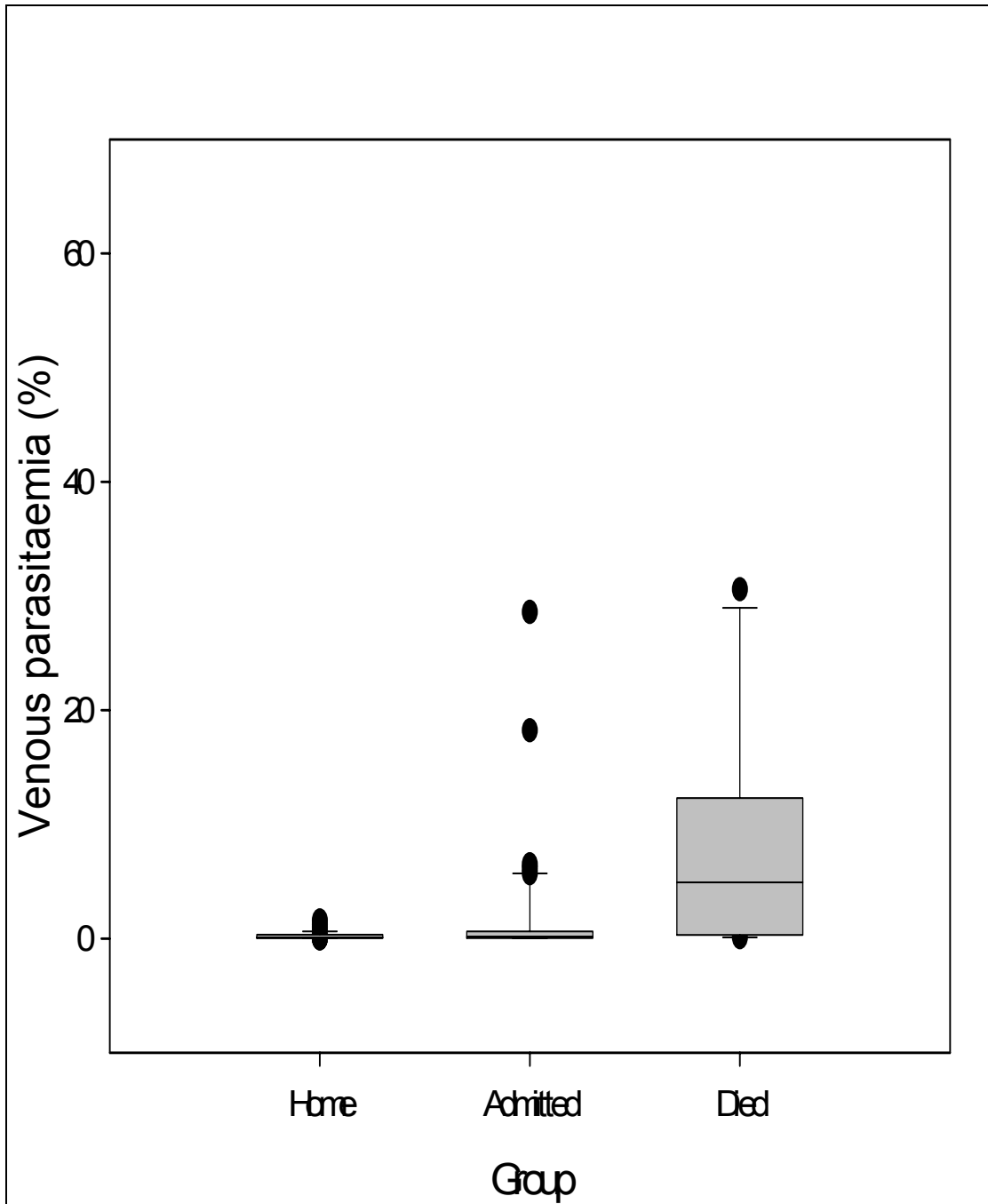


Figure 6c: Venous parasitaemias of the three outcome groups. Horizontal lines represent the median, boxes the interquartile range, whiskers the 10th and 90th percentiles, and circles the outside values. The difference was significant when the 10 dead dogs were compared with the 40 dogs that went home ($P = 0.0001$) and the 50 admitted dogs ($P = 0.002$). The difference between 'home' and the 'admitted' groups was not significant ($P = 0.2$).

6.3 Correlation between the ratio of capillary to venous parasitaemia and outcome

We were unable to find a statistical tool to determine which individual dogs had a capillary parasitaemia that was higher than could be explained by the error inherent in sampling and slide scoring. Further analysis was not performed for this reason.

6.4a Circulatory score and outcome

Nine of the 21 dogs with a clinically compromised circulation and 1 of the 79 dogs with a clinically normal circulation died. Dogs with a compromised circulation were therefore significantly more likely to die ($P < 0.0001$).

6.4b Circulatory score and parasitaemia

Dogs with a clinically compromised circulation had a significantly higher capillary (median 5.98%, range 0.09-71.6%, IQR 2.44-19.4%) and venous (median 2.81%, range <0.05-30.6%, IQR 0.17-9.03%) parasitaemia than dogs with a clinically normal circulation ($P < 0.0001$ for both). The latter had a median capillary parasitaemia of 0.38% (range <0.05-12.9%, IQR 0.16-1.42%) and a median venous parasitaemia of 0.096% (range 0-6.13%, IQR <0.05-0.33%) (Figures 6d and 6e). Thus median capillary parasitaemia was 16 times and median venous parasitaemia was 29 times higher in dogs with a clinically compromised circulation.

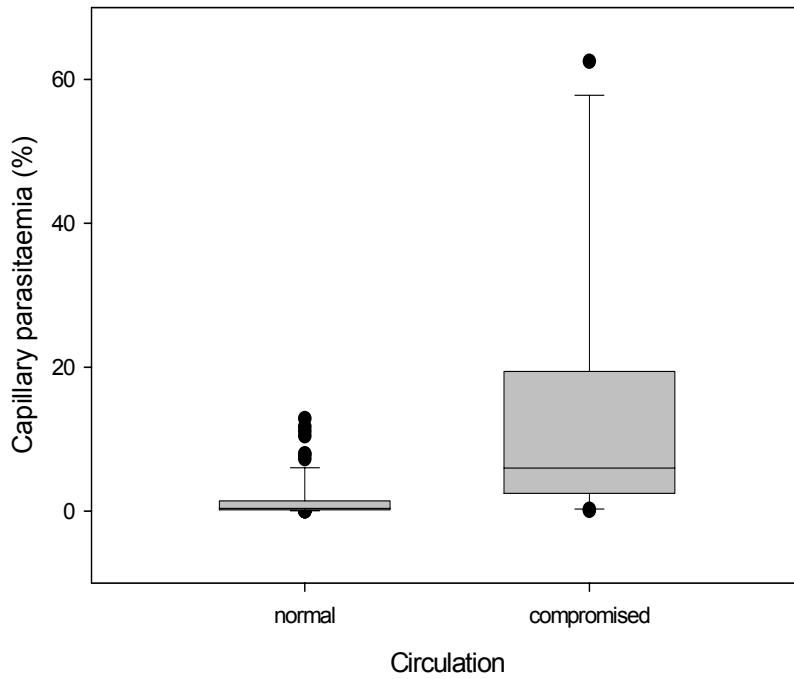


Figure 6d:
 Capillary parasitaemia of dogs with normal (n=21) and compromised circulation (n=79). Horizontal lines represent the median, boxes the interquartile range, whiskers the 10th and 90th percentiles, and circles the outside values. The difference between the two groups was significant ($P < 0.0001$)

