A flow cytometric assessment of the lymphocyte immunophenotypes in dogs naturally infected with *Babesia rossi*

By

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Date submitted: March 2017

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PROJECT TITLE	**Investigation of the inflammatory immune response by flow cytometry in South Africa canine babesiosis**
PROJECT NUMBER	**V091-13**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. Y Rautenbach**

STUDENT NUMBER (where applicable)	**22 001 388**
DISSERTATION/THESIS SUBMITTED FOR	**MMedVet**

ANIMAL SPECIES	**Dogs/Canine**	
NUMBER OF ANIMALS	**45**	
Approval period to use animals for research/testing purposes		**December 2013-December 2014**
SUPERVISOR	**Prof. A Leisewitz**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	15 January 2014
CHAIRMAN: UP Animal Ethics Committee	Signature	

Whoever gives heed to instruction prospers, and blessed is the one who trusts in the Lord

- Proverbs 16:20

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Acknowledgements

Without the help of the following people this dissertation would not have been possible. Heartfelt appreciation goes to:

Prof. Andrew Leisewitz, for being my promoter, providing financial assistance, and always being positive and enthusiastic about the research.

Prof. Amelia Goddard, for her advice during the planning of the protocol, input to and revision of my dissertation, and her thoroughness and attention to detail.

Prof. Peter Thompson, for doing the statistical analysis for this project.

Dr. Richard Mellanby, for his well-thought through contributions to the research topic.

Riaan, for your love and support over the past few years, even when we were separated by thousands of kilometers. Your support of my career and happiness means the world to me.

Mamma and Pappa, for your unwaivering support and unconditional love, and for teaching me that there is no limit to what I am able to achieve in this life.

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List of abbreviations

AKI	Acute kidney injury
ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
CBC	Complete blood count
CD	Cluster of differentiation
$CD3^+$	T lymphocytes
$CD4^+$ T lymphocytes	Helper T lymphocytes (T_H)
INFDIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IMHA	Immune-mediate haemolytic anaemia
GPI	Glycosylphosphatidylinositol anchors
NK	Natural killer

NO	Nitric oxide
PBLs	Peripheral blood leukocytes
PCR	Polymerase chain reaction
RLB	Reverse line blot
RNI	Reactive nitrogen intermediates
SPA	Soluble parasite antigens
SA	South Africa
TLR	Toll-like receptor
TNF	Tumour necrosis factor

Summary

A flow cytometric assessment of the lymphocyte immunophenotypes in dogs naturally infected with *Babesia rossi*

Rautenbach, Y. University of Pretoria, 2017

Keywords: babesiosis, canine, flow cytometry, lymphocyte, immunology

Immunity to *Babesia* infection requires both innate and acquired responses, including both cell mediated- and humoral responses. The aims of this study were to investigate the variation in selected peripheral blood lymphocyte phenotypes in dogs with virulent babesiosis at presentation and over time post treatment, and to determine whether these correlated with the severity of clinical signs.

Forty-four dogs naturally infected with *Babesia rossi* were studied and 5 healthy dogs were included as controls. *Babesia*-infected dogs were divided into complicated or uncomplicated groups based on clinical signs and in-house laboratory assays. Blood samples were collected from the jugular vein at admission, prior to any treatment, and at 24 hours and 48 – 72 hours. Leukocytes were incubated with canine specific, fluorochrome conjugated anti- cluster of differentiation (CD)3, anti-CD4, anti-CD8, and anti-B cell markers.

The percentage $CD3^+$ lymphocytes in the complicated group was significantly lower compared to the controls (P = 0.014) and uncomplicated group (P = 0.007). The percentage $CD4^+$ T lymphocytes in the complicated group was significantly lower compared to the controls (P = 0.027) and uncomplicated group (P = 0.014). Both the complicated and uncomplicated groups expressed a significantly lower percentage $CD8^+$ T lymphocytes compared to the control group (P<0.001 and P = 0.005, respectively). The percentage B lymphocytes was significantly higher in the complicated group at 48 - 72 hours compared to the percentage at presentation.

These findings could indicate the presence of a functional immune suppression in dogs with complicated babesiosis, secondary to increased apoptosis or redistribution of effector lymphocytes and/or a combination of other immune modulatory mechanisms induced by *B. rossi* infection.

Chapter 1 Introduction

1.1 Background

Haemoprotozoal diseases cause significant mortality and morbidity in mammals worldwide. Malaria is the single most important haemoprotozoal disease affecting humans with approximately three billion people living in countries where transmission occurs, resulting in around 2000 deaths per day (1). Babesiosis is probably the second most important haemoprotozoal disease infecting animals and causes massive economic loss where it occurs (2). It has also been described as an emerging zoonotic disease and is the most important blood transfusion transmitted disease in humans in the USA (3, 4). The pathobiology of malaria and babesiosis has been compared for decades (5). There is evidence to suggest that, similar to malaria, the disease caused by *Babesia* spp. is the result of an exuberant host response, which may lead to systemic inflammatory response syndrome, collateral organ damage and even death (6-10).

The usefulness of the dog in biomedical research and especially as a model system for human disease, is growing rapidly (11). A particularly relevant example here is the way it has been used in the study of sepsis (12). Animal models of malaria have provided significant insight into disease pathogenesis and have been an important aid in vaccine development (13-16). The traditional murine model of malaria has however been criticised and has deficiencies (17). There is a clear need to explore other

possible animal models of haemoprotozoal disease that may offer the research community an opportunity to explore the immunology and pathogenesis as well as possible supportive therapeutic interventions for these diseases (18, 19).

Immunity to *Babesia* infection depends on both innate and acquired responses, which are both T lymphocyte- and antibody-mediated (20). Resolution of acute *B. bovis* infection in immunologically naïve animals depends on a strong innate immune response that leads to activation of macrophages via interferon-γ (IFN-γ) and parasite derived products, and results in parasite elimination by phagocytosis and production of toxic macrophage metabolites, especially nitric oxide (NO) (21). Protection against clinical disease in persistently infected animals or in successfully immunised animals relies on activation of antigen-specific $CD4^+$ T lymphocytes (helper T lymphocytes [T_H]) that secrete IFN-γ and assist in the production of neutralising antibodies, which are critical to the adaptive immune response (20, 22, 23). At present, limited information of the host response against canine babesiosis is available. The *Babesia* parasite, more so after being damaged or inactivated by anti-babesial therapy, stimulates a rapid humoral response, which enhances the cellular immune response (24, 25). Clinical resolution of canine babesiosis has been suggested to be associated with the stimulation and intensity of the $CD4^+$ T lymphocyte driven cellular immunity (25).

1.2 Research questions

1.2.1 Do the percentages of selected peripheral blood lymphocyte phenotypes ($CD3^+$, $CD4^+$, $CD8^+$, B cell marker) at presentation correlate with disease severity (complicated versus uncomplicated) in dogs naturally infected with *B. rossi*?

1.2.2 Do the percentages of selected peripheral blood lymphocyte phenotypes ($CD3^+$, $CD4^+$, $CD8^+$, B cell marker) at 24 hours and 48 – 72 hours after presentation correlate with disease severity at presentation (complicated versus uncomplicated disease)?

1.3 Hypothesis

The research hypotheses of the study were:

1. The percentage of selected peripheral lymphocyte phenotypes at presentation would be lower in dogs with the complicated form of the disease compared to dogs with the uncomplicated disease form and healthy controls.
2. The change over time of the percentage of selected peripheral lymphocyte phenotypes would be different in dogs with the complicated form of the disease compared to dogs with the uncomplicated disease form.

1.4 Objectives

1.4.1 To investigate the variation in selected peripheral blood lymphocyte phenotypes at presentation in dogs naturally infected with *B. rossi*, and to determine the correlation with disease severity.

1.4.2 To monitor the changes over time in the selected peripheral blood lymphocyte phenotypes post treatment and correlate the changes with disease severity.

1.5 Benefits

1.5.1 This study provides important information about the role of the immune response in dogs with babesiosis caused by *B. rossi* and how it correlates with disease severity. The results may impact therapeutic decision-making and prognostication in future.

1.5.2 This study will provide additional information that can aid in the development of a vaccine against *B. rossi* and in immune-therapeutic approaches to appropriately manage these cases. Thus, it may aid in reducing the severity of disease in affected animals.

1.5.3 The results strengthen the case for the use of canine babesiosis as a model for certain aspects of malaria in humans.

1.5.4 This study has contributed to the literature available on the role of the immune response in haemoprotozoal diseases. A database of results is available for future studies.