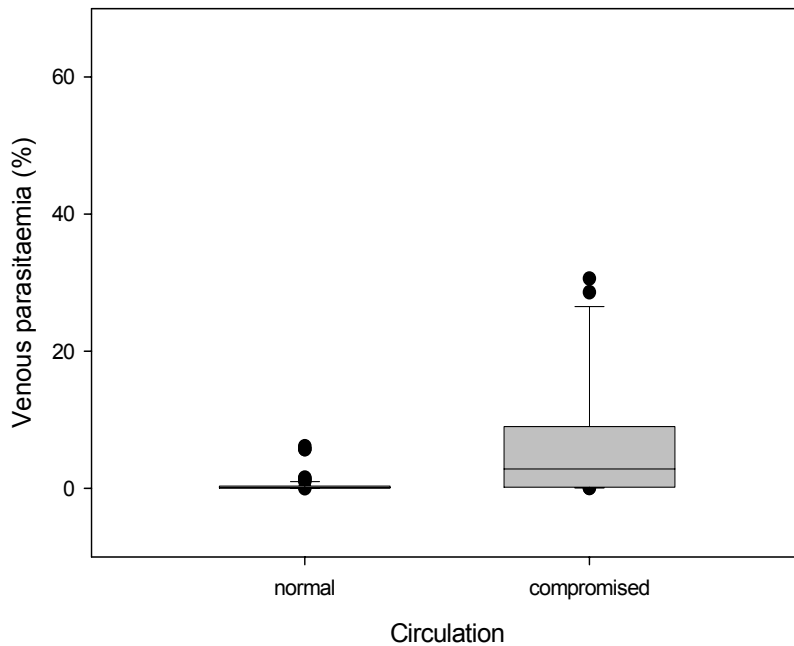


Figure 6e:
 Venous parasitaemia of dogs with normal and compromised circulation. Horizontal lines represent the median, boxes the interquartile range, whiskers the 10th and 90th percentiles, and circles the outside values. The difference between the two groups was significant ($P < 0.0001$)

6.5 Discussion

This study shows that that capillary parasitaemia is greater than venous parasitaemia in most *B. rossii* infected dogs. Thus parasites of the most pathogenic large canine babesia species accumulate in capillaries while the large babesia studied in the USA (assumed to be *B. vogeli*) is more prevalent in venous samples (Ewing, 1966; Birkenheuer *et al.*, 2005). This mirrors the observations in human malaria that the highly pathogenic *P. falciparum* results in higher capillary parasitaemias while the less pathogenic *P. vivax* appear with equal frequency in capillary and venous samples (Singh *et al.*, 2003). This observation is consistent with the theory that *B. rossii* sequesters. The fact that capillary parasitaemias were occasionally similar to or lower than venous parasitaemias suggests that sequestration, if present, occurs only in a proportion of *B. rossii* infected dogs, or only during certain stages of the evolution of the disease, or is spatially heterogeneous in dermal capillaries.

It means that capillary samples would be the ones most appropriately collected for diagnostic purposes as they are both quicker to collect and are likely to reveal *B. rossii* pRBC more readily. As only one venous slide had no detectable parasites during 15 minutes of scoring, it appears unlikely that many infections would be missed if only venous samples were analysed.

The higher capillary parasitaemias observed here need not necessarily be the result of sequestration. Parasite-induced increases in RBC membrane rigidity may specifically slow the transit of pRBC through capillaries and result in more pRBC being found there at any one time. Such changes in RBC membrane deformability have been demonstrated in falciparum malaria (Suwanarusk *et al.*, 2004) and septic shock (Hinshaw, 1996), a condition that bears some resemblance to complicated canine babesiosis and falciparum malaria (Clark *et al.*, 1998; Jacobson *et al.*, 2000; Welzl *et al.*, 2001). In addition, direct observations of capillary blood flow in 600 humans affected by a variety of diseases showed that RBC invariably lost their laminar flow

in people sufficiently ill to seek medical care and the passage of sedimented RBC through capillaries was retarded (Knisely *et al.*, 1947) (refer 1.3.2). It has been postulated that parasites within aggregated RBC could find themselves in a micro-environment more favourable to multiplication (Malherbe, 1956; Schetters *et al.*, 1998) and indirect evidence of local proliferation of canine babesias has been presented (Schetters *et al.*, 1998). Thus any mechanism (shock, sludging, rigid pRBC) that retards capillary flow could favour localized parasite proliferation and result in higher capillary parasitaemias.

When considering the correlation between capillary and venous parasitaemia, Figure 6a illustrates that for each individual venous parasitaemia, the capillary parasitaemia varies over a range of 2 logs i.e. over 100 times. Thus it is impossible to predict capillary parasitaemia if venous parasitaemia is known.

The correlation co-efficient shows that 63.5% of the variance in the capillary parasitaemia is explained by its relationship to venous parasitaemia (refer 6.1). The remaining 36.5% of the variance would include the error inherent in manual slide scoring. Further variance could be the result of variation in individual dogs' response to infection, different parasite strains and / or different stages in the evolution of the disease.

The results presented here show that *B. rossi* infected dogs that died had significantly higher capillary and venous parasitaemias than dogs that survived. A similar association has been shown in falciparum malaria (World Health Organisation, 2000). The only previous study that evaluated this aspect in canine babesiosis could not show a significant difference between the parasitaemias of survivors and non-survivors (Reyers *et al.*, 1995). The study was designed to assess the prognostic significance of haematological parameters in babesia-infected dogs. The venous samples used in the study were drawn in the first 24 hours after admission and treatment with an antibabesial. It was shown subsequently that parasitaemia will, on average, increase slightly during the first two hours after treatment with diminazene aceturate or trypan blue and

then fall dramatically by 6 hours post treatment (Jacobson *et al.*, 1996). Sampling animals at variable times post treatment probably obscured significant findings in the earlier study.

Despite the highly significant association between dogs that died and high parasitaemias, there is so much overlap between the groups (Figures 6b and 6c) that this finding is not clinically helpful. The reason parasitaemia does not correlate more closely with outcome may lie in the varied host response to infection. For most dogs, parasite-induced anaemia is the most serious and potentially life-threatening problem. A proportion develop complications, some of which (hepatopathy, immune-mediated haemolysis) typically extend hospital stay but do not affect mortality if appropriately treated (Jacobson *et al.*, 1994; van Zyl, 1995; Miller, 1999), while others (haemoconcentration, neurological signs not associated with hypoglycaemia, acute renal failure, pulmonary oedema) require early, aggressive and intensive therapy and carry a poor prognosis (Jacobson *et al.*, 1994; Reyers *et al.*, 1995; Welzl *et al.*, 2001; Keller *et al.*, 2004). Individual dogs may develop several different complications to varying degrees (Welzl *et al.*, 2001), thus disease manifestations form a continuum and affected dogs cannot be separated perfectly into uniform groups. An additional reason for the lack of a significant difference in parasitaemias between the two surviving groups is that the decision to hospitalise a dog was made by different people, as discussed in the materials and methods (refer 3.5). Some dogs were hospitalised overnight but were discharged without further treatment when, for example, urine production was maintained or the PCV did not drop precipitously. Others were treated as outpatients at the owner's request owing to cost constraints although hospitalisation was indicated. Lastly the varied times after infection that the dogs were presented for treatment may have affected parasitaemia (Lewis *et al.*, 1995).

The correlation between the ratio of capillary to venous parasitaemias and outcome was not explored as it was not possible to find a statistical tool to determine which individual dogs had a capillary parasitaemia that was higher than could be explained by the error inherent in the sampling and slide scoring and as there were problems associated with the use of such a ratio.

The main difficulty was that the errors induced by slide preparation and the operator could not be quantified in any meaningful way. Common sense allowed identification of some individuals which undoubtedly had higher capillary parasitaemias. Dogs with capillary and venous parasitaemias of 62.5 and 2.51%, and 71.6 and 30.6% were examples. Conversely, the six dogs that had higher venous parasitaemia scores probably did not have pRBC accumulating in capillary beds of the ear.

An additional problem was that when differences in capillary and venous parasitaemias were expressed as a percentage of venous parasitaemia, it exaggerated the difference at low parasitaemias. For example, a dog with a capillary parasitaemia of 0.14% and a venous one of 0.048% had a capillary parasitaemia that was 291.7% of the venous one. Yet most if not all of this difference could have been the result of the technique used in determining that parasitaemia (see 6.1). Conversely, the dog with a capillary parasitaemia of 43.2% and a venous one of 28.6% had a capillary parasitaemia that was only 151% of the venous one, yet there was almost certainly a real difference between these two results. The same difficulty was encountered when ratios of capillary and venous parasitaemias were calculated. It was also noted when scaled differences (also essentially a ratio) were calculated to allow comparison of the results on repeatability of smear analysis presented here with those presented in an earlier malaria paper (Prudhomme O'Meara *et al.*, 2005) (refer 5.6).

When differences in capillary and venous parasitaemias were expressed as an absolute difference, this tended to exaggerate differences at high parasitaemias. For example, the dog with a capillary parasitaemia of 1.05% and a venous one of 0.04% had an absolute difference in parasitaemia of 1.01%. Yet 21 parasites had been seen on the capillary smear and only one on the venous smear and this was outside the designated areas (refer Table VI). This was almost certainly a real difference. In contrast, the dog with a capillary parasitaemia of 7% and a venous one of 5.45% had a higher absolute difference between the two (1.55%). Yet the parasitaemias

are equivalent to seeing 140 pRBC on the capillary and 109 pRBC on the venous smear. It is much less certain that this difference was real.

The comparisons made between capillary and venous parasitaemias of groups of dogs are still valid despite these problems as it is reasonable to assume that errors in the method used to determine parasitaemia would affect capillary and venous samples to a similar extent.

There was a highly significant association between a higher circulatory score and outcome. This confirms prior work that clinical collapse and shock are poor prognostic signs in dogs with babesiosis (Maegraith *et al.*, 1957; Abdullahi *et al.*, 1990).

In the group of dogs with a clinically collapsed circulation, both capillary and venous parasitaemias were significantly higher than those of dogs with clinically normal circulation (Figures 6d and 6e). Capillary parasitaemia would be expected to be increased markedly and out of proportion to venous parasitaemia if parasite sequestration is more prevalent among animals with a collapsed circulation. This was not shown.

The association between higher parasitaemias and circulatory compromise prompts the question whether the two are causally related. Although canine babesiosis has been described as a form of sepsis (Jacobson *et al.*, 2000), high parasitaemias are unlikely to be the sole trigger of circulatory collapse in babesia infected dogs as some dogs with low parasitaemias developed signs of circulatory collapse. The type of immune response or inflammatory response mounted by babesia-infected dogs is likely to play a significant role (Jacobson *et al.*, 1994; Reyers *et al.*, 1998). In individual animals hypovolaemia (Jacobson *et al.*, 2000) and decreased myocardial function (Lobetti, 2005) could contribute toward the development of shock. Although these abnormalities are recognised as independent triggers of circulatory collapse, all could be related to the type of host inflammatory or immune response in babesiosis. The study of inflammatory mediators in babesia-infected dogs has not revealed any strong correlations with parasitaemia,

outcome or circulatory compromise yet (Vaughan-Scott, 2001; Jacobson *et al.*, 2002; Ulutas *et al.*, 2005). The small number of dogs studied and the global measures used to study inflammatory mediators that often have a short half-life and a local effect probably hampered detection of significant correlations. This aspect of canine babesiosis deserves further study.

Chapter 7: Conclusions

In summary, three of the four research questions were answered:

1. *B. rossi* pRBC were more numerous in capillary than in venous smears collected within 10 minutes of each other. Although this has long been suspected to be true for *B. rossi* infections, this study provides the first proof. The converse has been shown for an American large babesia assumed to be *B. vogeli* (Ewing, 1966; Birkenheuer *et al.*, 2005). This observation adds to the known differences between *B. vogeli* and *B. rossi* infections and proves that capillary smears are the most sensitive diagnostic sample when *B. rossi* infection is suspected.
2. Both capillary and venous parasitaemias were significantly higher in dogs that died of their infection. There was a wide overlap in the parasitaemias of the three outcome groups with the result that neither capillary nor venous parasitaemias appear prognostically useful.
3. The question whether the ratio of capillary to venous parasitaemias correlated with outcome was not answered.
4. Dogs that died were more likely to have a clinically compromised circulation than dogs that survived infection and dogs with a clinically compromised circulation had significantly higher capillary and venous parasitaemias than dogs with a normal circulation.

The following were additional useful observations: It was shown that the parasitaemias determined from repeated venous smears were more consistent than those determined from repeated capillary smears. Thus venous smears would be indicated if a serial indication of parasite burden is required.

The study showed that venous parasitaemias were associated with a wide range of capillary parasitaemias and that it is thus impossible to predict capillary parasitaemia if venous parasitaemia is known.

The association between circulatory collapse and poorer outcome shown in this study provides a rapid means of identifying patients in need of intensive monitoring and treatment. The association between circulatory collapse and parasitaemia provides a practical means of selecting patients likely to harbour high parasite burdens, should this be required for future studies.

The study reports on capillary parasitaemias of four times more *B rossi* infected dogs than previously recorded in the literature and venous parasitaemias of 3 times more dogs. Higher parasitaemias observed may have been affected by the different slide scoring method used, but could also be the consequence of this being by far the largest survey of parasitaemias in naturally infected dogs.

The following three findings are consistent with the hypothesis that sequestration plays an important role in the pathophysiology of *B rossi* infections: Firstly, capillary parasitaemias were generally higher than venous ones. Secondly, higher capillary parasitaemias were associated with a poorer outcome and circulatory collapse. Lastly, results from pairs of capillary smears were less repeatable than those of pairs of venous smears, suggesting that pRBC distribute unevenly in ear capillaries. The observations that higher venous parasitaemias were also associated with a poorer outcome and circulatory collapse suggest that the influence of total parasite burden on the severity of disease manifestations may be greater than that of (possible) sequestration. The observation that some dogs with low parasitaemias also died underscores the complexity of the factors affecting outcome in this disease.

Although this study provides evidence that RBC parasitised by *B rossi* accumulate in dermal capillaries and that this accumulation is not necessarily uniform, further research will be

necessary to determine the role parasite sequestration plays in *B. rossii* infections. Sequestration is by no means the only pathomechanism that could explain these observations. Electron-microscopic studies of pRBC in capillaries in skin biopsies of infected dogs may show that pRBC adhere to capillary walls. Molecular studies will be needed to prove that adhesion is the result of parasite-induced ligands binding to endothelial receptors.

The fact that venous babesia parasitaemias observed here were occasionally higher than capillary ones suggests that sequestration, if present, may only affect some individuals or may be associated with particular stages of infection. Thus accurate means of determining the presence or absence of significantly higher capillary parasitaemias in individual dogs and of determining total parasite burden are required. These are not available at present. The use of flow cytometry should be investigated as this technique has been used to detect much lower babesia parasitaemias than ones observed in this study (Bicalho *et al.*, 2004). No information on repeatability of the technique in babesia infected dogs was found, but results would be expected to be more accurate as the technique allows many more RBC to be scrutinised for parasites in a short period. In addition, the technique should be less susceptible to non-uniform distribution of pRBC within a sample as the entire sample may be analysed. Determination of total parasite burden in a host in which pRBC distribute non-uniformly is likely to be even more challenging. Lastly, intra-erythrocytic babesia parasites have a shorter lifecycle than *P. falciparum* (17 hours vs 48 hrs (Cooke *et al.*, 2005) and lack the morphological changes associated with the maturation of malaria parasites. This will make it more difficult to determine whether sequestration of babesia parasites is associated with particular stages of infection.

Addenda

The information presented in addendum A is presented because it is the first information on *B vogeli* infection of dogs living in the vicinity of the OVAH. The information presented in addendum B was collected as part of another study that was supposed to run concurrently but did not happen. The criteria discussed in the addendum C (lymphadenopathy, TSP above 80 g/l and petechiation) were initially amongst the exclusion criteria for this study as they were thought to be suggestive of concurrent ehrlichiosis. The dogs were sampled and blood submitted for PCR as a colleague (P.T. Matjila) had requested samples from any dog infected with large babesia species. When it became clear that the majority did not, in fact, harbour concurrent *E canis* their data was included in this study.