1.5.5 The research conducted partially fulfils the requirements of the principal investigator's MMedVet (CLD) degree.

Chapter 2 Literature review

2.1 Canine babesiosis

Babesiosis is the second most important haemoparasite in mammals (2), has a global distribution and causes significant morbidity and mortality. It is an emerging zoonosis and is considered to be the most important blood transfusion acquired disease in humans in the USA (3, 4). Of the six *Babesi*a parasite species that infect dogs, *B. rossi* is the most virulent and occurs predominantly in southern Africa (26). Infection with *B. rossi* results in a peracute as well as an acute disease with high morbidity and mortality (27). The severity of the disease has been reported to depend on the age and immune status of the host (26). Both intra- and extravascular haemolysis have been described to occur and anaemia is a consistent feature of the disease (26, 28). Complications associated with the disease include severe anaemia, cerebral pathology (characterised by seizures in normoglycaemic dogs) (28), hepatopathy and icterus, secondary immune-mediated haemolytic anaemia (IMHA), acute respiratory distress syndrome (ARDS) (29), hypoglycaemia (30), acute kidney injury (AKI) (29, 31), hyperlactataemia (32-34), haemoconcentration (9, 29), pancreatitis (35), and disseminated intravascular coagulation (DIC) (36).

There is evidence to suggest that, similar to malaria, the disease caused by *Babesia* spp. is the result of an exuberant host response, which may lead to systemic inflammatory response syndrome, collateral organ damage and even death (6-10).

Babesiosis and malaria share many similarities with regards to complex clinical signs, underlying pathophysiology and immunopathology with many of the disease features being attributed to the host response (5, 37-40). Both diseases are vector borne and caused by an intra-erythrocytic protozoan (5). Much of the pathology associated with these diseases is thought to be secondary to a marked pro-inflammatory milieu resulting in organ failure and death (5, 7, 29, 41). The parallels between human malaria, canine babesiosis, sepsis, and other systemic inflammatory states are well recognised (29, 42, 43).

2.2 Immunopathology in babesiosis

Several studies have evaluated the inflammatory and immune mechanisms in canine babesiosis. Preliminary studies have found that leukotriene B4 and prostaglandin E2 were markedly elevated in five dogs with babesiosis, as well as in half of the dogs without babesiosis; however, this healthy control group only consisted of two dogs (44). Tumour necrosis factor (TNF), a key mediator of endotoxic shock and a major cause of organ damage in multiple organ dysfunction syndrome, is associated with disease severity and was also found to be elevated in *Babesia*-infected dogs with secondary hypoglycaemia, and had a significant correlation with parasitaemia (29, 45). Nitric oxide is a secondary mediator and is the major effector of TNF-induced hypotension in the dog (29). Reactive nitrogen intermediates (RNI), nitrate and nitrite serve as surrogate measures for NO (46). With the exception of a few complicated *Babesia*-infected dogs, RNI did not differ significantly between babesiosis cases and healthy control dogs (46). However, one study did report that RNI were significantly

higher in dogs with babesiosis compared to tick-free healthy laboratory control dogs (46). The presence of an acute phase response is undeniable in canine babesiosis (41, 47, 48). The acute phase protein, α-1-glycoprotein, has been shown to be increased in the majority of dogs with babesiosis, yet the concentration did not differ between groups with differing disease severity (49). Similarly, it has been shown that both C-reactive protein and serum amyloid A are increased in babesiosis but are not useful as prognostic markers, suggesting that inflammation is not the only contributing factor of disease severity (41, 48). In dogs naturally infected with *B. gibsoni*, a prominent suppression of lymphocyte blastogenesis and anti-parasite antibody production is evident (50).

In bovine babesiosis, the complex interactions between pro- and anti-inflammatory responses have clearly been illustrated (51). Similarly in malaria, anti-inflammatory cytokines counterbalance the pro-inflammatory responses necessary for parasite control, in order to minimise immunopathology (52-55). In dogs infected with *B. rossi*, an excessive pro-inflammatory response has been reported (41). In addition, sepsis can result in both an excessive uncontrolled and unfocussed, as well as a hypo-inflammatory immune responses (56, 57). A hypo-inflammatory immune response might be just as detrimental as excessive inflammation, and improved outcome might be achieved if the individual immune status is known and treatment is tailored accordingly (57, 58).

Besides the altered immune status reported in babesiosis, additional characteristics of the disease include abnormal perfusion and tissue hypoxia, which have major

implications for the functional capacity of every organ and system in the body (34). Abnormal tissue perfusion in virulent babesiosis and malaria can be related to the fact that intra-erythrocytic parasitism is a feature of both diseases. This alters the erythrocyte membrane predisposing it to become tethered to the activated micro-vessel endothelium. This erythrocyte sequestration has been associated with marked intravascular, vascular and perivascular pathology (59-61). Potential contributing factors to the tissue hypoxia in canine babesiosis include microvascular sequestration, severe anaemia, altered erythrocyte deformability and haemoglobin function, auto-agglutination, endothelial activation and damage, increased endothelial permeability, DIC, pulmonary oedema, thromboembolism and myocardial dysfunction (44).

2.3 The immunology of babesiosis

2.3.1. The innate immune response

The innate immune response is the first line of defence and directs the nature and quality of subsequent acquired responses. In murine malaria severely immunodeficient mice (lacking T- and B lymphocytes) and athymic (nude) mice exhibit ascending and peak parasitaemia levels that are similar to mice with intact immune systems, demonstrating the importance of the initial innate immune response in early parasite control as specific immune responses don't affect early parasitaemia (62, 63). A mouse model depleted of natural killer (NK) cells or genetically deficient in NK cells demonstrated higher initial peak parasitaemia and a more severe course of infection,

suggesting that IFN-γ secretion by NK cells during early infection played a major role in protective immunity to blood-stage malaria (64).

Autonomous glycosylphosphatidylinositol anchors (GPI) molecules, found in abundance in some parasitic protozoa, mediate a strong immunomodulatory effect on the host immune system (65). Several studies have reported evidence that *Babesia* parasites have GPI molecules (65, 66). In general, lipopolysaccharide, lipoprotein, peptidoglycan, unmethylated CpG deoxyribonucleic acid (DNA) and pathogen antigens such as flagellin have been among the several well-described pathogen-associated molecular patterns (14, 67). Purified lipids from merozoites of two *B. bovis* strains have been implicated in the modulation of the innate immune response (68). The lipid composition of these two different *B. bovis* strains showed quantitative differences that could be the explanation for the distinct inflammatory response generated by each strain (68). These lipids are also recognised by toll-like receptor (TLR)-2, and therefore demonstrate the ability to function as relevant antigens (68). Through a TLR-2 dependent pathway, the lipids from an attenuated *B. bovis* strain were able to generate a strong inflammatory response, which resulted in the control of acute infection and host survival (68). Toll-like receptor-2 can be activated by a wide range of microbial molecules containing a lipid component, such as GPI molecules in *P. falciparum* (68).

The effector and regulatory functions of NK cells, such as cytotoxic activity and IFN-γ production, can be primed by pro-inflammatory cytokine producing accessory cells (e.g. dendritic cells) (67, 69). Accessory cell maturation can be induced by NK cells either by direct contact or in synergy with inadequate levels of cytokines and microbial

signals (69). The interaction between accessory cells and NK cells orchestrates the regulation of innate immunity, as well as the promotion of suitable downstream adaptive immune responses (69). For example, resolution of bovine babesiosis is dependent on a T_H1-biased immune response, in which the secretion of IFN-γ is a critical component of the response (69). Cytokine production by accessory cells is central to the secretion of IFN-γ from bovine NK cells, which implies that interaction of accessory cells with NK cells during the early phases of *Babesia* infection is necessary to direct the immune response towards a T_H1 phenotype (67, 70).

2.3.2. Humoral responses

During the erythrocytic life cycle stage of malaria it is assumed that the humoral immune response plays a key role in the host response (71). In murine models, B lymphocytes and antibodies are critical in eliminating parasites and also play a regulatory role in $CD4^+$ T lymphocyte subset responses (71, 72).

Presentation of *Babesia* parasitic antigen by antigen presenting cells to $CD4^+$ T lymphocytes is critical to direct the adaptive immune response during subsequent infection as well as to provide protection against clinical disease (21). However, the control of infection is mediated by antibodies directed against extracellular merozoites and the surface antigens of infected erythrocytes (21). The host humoral response has also been reported to affect the host susceptibility to *Babesia* infections. This is illustrated in murine studies, where high concentrations of interleukin (IL)-10 are secreted by B lymphocytes subsequent to *B. microti* infection, favouring parasite growth and survival due to inhibition of cell-mediated immunity and inflammation (73).

Parasite infectivity is also modulated by the humoral response. *Babesia bigemina* has been reported to use immunoglobulin (Ig) M to enter erythrocytes resulting in enhanced parasite survival (74). Moreover, IgM deficient mice have been reported to be resistant to *B. microti* infection as demonstrated by very low levels of parasitaemia (75).

2.3.3. Cell-mediated responses

In the murine model, T lymphocytes express the receptor (TCR)-CD3 complex and are further classified according to accessory CD4 and CD8 molecules – $CD4^+$ T cells are helper T lymphocytes and $CD8^+$ T cells are cytotoxic. In malaria, T lymphocytes dictate the magnitude of the parasite load entering the erythrocytic cycle by directly controlling the growth and development of the pre-erythrocytic *Plasmodium* stages, as well as the pathogenesis of the infection by directing the immune response elicited by the erythrocytic stage (76). Moreover, during the erythrocytic life cycle stages, $CD4^+$ T lymphocytes also contribute to parasite elimination through cytokine secretion, macrophage activation and direction of the humoral immunity (76, 77).

Although the immune response to *Babesia* infection has not been extensively studied, the specific role of cell-mediated immunity (chiefly that of T lymphocytes) against the intra-erythrocytic parasite, has been investigated in previous studies. An elevated and persistent *B. microti* parasitaemia has been reported in athymic (nude) mice, a finding that is not seen in thymus-intact mice (78, 79). Similarly, mice infected with *B. rodhaini* and treated with anti-thymocyte serum showed high levels of parasitaemia and mortality (80). In addition, the adoptive transfer of thymocytes from normal

immunocompetent BALB/c mice enabled immunodeficient recipient mice to control their *B. microti*-infection (81). A study on *B. microti*-infection in mice depleted of T_H lymphocytes, especially the IFN-γ producing $CD4^+$ T lymphocyte subset, established that intra-erythrocytic killing of the parasite as well as the development of resistance against challenge infection is T_H cell dependant (82). The investigation of the cytokine and antibody response during *B. microti*-infection demonstrated that a T_H1 mediated cellular immunity was dominant during the early inflammatory response and probably controlled the levels of acute parasitaemia, while a T_H2-associated response as well as the production of parasite-specific IgG followed (83).

In murine malaria models, $CD8^+$ T lymphocytes producing IFN-γ are crucial for host protection during the pre-erythrocytic life cycle stage (84). Previous studies have reported that $CD8^+$ T lymphocytes do not seem to play a role in parasite clearance during the acute phase of *Babesia* spp. infections (82, 85).

2. 4 Vaccine implications

Both innate and acquired responses, including T lymphocyte-mediated and antibody responses are involved in immunity to the protozoan parasite *Babesia* (6, 20, 86, 87). Vaccination of unprotected cattle with live attenuated strains is an effective control measure against *B. bovis* infection in bovids (88). Vaccines are generally effective, preventing outbreaks in areas of enzootic instability as well as providing protection for naive animals when moved into endemic areas (88). Subunit vaccines may be useful for the control of bovine babesiosis and overcome some of the disadvantages

associated with live attenuated vaccines (88). However, incomplete or no protection against *B. bovis* is provided by the use of single recombinant proteins obtained from known strongly immunogenic proteins such as merozoites surface antigen-1 (containing B-cell epitopes) and rhoptry associated-protein-1 (containing T-cell epitopes) (21, 88, 89). Effective control against *B. bovis* may be provided by a multi-antigen subunit vaccine that is characterised by the inclusion of several antigens or a combination of effective B- and T-cell epitopes obtained from multiple effective antigens (88). This was illustrated by the partial protection elicited by a recombinant vaccine based on the *B. bovis* antigens 11C5 and 12D3 (90). Members of the variable merozoites surface antigen, which contain neutralisation-sensitive B-cell epitopes, appear to be useful contenders for *B. bovis* subunit vaccines (88).

Despite the availability of several successful vaccines against the clinical manifestation of canine babesiosis (91), little is known of their mechanisms of protection. Antigenic diversity within *Babesia* species appears to be responsible for the variable success rates obtained by vaccines against bovine and canine babesiosis (91). An experimental vaccine consisting of live parasites of a South African *B. rossi* isolate provided protection against homologous challenges in dogs as long as the infection was not sterilised (92). In Europe, two commercially produced vaccines against canine babesiosis are available (93). The first contains a saponin-adjuvanted soluble *B. canis* parasite antigen (SPA) collected from parasite culture supernatant (93, 94). This product probably does not cross-protect against other *Babesia* species (93). Protection against the disease (not the parasite) caused by homologous and heterologous parasite challenge is possible with the bivalent vaccine saponin adjuvanted SPA from *B. canis* and *B. rossi* cultures (93, 95). A reduction in *B. rossi*

clinical signs, parasitaemia and plasma concentrations of SPA has been reported in beagle dogs vaccinated with this bivalent product and challenged with heterologous *B. rossi* (93, 94). However, a reduced level of parasitaemia was not reported in vaccinated dogs challenged with heterologous *B. canis*, suggesting different protective effects by this bivalent vaccine (93, 96). The licensed duration of immunity for this vaccine is six months (93, 96), however field efficacy data for this vaccine are limited (93). Recently, three rhoptry-associated proteins, which are expressed in the merozoite stage of *B. gibsoni*, have been identified as promising vaccine candidates or drug targets due to their highly homologous protein structure and binding affinity for canine erythrocytes (97). Currently tick control still remains the means of choice for the control of canine babesiosis (93).

2. 5 Flow cytometry in canine babesiosis

Flow cytometry characterises cells or particles based upon the light scattering properties of the analysed cells or particles and the fluorescence emissions of targeted antibodies or cellular probes (98). The most common clinical applications of flow cytometry in small animal medicine include: 1) quantitation of erythrocytes and leukocytes in automated haematology analysers; 2) detection of antibodies in immune-mediated diseases involving erythrocytes and platelets; 3) immunophenotyping of leukocytes affected in immunodeficiency syndromes; and 4) diagnosis of lymphoproliferative diseases and malignancies (98). Potential future applications for flow cytometry would include assays that make use of cell sorting and multiplexing using microspheres, which will depend on assay validation and enhancement of the

instrumentation footprint leading to more compact, less expensive, and user-friendly instruments (98).

A number of studies on canine babesiosis have made use of flow cytometry. It has been used to good effect to quantify the percentage of reticulocytes in the peripheral blood of dogs infected with *B. gibsoni* (99). The degree of parasitaemia in canine babesiosis has also been determined by flow cytometry (100). It has also been utilised to identify parasitised erythrocytes in the peripheral blood samples from dogs infected with *B. gibsoni*, after staining with hydroethidine (101). In addition, detection of *B. canis*-infected erythrocytes in peripheral blood samples using hydroethidine-flow cytometry (HE-FC) has been validated for diagnostic purposes in endemic areas for canine babesiosis (102). A flow cytometric investigation into the detection of anti-erythrocyte membrane binding IgG and/ or IgM in *B. canis* and *B. vogeli* at the time of diagnosis has also been conducted (103). Recently it was used to characterise the different lymphocyte phenotypes in dogs infected with *B. canis* at initial diagnosis and after treatment with imidocarb, as well as to identify platelet-leukocyte aggregate formation in dogs infected with *B. rossi* (25, 104).

Chapter 3 Materials and Methods

3.1 Experimental design

3.1.1 Study design

This was a prospective, descriptive longitudinal study which included client-owned dogs, naturally infected with *B. rossi* that presented for veterinary care to the Onderstepoort Veterinary Academic Hospital, South Africa, between January 2014 and December 2014. The research protocol was approved by the University of Pretoria's Animal Ethics Committee (V091-13; 15 January 2014).

Infection with *Babesia* parasites was diagnosed by demonstration of intra-erythrocytic trophozoites on stained thin blood smears, and was confirmed as *B. rossi* by polymerase chain reaction (PCR) and reverse line blot (RLB) (105). The control dogs included five healthy, client-owned blood donor dogs. The control dogs were not matched for age or sex. Owner information sheets were provided and owner consent was obtained for enrolment of all the dogs in this study (Appendix A & B).