Addendum A: *B vogeli*

One hundred and seventeen dogs were sampled. Eight dogs were found to be infected with *Ehrlichia* sp. Five of these were co-infected with *Babesia vogeli* and 3 with *B rossi*. No dog was infected with more than one babesia species. No dog was infected with *B vogeli* only. Dogs with *Ehrlichia* morula on capillary smear were not sampled. Thus less than 4.3% of the dogs that presented to the OVAH with large babesias and without *Ehrlichia* morula on capillary smear were infected with *B vogeli*. Canine parvoviral enteritis was later confirmed by faecal electronmicroscopy in one and suspected in another dog. As *E canis* and *B vogeli* share their tick vector (Uilenberg *et al.*, 1989), the exclusion of dogs with ehrlichia morula on capillary smear probably decreased the chance of finding *B vogeli* infected dogs.

In a convenience sample of approximately 300 additional dogs presented to the OVAH during the course of 2005, 1 additional dog infected with *B vogeli* was identified ^b.

^b T. Matjila, personal communication 2006

Three of the dogs infected with both *E canis* and *B vogeli* in this study were 6 and 14 months and 3 years old. The two with concurrent confirmed or suspected parvoviral enteritis were 2 and 10 months old. The five dogs had capillary parasitaemias between <0.05 and 0.38% and venous parasitaemias below 0.05%. Two venous smears were too damaged to score. All five dogs were admitted and one died. There were too few *B vogeli* infected dogs to allow meaningful comparisons with *B rossi* infected ones.

Elsewhere in the world, *B vogeli* commonly causes subclinical infection in adult dogs (Farwell *et al.*, 1982; Callow, 1984; Irwin *et al.*, 1991; Boozer *et al.*, 2003) with very low parasitaemias after initial infection (Bicalho *et al.*, 2004). It is possible that the concurrent ehrlichiosis resulted in detection of otherwise subclinical *B vogeli* in the adult dogs in this study, either because it increased the severity of the babesiosis (van Heerden *et al.*, 1983) or because the ehrlichiosis mimicked clinical signs of babesiosis and *B vogeli* parasites were then detected on blood smear.

B vogeli causes severe disease in puppies elsewhere in the world. In two studies, 26/32 (81%)(Irwin *et al.*, 1991) and 7/11 (Farwell *et al.*, 1982) dogs showing clinical signs of babesiosis were less than 4 months old. Only one of 5 *B vogeli* infected dogs in this study fitted into this age group. It seems unlikely that puppies in South Africa are less susceptible to *B vogeli* than elsewhere. It is possible that *B vogeli* infections in anaemic puppies are missed at the Outpatients' clinic if another cause for the anaemia is detected (e.g. severe helminthiasis, severe flea infestation, parvoviral enteritis) as PCR is not routinely performed on these dogs. Alternatively, exposure to the pathogen could be lower in puppies around the OVAH for some reason. Lastly, the impression of a differing age distribution could be the random result of the small sample size. A random sample of clinically healthy adult township dogs that are mildly anaemic but aparasitaemic on capillary smear, or of aparasitaemic but anaemic puppies may reveal a higher prevalence of *B vogeli* when tested with PCR.

Addendum B: Correlation of clinical and historical data with parasitaemia and outcome

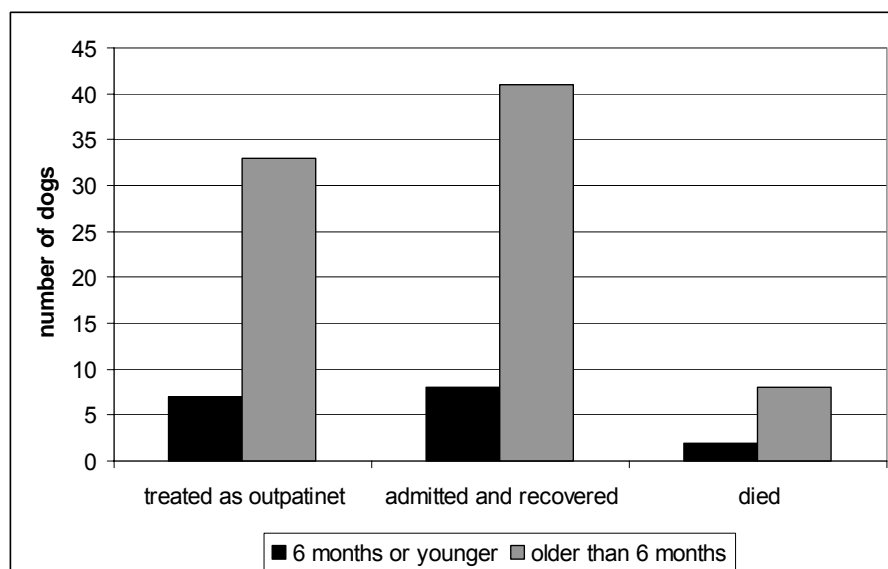
b.1 Correlation of the age of dog with severity of parasitaemia, outcome and PCV

There were 82 dogs older than 6 months and 17 dogs aged six months or younger. One dog's age was not recorded. The median capillary parasitaemia in the older group was 0.55% (range 0.04-62.61%, IQR 0.2-2.42%) and their median venous parasitaemia was 0.099% (range 0 – 14.3%, IQR 0.046 – 0.36%). The median capillary parasitaemia of the younger group was 3.55% (range 0.04-71.6%, IQR 0.26-16.9%) and their median venous parasitaemia was 0.64% (range 0.04-30.6%, IQR 0.14-8.32%). The Wilcoxon rank sum test showed that capillary and venous parasitaemias of dogs aged six months or younger were significantly higher ($P = 0.019$ and 0.006 respectively) than those of older dogs.

Previous South African workers found that between 38% and 53% of infected dogs were younger than 1 year (Jacobson *et al.*, 1994; van Zyl, 1995a; Keller *et al.*, 2004). In the group studied here, 38% of dogs were of this age. Thus dogs younger than 1 year of age were over-represented amongst dogs presenting to the Outpatients' clinic with babesiosis. It cannot be concluded from these observations that young dogs are more susceptible to babesiosis in South Africa as no study has compared the age distribution of babesiosis patients with that of the general patient population or, better still, that of the local dog population.

Of the dogs older than 6 months, 33 went home after the consultation (group H), 41 were admitted (group A) and 8 died. In the younger group, 7 dogs went home after the consultation, 8 were admitted for further treatment and recovered and 2 dogs died (see Figure B i). The two surviving groups were combined to increase the likelihood of finding an association between age and outcome. A Fisher's exact test could demonstrate no such association ($P = 0.68$).

Figure B i: Outcome of infection amongst dogs older than 6 months of age and younger dogs



Others found that when older dogs became infected, the disease was more often complicated and resulted in a higher mortality (Jacobson *et al.*, 1994; Reyers *et al.*, 1998). Jacobson and Clark (1994) report the highest mortality in dogs aged 1 to 5 years. Reyers and others (1998) showed that non-anaemic dogs had a higher median age (2.66 years) and a two to three times higher mortality than moderately or severely anaemic groups (median of 1.29 and 0.83 years respectively). A Fisher's exact test was thus used to compare the prevalence of death amongst the 67 dogs less than 3 years old (6 died) and the 32 older dogs (4 died). There was still no significant association between age and survival ($P = 0.72$). Both of these studies included significantly larger numbers of dogs. The smaller numbers sampled for this study may explain its discordant findings.