3.1.2 Study population

Dogs diagnosed with babesiosis were included in the study if they fulfilled a defined set of selection criteria. Suitable dogs were of any breed and either sex, provided that they were ≥12 weeks of age, weighed ≥3 kg, and had a demonstrable parasitaemia on a stained thin peripheral blood smear. Dogs were excluded if they were co-infected with *B. vogeli* or *Ehrlichia canis* based on PCR and RLB assay results. Dogs were also excluded if any signs of concurrent chronic or inflammatory disease conditions, any obvious infections or wounds, or any signs of trauma were present. Vaccination, glucocorticoid therapy, or any unrelated metabolic illness or babesiosis within the four weeks prior to presentation were also reasons for exclusion. Cases presenting with only mild or moderate anaemia (haematocrit >12%), that were ambulatory and had no historical or clinical indication of significant concurrent organ involvement, were considered uncomplicated and were treated as outpatients. Dogs were classified with complicated disease when one or more of the following were identified: severe anaemia (haematocrit ≤12%) necessitating transfusion with packed red cells; AKI (oliguria/anuria and persistently elevated serum creatinine concentration, despite appropriate fluid therapy); cerebral babesiosis (neurological signs in the face of normoglycaemia and that could not be attributed to any other cause); hepatopathy and/or icterus (elevated alanine aminotransferase and alkaline phosphatase activities with/without indications of cholestasis such as biliruburia and/or bilirubinaemia; or elevated bile acids on a starved sample in the absence of any signs of cholestasis); hypoglycaemia (blood glucose <3.3 mmol/L); hyperlactataemia (blood lactate >2.5 mmol/L); secondary IMHA (positive warm in-saline agglutination or Coombs-positive,

and/or marked spherocytosis); ARDS (dyspnoea, adventitious lung sounds, radiological evidence of lung consolidation or oedema, and blood-gas evidence of ventilation-perfusion mismatch); haemoconcentration (haematocrit >37% in association with intravascular haemolysis, low total protein, with concurrent clinical collapse); pancreatitis (elevated serum lipase activity, or a positive SNAP test for canine-specific pancreatic lipase (cPLI; IDEXX Laboratories) associated with abdominal pain, vomiting, ultrasonographic evidence of acute pancreatitis); and DIC (thrombocytopaenia with concurrent prolonged prothrombin and activated partial thromboplastin time, increased D-dimer and fibrin/fibrinogen degradation products concentrations, and decreased antithrombin activity).

The dogs received standard care for canine babesiosis, which included antibabesial treatment with diminazene aceturate (Berenil® RTU 0.07 g/mL, Intervet Schering-Plough Animal Health SA, Isando, Johannesburg, SA, dosed at 3.5 mg/kg once off), transfusion with packed red cells and intravenous fluids as needed. Dogs with secondary IMHA were treated with glucocorticoids (Lenisolone® 5mg, Aspen Pharmacare, Woodmead, Sandton, SA, dosed at 2 mg/kg per os twice a day). Additionally, any further related complications were treated accordingly at the discretion of the attending clinician. Control dogs were considered healthy based on history, a full clinical examination, peripheral blood smear evaluation, complete blood count (CBC), as well as PCR and RLB assays to rule out parasitaemia.

3.2 Experimental procedures

3.2.1 Sample collection

Peripheral venous blood was collected at presentation prior to any treatment, and at 24 hours and 48 - 72 hours after presentation. Blood samples were collected from the jugular vein from each dog with a 21-gauge needle by careful venipuncture with minimal stasis. The blood samples were collected into serum and ethylenediaminetetraacetic acid (EDTA) vacutainer plastic tubes (BD Vacutainer tubes). The EDTA sample was used to perform a CBC, PCR and RLB assays, and flow cytometric evaluation.

3.2.2 Reagents and antibodies for flow cytometric analysis

Red blood cell lysing solution (BD FACS Lysing Solution, catalog no. 349202, Franklin Lakes, New Jersey, USA), pH-balanced phosphate-buffered saline (BD CellWASH, catalog no. 349524, Franklin Lakes, New Jersey, USA) and calibration beads (SPHERO™ Rainbow Calibration Particles (6 and 8 Peaks), BD Biosciences, catalog no. 653144 and 653145, Franklin Lakes, New Jersey, USA) were used. The fluorochrome-labelled monoclonal antibodies for the surface markers of canine lymphocytes included APC-conjugated anti-Dog Pan T cell (Clone LSM 8.358; isotype mIgM) as the marker for T-lymphocytes; PE-conjugated anti-Dog CD4 (Clone LSM 12.125; isotype mIgG1) as the marker for $CD4^+$ T lymphocytes; FITC-conjugated anti-

Dog CD8 (Clone LSM 1.140; isotype mIgG1) as the marker for $CD8^+$ T lymphocytes (Canine T Lymphocyte Cocktail, BD Biosciences, material number 558699, Franklin Lakes, New Jersey, USA); and PE-conjugated anti-B cell marker (Clone LSM 11.425; isotype mIgG1) as the marker for B lymphocytes (Canine Activated T Lymphocyte Cocktail, BD Biosciences, material number 558704, Franklin Lakes, New Jersey, USA) (106).

3.2.3 Sample preparation and staining method for identification of lymphocyte phenotypes

Lymphocyte counts were determined, within 30 minutes of collection, on the ADVIA 2120 (Siemens, Munich, Germany) through 2-dimensional laser light scattering. Based upon the peripheral lymphocyte count, a calculated volume of EDTA-anticoagulated whole blood, containing 10^6 lymphocytes, was centrifuged at 1520 *g* for 10 minutes at room temperature within one hour after blood collection to separate peripheral blood cells from plasma. This was to include sufficient cells in the tube to ensure that enough peripheral blood leukocytes (PBLs) could be harvested, as well as effective lysis of the red blood cells in the sample tubes, as large volumes of blood were often required to obtain ~ 10^6 lymphocytes, because of peripheral leukopenia. The plasma was removed and 2 mL of BD FACS lysing solution was added to the cellular pellet and left for 15 minutes at room temperature to lyse all red blood cells after resuspending the cellular pellet. Thereafter the solution was centrifuged at 450 *g* for 20 minutes at 4˚C to concentrate the PBLs into a pellet, and the supernatant was removed with a pipette and sterile plastic tip. The pelleted PBLs were then resuspended and washed

twice by the addition of 2 mL of cooled staining buffer (BD CellWASH) followed by centrifugation at 450 *g* for 20 minutes at 4˚C. Peripheral blood leukocytes, containing 10^6 lymphocytes, were then resuspended in 500 µL staining buffer, from which two tubes containing 100 µL of the suspension were prepared and stained with 4 µL (20 µL of antibody per 10^6 cells) of the primary antibody mix (canine T lymphocyte cocktail; canine activated T lymphocyte cocktail) for 15 minutes in the dark at 4˚C. Once stained, the cells were washed once, as above, and resuspended in 500 µL staining buffer.

3.2.4 Flow cytometric analysis

Flow cytometric analysis was performed by the primary investigator, within two to three hours of collection, on the Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA) interfaced with a computer and analysed using BD CSampler Software (BD Biosciences, Franklin Lakes, New Jersey, USA). The instrument was calibrated for sample acquisition using SPHERO™ Rainbow Calibration Particles (6 Peaks and 8 Peaks). Compensation was performed on all dual labelled samples to ensure adequate separation of events.

Unstained cells were used to define the lymphocyte population, based on their forward-angle and side-angle light scatter characteristics (Fig. 1a). Cells in the unstained lymphocyte gate were used to formulate the next plot and subsequently, the lymphocytes were gated based on their CD3 (APC), CD4 (PE), CD8 (FITC) or B cell marker (PE) expression (Fig. 1b - d). Cells that were dual positive for both CD3 and CD4 as well as CD3 and CD8 were defined as $CD4^+$ T lymphocytes and $CD8^+$ T

lymphocytes, respectively. A minimum of 5000 lymphocytes were acquired for the initial gated population. Results were expressed as the percentage cells of the different phenotypes (T lymphocytes, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and B lymphocytes) within the lymphocyte population.

3.2.5 DNA extraction and PCR

DNA was extracted from 200 µL EDTA-anticoagulated whole blood using a blood and tissue extraction kit (QIAmp blood and tissue extraction kit, Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Molecular diagnosis of *B. rossi* and exclusion of other *Babesia*, *Theileria*, *Ehrlichia* and *Anaplasma* spp. were performed using PCR and RLB. 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. The membrane used for RLB included probes for *B vogeli*, *B. rossi*, *B. canis*, and *E. canis* (105).