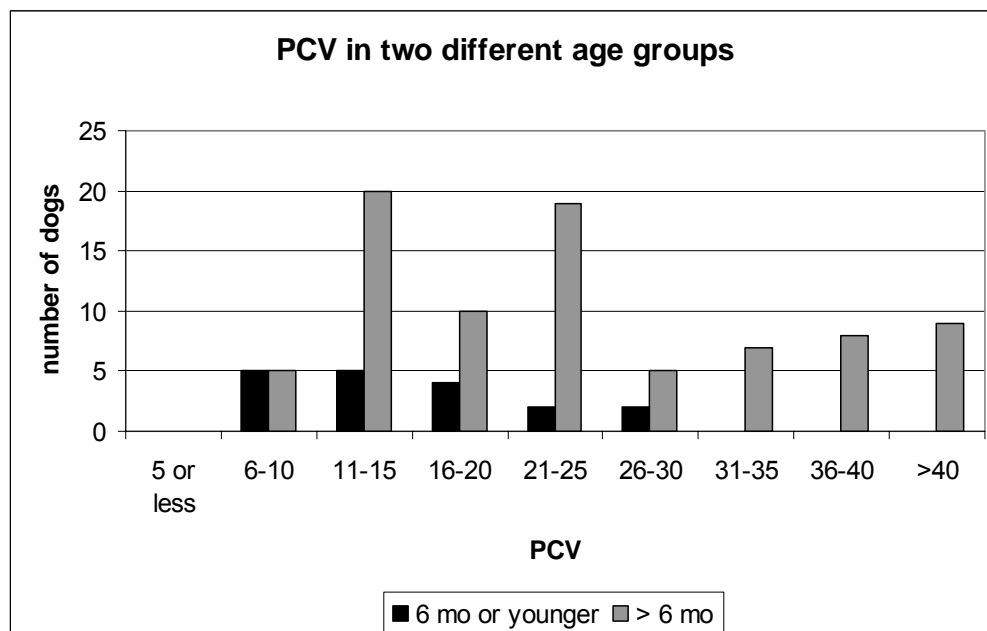
B vogeli has been shown to cause more severe clinical signs and increased mortalities in dogs less than 4 months old (refer Addendum A). Only 4 of the *B rossi* infected dogs sampled in this study were of this age. One was excluded from the final study group as it suffered from concurrent demodeciosis. All three of the remaining puppies were admitted but all survived. A Fisher's exact test showed that here was no significant difference in the outcome of these

puppies when compared to the remainder of the group ($P = 1$), but there were too few puppies less than 4 months old to be able to state with confidence that the lack of a statistically significant difference in the groups means that findings would be similar in a larger group of puppies.

The exclusion criteria used did not bias against the inclusion of puppies (refer 3.1). It could not be shown that puppies less than 4 months of age were more commonly infected with *B. rossii*. It is not clear whether this apparent difference between *B. rossii* and *B. vogeli* is the result of differences between the parasites, differences in puppies' susceptibility to infection with the two parasites or merely the result of different exposure patterns.

Lastly, the association between age and PCV was explored (Figure B ii). The median PCV of the group of dogs aged 6 months or less was 14% (range 6-28%, IQR 10-22%). The median PCV of the older group was 23% (range 8-54%, IQR 14-32%). A Wilcoxon rank sum test showed that the difference was statistically significant ($P = 0.005$).

Figure B ii: Comparison of PCV in dogs older than 6 month of age and younger dogs

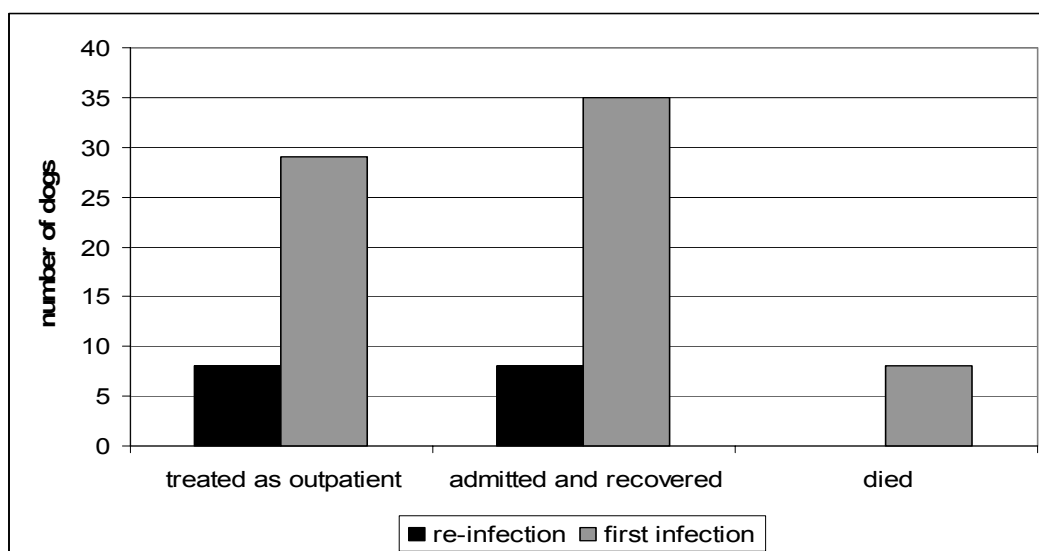


This confirmed previous findings that young dogs are more likely to have a severe anaemia (Jacobson *et al.*, 1994; Reyers *et al.*, 1998).

b.2 Correlation of previous exposure with severity of parasitaemia, outcome and PCV

There were 72 dogs presenting with babesiosis for the first time. They had a median capillary parasitaemia of 1.03% (range 0.04-71.6%, IQR 0.23-5.8%) and a median venous parasitaemia of 0.19% (range 0-30.58%, IQR 0.048-0.9%). Sixteen dogs had been infected previously. They had a median capillary parasitaemia of 0.25% (range 0.04-7.96%, IQR 0.11-0.51%) and a median venous parasitaemia of 0.047% (range 0.04-0.98%, IQR 0.04-0.048%). The information on prior exposure was not known for 12 dogs. A Wilcoxon rank sum test showed capillary and venous parasitaemia of previously exposed dogs was significantly lower than parasitaemias of dogs infected for the first time ($P = 0.012$ and 0.0007 respectively).

Figure B iii: Comparison of outcome amongst dogs infected for the first time and re-infected dogs



Of the 72 dogs infected for the first time, 29 were discharged immediately, 35 were admitted for further treatment and survived and 8 died. Of the 16 dogs that had been infected before, 8 were immediately discharged and 8 were admitted. None died (See Figure B iii). The Fisher's exact

test showed that there was no significant association between survival and prior exposure to babesiosis ($P = 0.34$). It is possible that the relatively small numbers of previously exposed dogs may have been insufficient to demonstrate an association.

The median PCV of the dogs infected for the first time was 21.5% (range 6-54%, IQR 12.25-27.5%). The median PCV of the dogs previously infected was 27% (range 10-48%, IQR 17-38.5%). A Wilcoxon rank sum test showed that these groups were significantly different ($P = 0.04$). It was considered likely that dogs exposed for the first time would also be younger dogs. Thus a multiple regression analysis was applied to estimate the relative effects of age and previous exposure on PCV. This showed that PCV increased by a mean of 0.09% for every month increase in age ($P = 0.034$). The effect of previous exposure on PCV did not reach significance ($P = 0.089$). Again, it is possible that a study that includes a larger number of previously infected dogs may detect significant findings.

Previous investigators proposed that the first infection with *B. rossi* in a young dog leads to severe anaemia as a result of haemolysis. They proposed older dogs experiencing subsequent infections were more likely to mount a massive, inappropriate inflammatory response, resulting in severe complications like haemoconcentration (Reyers *et al.*, 1998), explaining the greater average age in their non-anaemic group of dogs. The data from this group of 100 dogs did not support an association between outcome and prior exposure.

It has been suggested that severe complications develop more readily when a hereditary predisposition is present in a dog that has been previously exposed to babesiosis. Fighting breeds were over-represented among non-survivors in one study (Reyers *et al.*, 1998). The data from this study could not support this theory as only 4 dogs of fighting breeds (English, Staffordshire or American Pit Bullterriers) were included. They were all hospitalised but all recovered. It is possible that increased awareness of possible complications in fighting breeds

amongst OVAH clinicians lead to more intensive observation and treatment following the above study, resulting in apparently improved results.

b.3 Correlation of body temperature with parasitaemia and outcome

Fifty three dogs had a rectal temperature above 39.5°C. Forty dogs' temperatures were between 37.5 and 39.5°C and 6 dogs had temperatures below 37.5°C. The temperature was not recorded for 1 dog. The parasitaemias for these three groups are presented below.

Table B i: Capillary and venous parasitaemias of hyperthermic, normothermic and hypothermic dogs

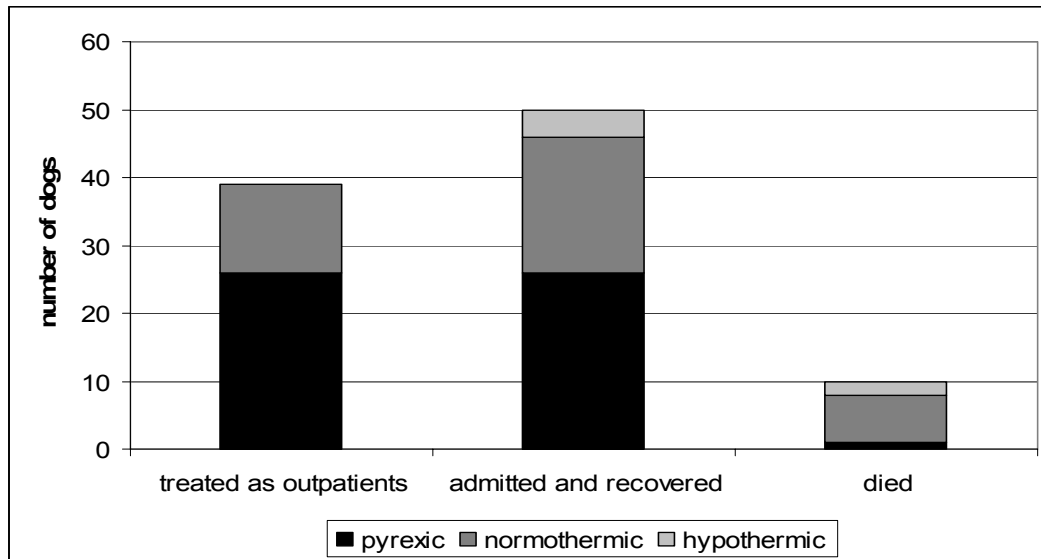
	> 39.5°C	37.5-39.5°C	< 37.5°C
median capillary parasitaemia	0.52	0.705	8.778
range capillary parasitaemia	0.04-23.72	0.04-62.51	1.16-71.55
median venous parasitaemia	0.14	0.16	0.445
range venous parasitaemia	0.04-18.23	0-28.55	0.04-30.58

The data were not normally distributed, so Spearman's correlation co-efficients were calculated. These showed that there was no significant correlation between body temperature and capillary or venous parasitaemias (Spearman's $r = -0.17$ and -0.05 respectively; $P = 0.1$ and 0.62 respectively)

Of the 53 pyrexia dogs, 26 were immediately discharged, 26 were admitted for further treatment and survived and 1 died. Of the 40 normothermic dogs, 13 were immediately discharged, 20 were admitted for further treatment and survived and 7 died (Figure B iv). Of the 6 hypothermic dogs, none was discharged immediately, 4 were hospitalised but recovered and two died. A Fisher's

exact test showed that pyrexia was associated with a decreased likelihood of death ($P = 0.0052$). A correlation between hypothermia and an increased risk of death could not be shown ($P = 0.11$).

Figure B iv: Outcome of hyperthermic, normothermic and hypothermic dogs



Prior studies have shown that in experimental canine babesiosis, hyperthermia develops around the time that parasitaemia first becomes evident and that parasitaemia decreases as hypothermia develops later in the course of the disease (Graham-Smith, 1905). No other information on the association between body temperature and parasitaemia or outcome was discovered.

The observation that pyrexia appeared to be associated with a decreased mortality was unexpected. The theory that pyrexia is associated with early infection and early treatment results in the decreased mortality observed was explored. When the time taken by owners to present dogs after they first observed clinical signs was compared for the pyrexia and the two other groups, a student's t-test showed no significant difference in the groups ($P = 0.36$, data not shown). It is possible that the nature of the immune response differed in the pyrexia dogs, resulting in an improved outcome. Further studies will be necessary to explore this theory.

b.4 Correlation of PCV with parasitaemia, outcome and circulatory status

There were 35 dogs with a PCV below 16% in this study. Ten of these had a PCV below 11%. Of the remaining dogs, 50 had a PCV from 16 to 36% and 15 had a PCV above 36%.

The median capillary and venous parasitaemias and their ranges are presented in Table B ii. Spearman's correlation co-efficients were calculated as data was not normally distributed. There was a significant negative correlation between capillary parasitaemia and PCV (Spearman's $r = -0.38$, $P < 0.0001$). The same was true for PCV and venous parasitaemia (Spearman's $r = -0.25$, $P = 0.012$). This is not surprising as the RBC available for babesia parasites to multiply in are severely restricted in very anaemic animals. In other words, the same total body parasite burden would result in a markedly higher percentage parasitaemia in very anaemic individuals. There is thus an argument for using total body parasitaemia when looking for associations between

Table B ii: Capillary and venous parasitaemias, circulatory compromise and exposure status of dogs grouped according to PCV

PCV	10% or less	11-15%	16-20%	21-25%	26-30%	31-35%	36-40%	>40
number of dogs	10	25	13	21	7	7	8	9
median capillary parasitaemia	13.68	1.42	0.32	0.56	0.14	0.59	0.243	0.59
median venous parasitaemia	0.831	0.238	0.18	0.09	0.09	0.045	0.074	0.15
range capillary parasitaemia	0.52-71.6	0.097-22.2	0.04-18.5	0.04-5.98	0.04-15.7	0.04-6.02	0.093-1.70	0.04-0.43
range venous parasitaemia	0.04-30.6	0.04-10.9	0.04-14.6	0.04-3.15	0-1.54	0.04-6.13	0.04-11.1	0.04-1.41
dogs with a compromised circulation	8	6	2	2	1	1	0	1
Previously exposed	1/9	0/22	6/10	0/19	2/7	2/6	2/7	3/8

parasitaemia and outcome, circulatory status, as well as signalment, clinical signs and haematological data. No means of determining total parasite burden has been validated for *B. rossi* infected dogs. The non-uniform distribution of pRBC in dogs shown in this study (refer 6.1) suggests that this will not be a simple task. One prior study looked at this aspect of babesiosis and was unable to show an association between haematocrit and parasitaemia (Jacobson *et al.*, 1996). The small numbers included in the study and the exclusion of dogs with PCVs below 15% probably explain this discrepancy.

A prior study of 90 severe or complicated babesia cases found that the mean PCV of survivors and non-survivors was almost identical (Nel *et al.*, 2004). These results differ from those of a study of 662 dogs where non-anaemic dogs had a significantly increased risk of mortality (Reyers *et al.*, 1998). A third study documented a 40% mortality among dogs with a PCV above 45% (van Zyl, 1995b). It appeared possible that the risk of death increases in dogs with very high and very low PCVs. For this reason, the association between outcome and PCV was explored for dogs with a PCV 37% or less and 20% or more. Logistic regression revealed no significant association between PCV and death in either group ($P = 0.37$ and $P = 0.23$, respectively).

The difference between the findings of this study and prior studies may be the result of the small number of patients included in this group. In addition, 2 of the 14 cases with a haematocrit above 36% died and 4 were hospitalised for more than a day, suggesting that true haemoconcentrating cases were rare in the dogs included in this study.

The theory that many local clients had learnt to present their pets as soon as they noticed clinical signs and before severe anaemia had developed was explored. Original records showed that the 4 dogs with PCVs above 45% had been ill for 1, 3, 7 and 7 days respectively and only 3 of the 10 dogs with PCVs between 37 and 44% had been presented within 24 hours of the owner first noting clinical signs. Thus this seemed an unlikely explanation

In contrast, a Fisher's exact test showed that there was a significant association between severe anaemia and circulatory collapse (Table B iii). This is not unexpected as severe anaemia must eventually result in tissue hypoxia, which will lead to shock and circulatory collapse. This study cannot answer whether circulatory collapse is the direct consequence of homeostatic attempts to adapt to this tissue hypoxia or whether severe anaemia and shock are the result of some other underlying stimulus.