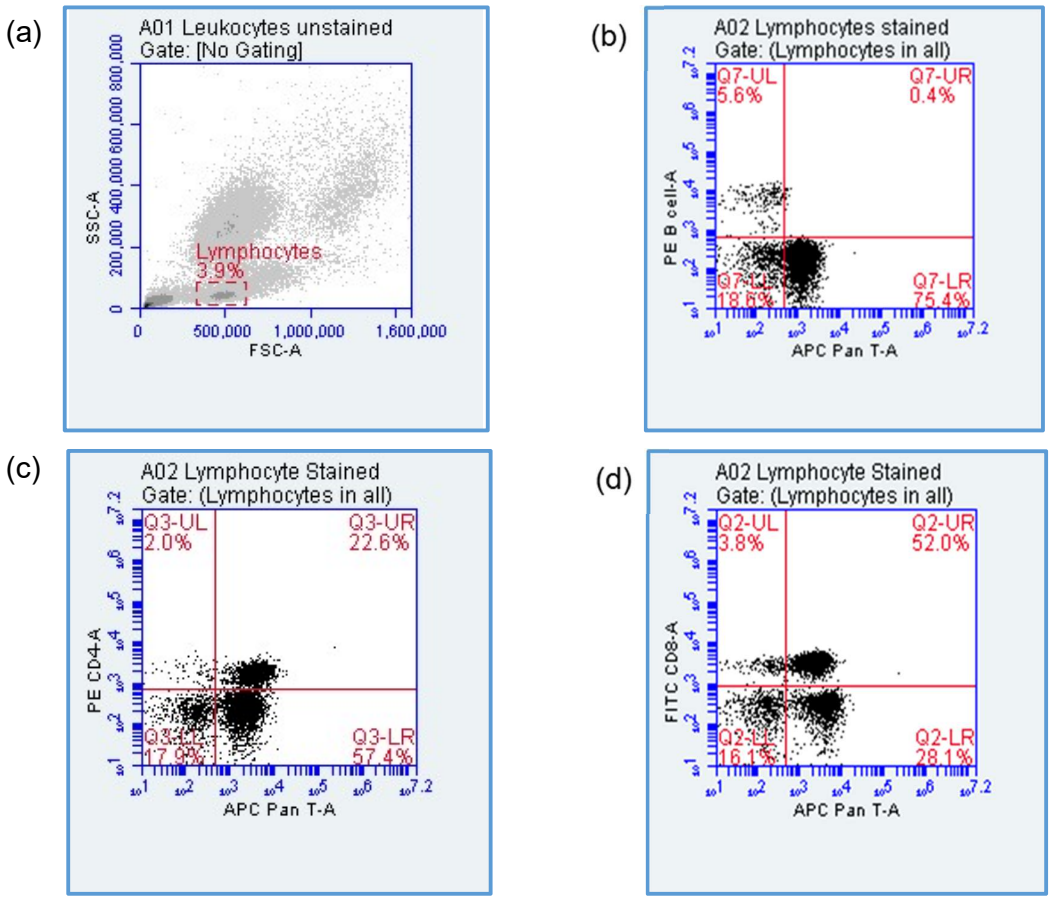

Figure 1 Flow cytometric analysis of peripheral lymphocyte phenotypes in a control dog:

(a) The lymphocyte population was identified and gated based on forward angle and side-angle light scatter characteristics of unstained cells; Subsequently the lymphocytes were gated based on their CD3 (APC) (b), B cell marker (PE) (b), CD4 (PE) (c), or CD8 (FITC) (d) expression. Values in the quadrants indicate the percentage of cells.

3.3 Observations

3.3.1 Signalment, history, clinical data disease phenotyping (Appendix C).

3.3.2 Recordings of results of minimum database and clinicopathological findings (Appendix C).

3.3.3 Immunophenotyping results generated by flow cytometry generated as scatter plots and reported as percentages.

3.4 Data capture and statistical analysis

All data generated were captured on a spreadsheet database (Microsoft Excel® 2016, Microsoft Corp. USA). The data was stored on the primary investigator's desktop computer and was regularly backed up onto an external hard disc. In addition, the electronic files of each research case were emailed to the primary supervisor and printouts of each case were also stored in the primary investigator's office.

The normality assumption was evaluated using the Shapiro-Wilk test and the total leukocyte and lymphocyte counts were log-transformed to approximate normality for between-group analysis. Age and weight were compared between the groups (complicated, uncomplicated and control) using analysis of variance (ANOVA) and gender proportions were compared using the Fisher's exact test. One-way ANOVA was used to assess differences between groups at presentation; if significant differences were present, the Bonferroni multiple comparison test was used as *post*

hoc analysis. Differences between groups (complicated and uncomplicated) in the lymphocyte phenotypes over time was evaluated using linear mixed models with animal as random effect and time as a repeat measure, while adjusting for glucocorticoid therapy and packed red cell transfusion. Statistical analyses were performed using commercial software packages (SPSS Statistics 23.0 Software, Stata 12.1 (StataCorp, College Station, TX, U.S.A.) and NCSS 2007 (NCSS, Kaysville, UT, U.S.A.)); $P \leq 0.05$ was considered significant.

Chapter 4 Results

4.1 Study population

Of the 48 dogs naturally infected with *B. rossi* that were sampled, 44 dogs were included in the study: two dogs were co-infected with parvovirus, one dog was co-infected with *E. canis*, and one dog was only infected with *B. vogeli*. Twenty-two *Babesia*-infected dogs were included in each of the complicated and uncomplicated disease groups. Complications included: severe anaemia (*n* = 11); AKI (*n* = 1); cerebral babesiosis (*n* = 1); hepatopathy and/or icterus (*n* = 11); hypoglycaemia (*n* = 5); hyperlactataemia (*n* = 10); concurrent hypoglycaemia and hyperlactataemia (*n* = 4); secondary IMHA (*n* = 8); ARDS (*n* = 2), and haemoconcentration (*n* = 2). The mean age (range) and weight (range) of the dogs in the complicated disease group were 26 months (3 - 91) and 16.5 kg (3.4 - 45.6), respectively, and included 17 male and five female dogs. The breeds included consisted of mixed breed (*n* = 8), Jack Russell terrier (*n* = 6), German shepherd dog (*n* = 3), Boerboel (*n* = 2), and one of each of the following, Siberian husky, chow chow, smooth-haired fox terrier, and American pit bull terrier. The mean age (range) and weight (range) of the dogs in the uncomplicated disease group were 27 months (4 - 84) and 13 kg (3.0 - 32), respectively, and included 12 male and 10 female dogs. The breeds included consisted of mixed breed (*n* = 8), Jack Russell terrier (*n* = 4), Labrador retriever (*n* = 3), and one of each of the following; basset hound, smooth-haired dachshund, wire-

haired fox terrier, rottweiler, Yorkshire terrier, and cockerspaniel. The age, weight, sex and breed of the 44 *Babesia*-infected dogs are summarised in Appendix D.

Five control dogs were sampled. The control dogs included two males and three females, with a mean age (range) and weight (range) of 52 months (24 - 76) and 28.2 kg (20.0 - 35.0), respectively. The breeds included German shepherd dog (n = 2), and one each of the following, standard French poodle, golden retriever, and a mixed breed. The age, weight, sex and breed of the 5 healthy control dogs are summarised in Appendix E. The weight of the healthy control dogs was significantly higher (P = 0.011) compared to the uncomplicated disease group. There were no significant differences in age or gender between the three groups.

4.2 Comparison of total leukocyte count, lymphocyte count, and associated flow cytometric variables, between groups at presentation

Table 1 contains a summary of the variables for the groups at presentation. Results for 22 dogs in both the complicated and uncomplicated disease groups were available. There were no significant differences between groups for the mean total leukocyte count; however, compared to the controls, the mean lymphocyte count was significantly lower in both the complicated and uncomplicated disease groups (P = 0.008 and P = 0.003, respectively). The mean $CD3^+$ lymphocytes percentage in the

complicated disease group was significantly lower compared to the controls (*P* = 0.014) and uncomplicated disease group (*P* = 0.007) (Fig. 2). Similarly, the mean $CD4^+$ T lymphocytes percentage in the complicated disease group was significantly lower compared to the controls (*P* = 0.027) and uncomplicated disease group (*P* = 0.014) (Fig. 3). Both the complicated and uncomplicated disease groups had a significantly lower mean $CD8^+$ T lymphocytes percentage compared to the control group (*P*< 0.001 and *P* = 0.005, respectively) (Fig. 4); however, there was no significant difference between the two disease groups. There were no significant differences between the groups for the mean B lymphocytes percentage.

Table 1 Haematological and lymphocyte phenotype data in healthy control dogs and in dogs with complicated and uncomplicated babesiosis at presentation.

Variable	Controls (*n* = 5)	*Babesia*-infected (*n* = 44)	Complicated *Babesia*-infected (*n* = 22)	Uncomplicated *Babesia*-infected (*n* = 22)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Leukocyte count ($x10^9$/L)	10.08 ± 2.70	9.32 ± 15.20	12.46 ± 21.06	6.18 ± 2.98
Lymphocyte count ($x10^9$/L)	2.71 ± 1.07 [a,b,c]	1.33 ± 0.80 [a]	1.41 ± 1.0 [b]	1.27 ± 0.61 [c]
$CD3^+$ lymphocytes (%)	65.24 ± 15.33 [b]	43.64 ± 24.70	32.96 ± 23.04 [b,d]	54.32 ± 21.90 [d]
$CD3^+/4^+$ lymphocytes (%)	40.56 ± 15.38 [b]	26.76 ± 16.57	19.91 ± 15.37 [b,d]	33.61 ± 15.10 [d]
$CD3^+/8^+$ lymphocytes (%)	21.62 ± 17.27 [a,b,c]	8.10 ± 5.40 [a]	6.25 ± 5.58 [b]	9.95 ± 4.64 [c]
B lymphocytes (%)	9.22 ± 4.00	6.16 ± 5.10	4.88 ± 3.28	7.44 ± 6.19