Table B iii: Association between PCV and circulatory status

PCV	number of dogs with a compromised circulation (score 1)	number of dogs with a normal circulation (score 0)	<i>P</i>
10% or less	8	2	<0.0001
above 10%	13	77	
15% or less	14	21	0.015
above 15%	7	58	

b.5 Correlation of in-saline agglutination (ISA) status with parasitaemia, outcome, circulatory status, PCV and previous exposure

There were 12 dogs whose red cells agglutinated in saline and 88 dogs that were ISA negative. The median capillary parasitaemia of the ISA positive dogs was 0.46% (range 0.09-18.53%, IQR 0.15-7.64%), while that of the ISA negative dogs was 0.62% (range 0.04-71.6%, IQR 0.23-3.7%). The Wilcoxon rank sum test showed that there was no significant difference between these two groups ($P = 0.95$). The median venous parasitaemia of the ISA positive dogs was 0.12% (range 0.04-14.63%, IQR 0.046-4.18%) while that of the ISA negative dogs was 0.17% (range 0-30.6%, IQR 0.047-0.45%). The same test showed no significant difference between the two groups ($P = 0.98$).

Three of the 12 ISA positive dogs died and 9 survived. The surviving dogs were not divided further as ISA positive dogs would usually be admitted. Seven of the 88 ISA negative dogs died.

The Fisher's exact test revealed no significant association between ISA status and survival ($P = 0.1$). This corroborates prior findings (van Zyl, 1995). In addition, 4 of the 12 ISA positive and 17 of 88 ISA negative dogs showed signs of circulatory compromise. A Fisher's exact test revealed no significant association between ISA status and circulatory compromise ($P = 0.27$).

ISA positive dogs had a mean PCV of 14.7% (standard deviation 4.35%). ISA negative dogs had a mean PCV of 23.7% (+/- 11.2%). The Wilcoxon rank sum test showed that ISA positive dogs had a significantly lower PCV ($P = 0.005$). This association is expected as auto-agglutinating dogs would be expected to lyse RBC faster than other babesia-infected dogs, resulting in a more severe anaemia.

Three of 10 ISA positive dogs had had previous episodes of babesiosis. The same was true for 13 of 78 ISA negative dogs. The exposure status was unknown for 12 dogs. The Fisher's exact test showed no association between ISA status and prior exposure to babesia parasites ($P = 0.38$). It has been theorised that babesia parasites alter RBC membrane antigens. An immune response targeted against the altered antigens results in haemolysis (Jacobson *et al.*, 1994). It seemed likely that prior exposure to babesia parasites would prime the immune system and thus increase the likelihood of secondary immune-mediated haemolysis. This does not appear to be the case.

Addendum C. Prevalence of clinical signs and serum protein changes traditionally considered suggestive of *Ehrlichia canis* infection and their association with parasitaemia and outcome of infection

c.1. Prevalence of lymphadenopathy and its association with parasitaemia and outcome

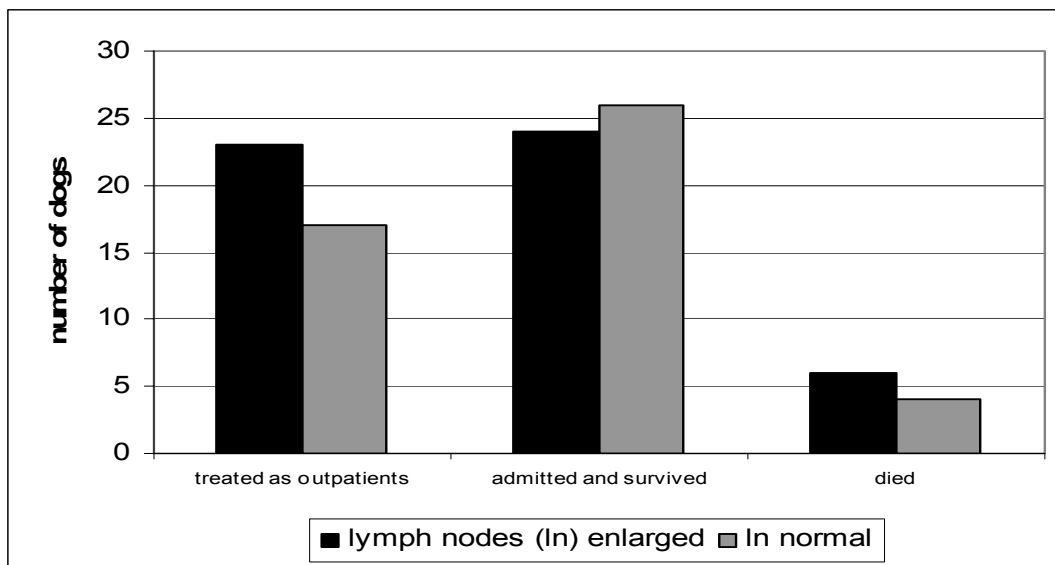
Traditionally, lymphadenopathy in babesia infected dogs has been considered suggestive of concurrent ehrlichiosis at the OVAH. Among the 100 dogs of this study, there were 53 dogs with lymphadenopathy and 47 without. Lymphadenopathy was usually mild. The median capillary parasitaemia of the dogs with lymphadenopathy was 0.98% (range 0.04-62.5%, IQR 0.25-5.89%) and the median venous parasitaemia was 0.15% (0.04-18.2%, IQR 0.048-0.6%). The median capillary parasitaemia of the dogs without lymphadenopathy was 0.41% (range 0.04-71.6%, IQR 0.18-2.02%) and the median venous parasitaemia was 0.14% (0-30.6%, IQR 0.045-0.33%). The Wilcoxon rank sum test showed no significant difference in the capillary and venous parasitaemias of these two groups ($P = 0.17$ and 0.51 respectively).

Of the 53 dogs with enlarged lymph nodes, 23 were treated as outpatients (group H), 24 were hospitalised (group A) and survived and 6 died (group D). Amongst the 47 dogs with normal lymph nodes, there were 17, 26 and 4 in groups H, A and D respectively (see Figure C i). Groups A and H were not separated when considering the effect of lymphadenopathy, TSP or petechiation on outcome as these parameters may have influenced individual clinicians' decisions whether to hospitalise the case or not. The Fisher's exact test showed that dogs with lymphadenopathy were no more likely to die than dogs without lymphadenopathy ($P = 0.75$).

Two prior papers reported lymphadenopathy in dogs infected with large babesias (Dorner, 1969; Abdullahi *et al.*, 1990). One author performed a post mortem study of experimentally infected dogs. He described lymph node hyperplasia but did not report its prevalence (Dorner, 1969). A group reported that 35 of 65 (54%) naturally infected dogs had enlarged lymph nodes (Abdullahi *et al.*, 1990). Neither made any attempt to specifically exclude *E canis* co-infections, though these

appear unlikely in the experimentally infected dogs. Lymphadenopathy was reported in 64% of 120 dogs naturally infected with *E canis* (van Heerden, 1982). The above reports as well as the observations presented here suggest that lymphadenopathy is not specific to canine ehrlichiosis. It should be noted that this study was not designed to assess lymphadenopathy in canine babesiosis. Although an objective way of quantifying the degree of lymph node enlargement has not been devised, fine needle aspirate cytology could be used in future studies to provide objective evidence of lymphoid hyperplasia.

Figure C i: Outcome of dogs with and without lymphadenopathy

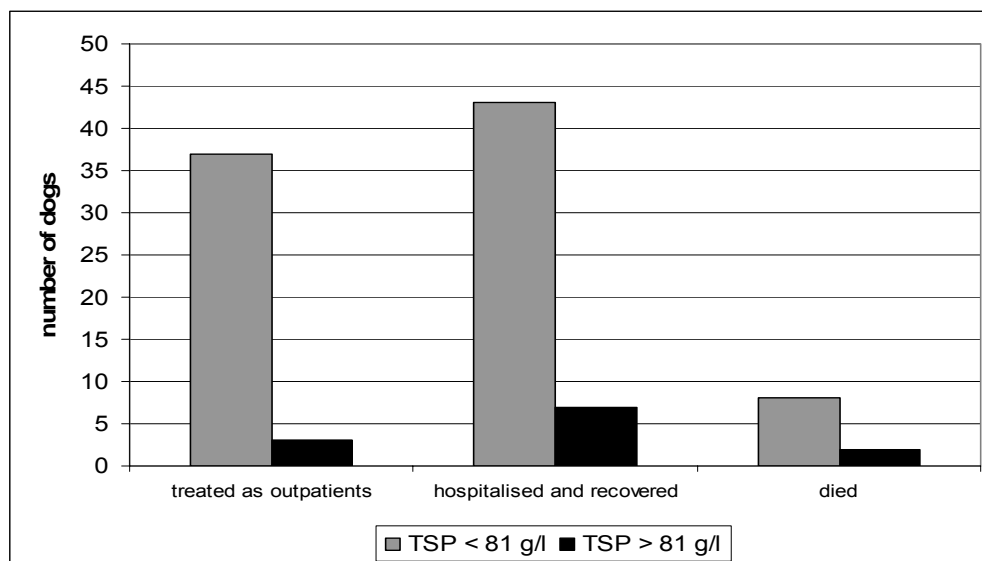


c.2. Prevalence of elevated total serum protein (TSP) and its association with parasitaemia and outcome

Traditionally, a TSP above 80 g/l has been considered suggestive of ehrlichiosis at the OVAH. The dogs in this group were divided into those with a TSP above and below 81g/l as there were 10 dogs with a TSP of 80 g/l and none with a TSP of 81 g/l. The slightly higher cut off parasitaemia chosen to separate the groups should magnify any difference in outcome associated with higher TSP. There were 12 dogs with a TSP above 81 and 88 with a TSP below this cut-off.

The median capillary parasitaemia of the dogs with TSP below 81 g/l was 0.79% (range 0.04-71.6%). Their venous parasitaemia was 0.19% (0-30.6%). The median capillary parasitaemia of the group with higher TSP was 0.33% (0.04-5.8%) and the median venous capillary parasitaemia was 0.28% (0.04-5.4%). The Spearman's correlation co-efficient was calculated to explore the association between TSP and parasitaemia as data was not normally distributed. There was a significant negative correlation between TSP and capillary parasitaemia (Spearman's $r = -0.27$; $P = 0.007$). The same was true for venous parasitaemia (Spearman's $r = -0.24$; $P = 0.018$). Thus TSP decreases slightly as parasitaemia increases. This does not prove causality. Low TSPs are commonly observed in puppies presented to OVAH owing to concurrent severe helminthosis. Dogs aged 6 months or younger also had higher parasitaemias (refer b.1). The mean TSP of dogs aged 6 months or younger (67.2 +/- 7.1 g/l) was thus compared with that of the older dogs (70.9g/l +/- 8.9g/l). A student's t-test was unable to show a significant difference ($P = 0.11$). Thus the negative association between TSP and parasitaemia was probably not confounded by age.

Figure C ii: Comparison of outcome of dogs with a total serum protein below and above 81 g/l



Of the 88 dogs with a TSP below 81 g/l, 37 were treated as outpatients (group H), 43 were admitted to the hospital and recovered (group A) and 8 died (group D). There were 3, 7 and 2

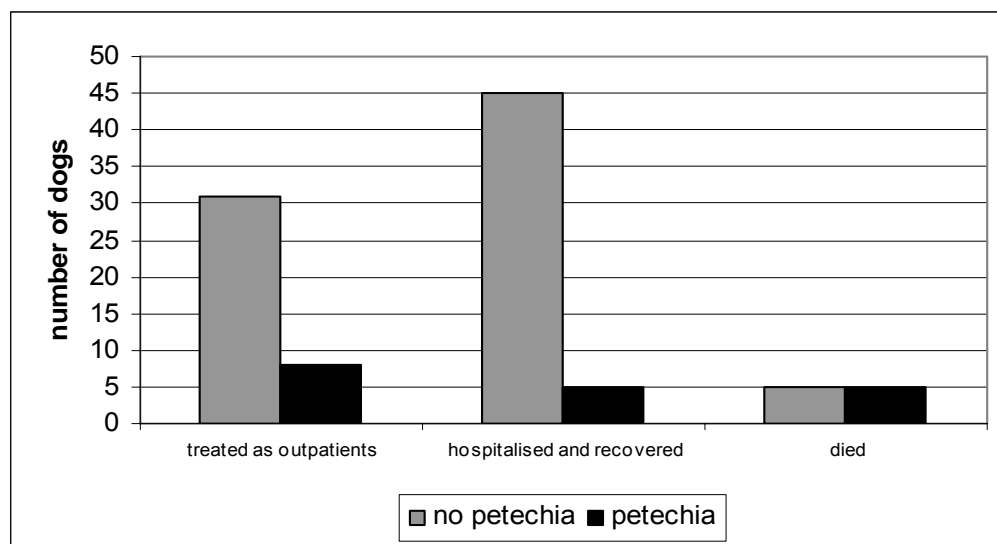
dogs in groups H, A and D respectively amongst the 12 dogs with a TSP above 81 g/l (see Figure C ii). A Fisher's exact test showed no significant association between high TSP and death ($P = 0.34$). None of the 10 dogs with a TSP of 80 g/l died.

No prior reports on the range of TSP in babesia-infected dogs were found. It appears that TSPs above 80 g/l are not uncommon in babesia-infected dogs that are PCR negative for concurrent ehrlichiosis.

c.3 Prevalence of petechiation and its association with parasitaemia and outcome

There were 18 dogs with petechiae, 81 without and 1 dog for which this information was not recorded. The median capillary parasitaemia of the dogs with petechiae was 1.16% (range 0.095-71.6%, IQR 0.34-8.84%) while their median venous parasitaemia was 0.31% (0.04-30.6%, IQR 0.048-5.51%). The median capillary parasitaemia of the dogs without petechiae was 0.53% (0.04-62.51%, IQR 0.17-2.42%) and their median venous parasitaemia was 0.13% (0-28.6%, IQR 0.043-0.39%). The Wilcoxon rank sum test showed that difference in the capillary and venous parasitaemias of these two groups approached significance ($P = 0.09$ and 0.06 respectively).

Figure C iii: Comparison of outcome of dogs with and without petechiation



Eight of the dogs with petechiae were treated as outpatients (group H), 5 were hospitalised and recovered (group A) and 5 died (group D). Amongst the dogs without petechiae, there were 31, 45 and 5 dogs in groups H, A and D respectively (Figure C iii). The Fisher's exact test showed petechiation was significantly associated with death ($P = 0.016$). In addition, 8 of 18 dogs with petechiae but only 12 of 81 dogs without petechiae had a clinically collapsed circulation. A Fisher's exact test showed that petechiation was significantly associated with circulatory collapse ($P = 0.0088$).

Dogs infected with *B. rossii* are almost always thrombocytopenic, often markedly so (Kettner *et al.*, 2003). In the group of dogs studied here, petechiae were most commonly noted on the ventral abdomen. Affected dogs were also commonly flea infested or had adherent ticks. Tick attachment sites were not unusually surrounded by a small area of haemorrhage. Thus many cases had an additional challenge to their coagulation system in the form of insect bites. This additional challenge may have converted a formerly asymptomatic dog into one with petechiae.

In addition, DIC is described as a complication of canine babesiosis (Malherbe *et al.*, 1951; Furlanello *et al.*, 2005). DIC was suspected in 1 and confirmed in a second case in this series. When these two dogs were excluded from analysis, both the association between petechiation and outcome and the association between petechiation and circulatory collapse are no longer significant ($P = 0.3$ and $P = 0.071$ respectively).

These observations underline the fact that dogs with babesiosis may develop petechiae as a result of different underlying pathologies. It is possible that only underlying DIC is associated with a poorer prognosis. It is possible that a larger study including more dogs with petechiation could find significant associations. This aspect of canine babesiosis has received very little attention, which should be remedied. Although all babesia species appear to cause thrombocytopenia, a prior study has shown that large canine babesia species differ in their effect on the coagulation system (Schetters *et al.*, 1997).

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