[a] Significant difference between the control and *Babesia*-infected groups ($P < 0.05$)
[b] Significant difference between the control and complicated *Babesia*-infected groups ($P < 0.05$)
[c] Significant difference between the control and uncomplicated *Babesia*-infected groups ($P < 0.05$)
[d] Significant difference between the complicated and uncomplicated *Babesia*-infected groups ($P < 0.05$)

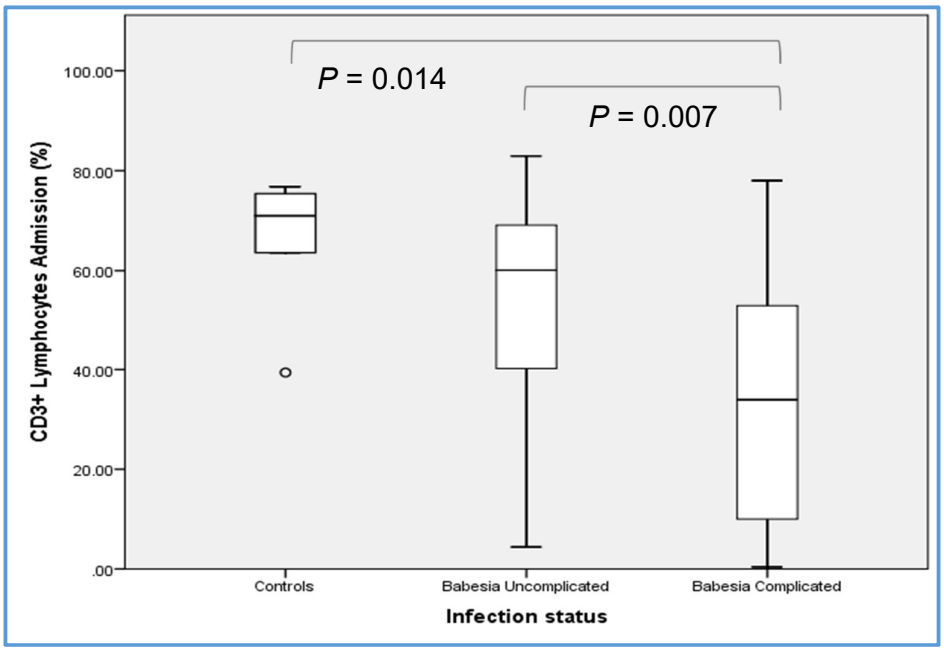

Figure 2 Box plot of $CD3^+$ lymphocytes in the healthy control group, uncomplicated and complicated *Babesia*-infected groups at presentation. The box represents the IQR (i.e. the middle 50% of the observations with the line inside the box as the median). The whiskers represent the main body of the data, indicating the range of the data. Outliers, values that are 1.5 times removed from the IQR, are plotted as open circles.

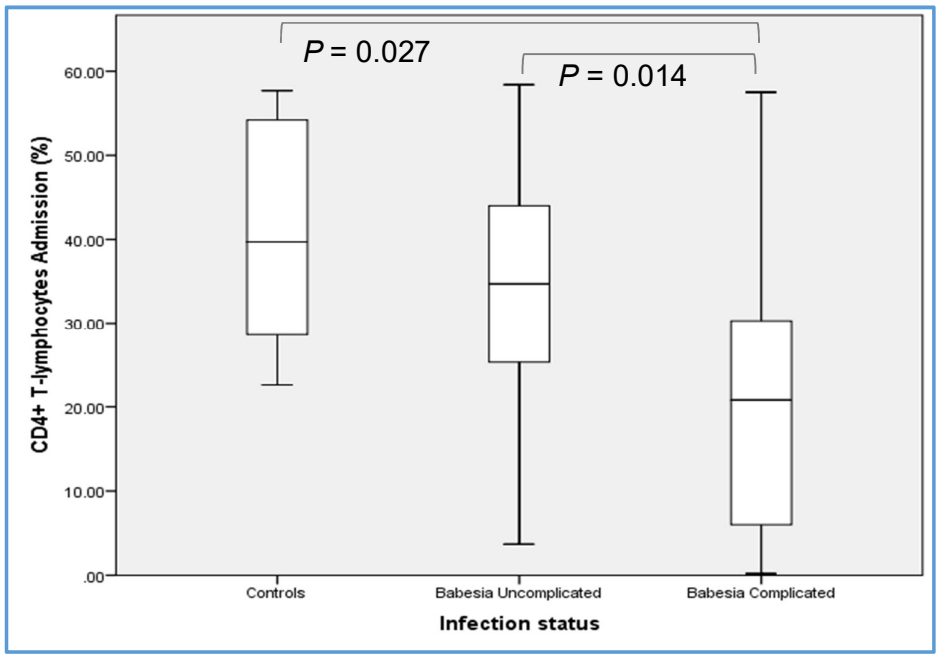

Figure 3 Box plot of $CD4^+$ T lymphocytes in the healthy control group, uncomplicated and complicated *Babesia*-infected groups at presentation. The box represents the IQR (i.e. the middle 50% of the observations with the line inside the box as the median). The whiskers represent the main body of the data, indicating the range of the data.

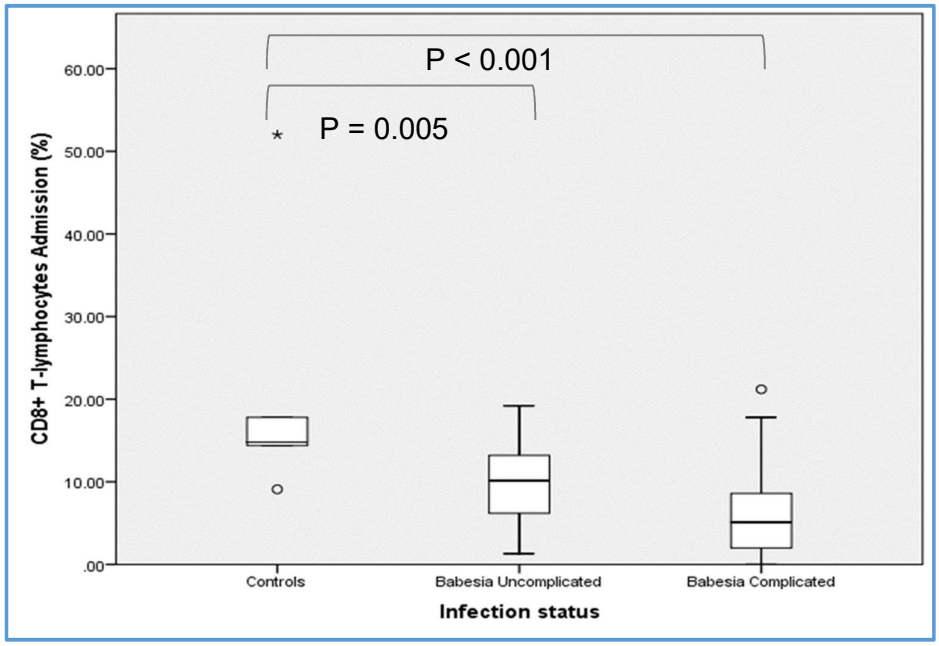

Figure 4 Box plot of $CD8^+$ T lymphocytes in the healthy control group, uncomplicated and complicated *Babesia*-infected groups at presentation. The box represents the IQR (i.e. the middle 50% of the observations with the line inside the box as the median). The whiskers represent the main body of the data, indicating the range of the data. Outliers, values that are 1.5 times removed from the IQR, are plotted as open circles. Asterisks represent extreme outlier values that are 3 times removed from the IQR.

4.3 Change over time for total leukocyte count, lymphocyte count, and lymphocyte phenotypes, as well as differences between groups at 24 and 48 - 72 hours post-presentation

Table 2 contains a summary of the variables for the complicated and uncomplicated disease groups at 24 hours as well as 48 - 72 hours post-presentation. Results of 18 dogs in both the complicated and uncomplicated disease groups were available for the 24 hour post-presentation period, and results of 13 dogs in both the complicated and uncomplicated disease groups were available for the 48 - 72 hours post-presentation period. The mean total leukocyte count was significantly increased at 24 hours and 48 - 72 hours compared to the values at presentation for the complicated (P = 0.016 and P = 0.002, respectively) and uncomplicated (P < 0.001 for both) disease groups. Similarly, the mean lymphocyte count was significantly increased at 24 hours and 48 - 72 hours compared to the values at presentation for the complicated (P < 0.001 for both) and uncomplicated (P < 0.001 for both) disease groups. Treatment with packed red blood cells or daily glucocorticoids post-presentation did not have any significant effect on the total leukocyte or lymphocyte counts.

Evaluation of the mean $CD3^+$ T lymphocyte phenotype percentages for the complicated and uncomplicated disease groups at 24 hours and 48 - 72 hours revealed no significant difference between groups or over time. The mean $CD4^+$ T lymphocyte percentage was significantly decreased in the uncomplicated disease

group at 48 - 72 hours compared to the percentage at presentation (P = 0.019). The mean $CD8^+$ T lymphocyte percentage was significantly increased in the uncomplicated disease group at 24 hours compared to the percentage at presentation (P = 0.047). In addition, the mean $CD8^+$ T lymphocyte percentage was significantly higher in the uncomplicated disease group compared to the complicated disease group at 24 hours (P = 0.014) (Fig. 5). The mean B lymphocyte percentage was significantly increased in the complicated disease group at 48 - 72 hours compared to the percentage at presentation (P = 0.041). Treatment with packed red blood cells had no effect on any of the lymphocyte phenotype variables; however, dogs treated with glucocorticoids post-presentation had a significantly higher percentage of B lymphocytes (P = 0.020) at the time points post-presentation. The haematological and lymphocyte phenotype results for the 5 healthy control dogs and the 44 *Babesia*-infected dogs are summarised in Appendix F.

Table 2 Haematological and lymphocyte phenotype data in healthy control dogs and in dogs with complicated and uncomplicated babesiosis at 24 hours and 48 - 72 hours post-presentation.

Variable	Complicated *Babesia*-infected (*n* = 18)	Uncomplicated *Babesia*-infected (*n* = 18)	Complicated *Babesia*-infected (*n* = 13)	Uncomplicated *Babesia*-infected (*n* = 13)
	24 hours		48 - 72 hours	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Leukocyte count ($x10^9$/L)	15.68 ± 12.11 [a]	12.09 ± 5.63 [c]	19.94 ± 9.27 [b]	18.25 ± 10.20 [d]
Lymphocyte count ($x10^9$/L)	2.35 ± 1.20 [a]	3.53 ± 1.88 [c]	3.33 ± 1.43 [b]	4.63 ± 3.61 [d]
$CD3^+$ lymphocytes (%)	40.44 ± 16.96	50.31 ± 17.63	45.57 ± 12.52	42.88 ± 19.02
$CD3^+/4^+$ lymphocytes (%)	24.98 ± 11.07	30.12 ± 12.26	26.51 ± 9.22	25.11 ± 9.49 [d]

$CD3^+/8^+$ lymphocytes (%)	8.12 ± 6.06 [e]	13.02 ± 7.28 [c,e]	8.18 ± 3.64	12 ± 7.71
B-cell lymphocytes (%)	6.64 ± 7.12	6.57 ± 5.46	12.64 ± 9.70 [b]	9.90 ± 6.40

[a] Significant difference between values at presentation and 24 hours post-presentation in the complicated *Babesia*-infected group ($P < 0.05$)
[b] Significant difference between values at presentation and 48 - 72 hours post-presentation in the complicated *Babesia*-infected group ($P < 0.05$)
[c] Significant difference between values at presentation and 24 hours post-presentation in the uncomplicated *Babesia*-infected group ($P < 0.05$)
[d] Significant difference between values at presentation and 48 - 72 hours post-presentation in the uncomplicated *Babesia*-infected group ($P < 0.05$)
[e] Significant difference between the complicated and uncomplicated *Babesia*-infected groups at specified time point ($P < 0.05$)

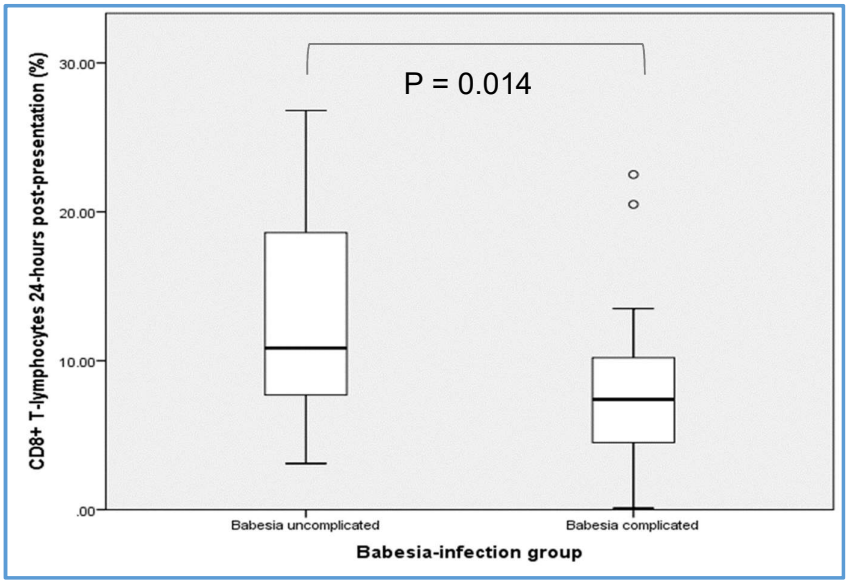

Figure 5 Box plot of $CD8^+$ lymphocytes in the uncomplicated and complicated *Babesia*-infected groups 24 hours post presentation. The box represents the IQR (i.e. the middle 50% of the observations with the line inside the box as the median). The whiskers represent the main body of the data, indicating the range of the data. Outliers, values that are 1.5 times removed from the IQR, are plotted as open circles.

Chapter 5: Discussion

This study reported a reduced percentage of T lymphocytes ($CD3^+$), specifically $CD4^+$ and $CD8^+$ T lymphocytes, in the peripheral blood of dogs infected with *B. rossi*. In addition, the study also showed that the percentage T lymphocytes within the group of dogs with the complicated form of the disease was significantly lower compared to dogs that suffered from the uncomplicated form of babesiosis. A significant reduction in the percentage of the T_H lymphocytes ($CD3^+$ $CD4^+$) was evident in the complicated *Babesia*-infected dogs compared to both the healthy control dogs and uncomplicated *Babesia*-infected dogs.

The reduced percentage of T lymphocytes, especially T_H ($CD4^+$) lymphocytes, at presentation in dogs infected with *B. rossi* may be indicative of a functional immunosuppression. Similarly, the lymphoproliferative response in dogs naturally infected with *B. gibsoni*, in particular those with subclinical infections or relapse infection, revealed depression of lymphocyte blastogenesis (50). In acute human malaria infection, defective metabolism in the second messenger adenosine 3', 5'-monophosphate (cAMP) in peripheral blood lymphocytes appeared to correlate functionally with a reduction in the proliferative response of the lymphocytes (107). Reduced amounts of CD3 and TCRαβ at both RNA and protein expression level due to T lymphocyte apoptosis and reduction of T lymphocyte specific gene expression have been reported in children with *P. falciparum*-infection (108). In humans, naturally infected with *P. vivax*, a low peripheral blood lymphocyte count associated with a high

percentage of $CD4^+$ T lymphocytes in early and/or late apoptosis demonstrated that *P. vivax* infection induced apoptosis of this subset of lymphocytes via the intrinsic as well as the extrinsic apoptotic pathway (109).

A reduction in the percentage of $CD4^+$ T lymphocytes was noted in the uncomplicated *Babesia*-infected dogs at 48 - 72 hours post-presentation compared to the percentage at presentation. In contrast, the percentage of $CD4^+$ T lymphocytes was not significantly different at either time point post-infection compared to the percentage at presentation in the complicated *Babesia*-infected dogs. These findings differ from a recent study that showed an increased percentage of $CD4^+$ T lymphocytes after treatment with imidocarb in dogs naturally infected with *B. canis* (25). However, the study did not differentiate between complicated and uncomplicated cases. Moreover, the infected dogs in our study were treated with diminazene aceturate, a minor-groove-binding agent that acts preferentially at the level of the mitochondrial DNA, thus influencing cell proliferation, ultrastructure, and mitochondrial activity of the targeted parasite (110). In addition to its anti-parasitic effect, it has been shown that diminazene aceturate administration during infection also modulates the host immune response by diminishing T lymphocyte and macrophage hyperactivation, lowering the percentage expression of FoxP3 regulatory T lymphocytes, as well as lowering the systemic pro-inflammatory cytokine concentrations (111). We suggest that in our population of dogs, the combination of infection with *B. rossi* (which reduces the proportion of T_H lymphocytes), together with the reported immunosuppressive effect of diminazene aceturate, resulted in host immunosuppression. *Babesia canis* and *B. gibsoni* are also less virulent parasites and hence may not have the same effect on the immune system as *B. rossi*.

In our study the percentage of $CD8^+$ T lymphocytes was significantly reduced in both the complicated and uncomplicated *Babesia*-infected dogs at presentation compared to the healthy control dogs. In mice, infected with *P. yoelii*, lower numbers of effector $CD8^+$ T lymphocytes were found to be mainly due to increased apoptosis rather than reduced recruitment or proliferation rates of naïve T lymphocytes (112). Similar mechanisms resulting in slower expansion kinetics of antigen-specific $CD8^+$ T lymphocytes may be involved in *B. rossi*-infected dogs. Furthermore, unlike malarial parasites, *Babesia* spp. do not have a tissue stage in the mammalian host which may also account for the lack of requirement of $CD8^+$ T lymphocytes in the immune response against the early phase of the infection (20).

The percentage of $CD8^+$ T lymphocytes was significantly higher in the uncomplicated *Babesia*-infected dogs at 24 hours post-presentation. Similar findings have been reported in dogs infected with *B. canis* and *B. gibsoni* (24, 25). In *Babesia*-infections the central role of $CD4^+$ T lymphocytes is to aid in cell mediated immunity, including the proliferation of $CD8^+$ T lymphocytes, activation of macrophages, or the production of cytokines, as well as the humoral immune response (113). Therefore, the unchanged percentage of $CD8^+$ T lymphocytes at 24 hours post-presentation in the complicated *Babesia*-infected dogs compared to uncomplicated *Babesia*-infected dogs may be as a result of the significantly reduced proportion of $CD4^+$ T lymphocytes in the complicated *Babesia*-infected dogs at presentation.

Compared to the uncomplicated group, the complicated *Babesia*-infected dogs had a significantly reduced percentage of circulating T lymphocytes, in particular T_H lymphocytes. Dogs with complicated disease have a reported mortality rate of 10%, with 80% dying within the first 24 hours of hospital admission (30, 32, 114). Increased concentrations of pro-inflammatory cytokines, in particular IL-6 and monocyte-chemotactic protein-1 (41), and a high parasitaemia (115) have been associated with increased risk of mortality. The lower proportion of circulating lymphocyte subtypes noted in the complicated disease group in our study suggests a specific profile of inflammatory and modulatory cytokines, potentially related to a higher parasite burden, resulting in a unique host immune response in this particular group of *Babesia*-infected dogs.

No significant differences were noted at presentation in the percentage of B lymphocytes in the *Babesia*-infected dogs and the healthy control dogs. This is similar to what has been reported in dogs infected with *B. canis* (25). This is not an unexpected finding since it is doubtful that antibody dependent mechanisms of immunity play a role in the first few rounds of parasite multiplication within an infection. However, it has been shown that antibody mediated neutralisation does play a role in the short period between the tick bite and erythrocyte invasion during which time the *Babesia* parasite is extracellular and exposed (116, 117). Contrary to the group of dogs infected with *B. canis*, where the percentage of $CD21^+$ B lymphocytes remained unchanged throughout the entire study period (25), the percentage of B lymphocytes in our study increased at 48 - 72 hours post-presentation in the complicated *Babesia*-infected group. In dogs infected with *B. gibsoni*, the $CD21^+$ B lymphocytes in treated animals showed the first significant increase 14 days after infection, which

corresponded to 7 days after initiation of clindamycin therapy (24). The findings of an upregulation of T helper response, accompanied by an IgG response, in mice infected with *B. microti* (83), suggest that, similar to malaria, antibody responses are crucial for controlling parasitaemia after the acute infection is resolved (118, 119). Therefore, the increased percentage B lymphocytes in the *Babesia*-infected dogs may be representative of a later T_H2 aided B lymphocyte dependant type response.

Although the mean blood lymphocyte count for both uncomplicated and complicated *Babesia*-infected dogs was within the laboratory reference interval (1 – 4.8 $x10^9$/L) throughout the study, similar to a previous study of dogs infected with *B. rossi* (120), it was significantly lower in the group of infected dogs compared to the healthy control dogs. The lymphocyte count also increased significantly at 24 hours and 48 – 72 hours post-presentation in both the complicated and uncomplicated disease groups compared to values obtained at presentation. Lymphocyte migration and retention in several organs, especially the spleen and liver, have been demonstrated during peak parasitaemia in murine *P. chabaudi* infections (121, 122). Furthermore, the observed peripheral leukocytosis (primarily due to a lymphocytosis) two to five days after peak parasitaemia in murine malaria, was determined to be due to a redistribution of the lymphocyte pool (121). Redistribution of splenic cells is central to the acute immune response of naïve animals to haemoparasitic infection and remarkable similarities of this acute splenic response have been demonstrated in both *B. bovis*-infected calves (123) as well as *P. chabaudi*-infected mice (124). Investigation of the lymphocyte trafficking patterns in canine babesiosis might provide valuable insights into the protective role of the spleen against babesiosis.

In general, the effects of glucocorticoids on lymphocytes are variable and dependant on the glucocorticoid dose, as well as on the lymphoid subpopulation and activation state (125). In humans, the effect of glucocorticoids on B lymphocytes is characterised by a reduced proliferation of precursor B lymphocytes, whereas it results in increased production of certain antibody isotypes by mature plasma cells (126). A significant decrease in the number of circulating B lymphocytes in dogs treated daily for 14 days with prednisone has been reported (127). In addition, immunosuppressive dosages of prednisone given to dogs for three consecutive days resulted in marked decreases in the percentage of lymph node $CD21^+$ B lymphocytes at day 1, 3 and 38 after initiation of therapy (125). In our study we observed that *Babesia*-infected dogs that received glucocorticoids showed a significantly higher percentage circulating B lymphocytes at the time points post-presentation compared to *Babesia*-infected dogs that did not received glucocorticoid therapy. The subset of dogs that did receive glucocorticoid therapy in our study were all classified as having secondary IMHA as a complication. It therefore appears that the increase in the percentage of B lymphocytes noted in the dogs that received glucocorticoid therapy, may be attributed directly to increased antibody production in IMHA, or it may be as a result of variation in the B lymphocyte response to glucocorticoids in infected dogs with an associated secondary IMHA. It may also represent a delayed effect of glucocorticoids on the B lymphocyte population. Interestingly, no statistically significant effect of glucocorticoid therapy was noted on the T lymphocytes in our study. This is in contrast to a reported decrease in circulating $CD4^+$ and $CD8^+$ T lymphocytes in healthy dogs receiving prednisone at a daily dosage of 2 mg/kg for 14 days (127). Prednisone has been reported to induce *in vitro* apoptosis of peripheral T lymphocytes in a time- and dose-

dependent fashion (128). The lack of a glucocorticoid effect noted on the T lymphocytes and subsets in our study may reflect a delayed effect of glucocorticoids on these lymphocytes, or reflect that the dosage used was not adequate to induce a decrease in the circulating T lymphocytes.

Limitations to this study included its clinical nature which prohibited standardisation of the time period of infection prior to presentation. Dogs were therefore at different stages of infection when presented, which could have influenced the results. Another limitation is the lack of specific isotype controls to define fluorescence gates for the control population and to detect the presence of non-specific staining. Furthermore, antibody saturation curves were not performed to determine the optimal concentration to use for each sample, nonetheless the concentration of lymphocytes in each sample was known which allowed the calculation of an appropriate volume of antibody based on the manufacturer's recommendation.

Chapter 6: Conclusion

This study has demonstrated a significantly reduced percentage of both $CD4^+$ and $CD8^+$ T lymphocytes in the peripheral blood of dogs naturally infected with *B. rossi*, particular in the dogs with the complicated form of the disease. These findings could be consistent with the presence of a functional immune suppression secondary to increased apoptosis or redistribution of effector T lymphocytes and/or a combination of other immune modulatory mechanisms caused by *B. rossi* infection. Our results suggest that additional studies are required to further elucidate the immune response caused by *B. rossi*-infection, particularly in the spleen. A hypo-inflammatory immune response might be just as detrimental as an excessive systemic inflammatory response. A better understanding of the canine host immune response to *Babesia* infection may aid in vaccine development as well as immunotherapeutic approaches tailored to the individual immune status, resulting in appropriate regulation of the immune response which might be the key to improved outcomes in these dogs. The presence of a potential functional immune suppression that appears to play a role in the severity of the disease requires further investigation, including measurement of a wide spectrum of cytokines to compare with the lymphocyte changes. Evaluation the splenic lymphocyte immunophenotypes in *Babesia* infected dogs and comparing it with that found in peripheral blood should also be investigated in the future.

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Appendices

Appendix A Client consent form

FORM FOR INFORMED CONSENT:

(To be completed by the patient's owner / authorised agent)

Project title: Investigation of the inflammatory immune response by flow cytometry in South African canine babesiosis

Encircle Yes or No where necessary

1. Have you read the information sheet on canine babesiosis?

   Yes / No

2. Have you had the opportunity to ask questions about the research project?

   Yes / No

3. Have you received satisfactory answers to your questions?

   Yes / No

4. Have you received enough information about this study?

   Yes / No

5. Supply the name of the person to whom you have spoken to:

   ……

6. 6. Do you grant consent that blood and urine samples can be drawn from your dog?

   Yes / No

7. Do you grant consent that a post mortem examination can be performed in the case of death?

   Yes / No

I, ……, hereby give permission that my dog …….., a ……. may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.

I understand that this study will in no way harm my dog. Furthermore, I understand that the costs of the additional tests will be borne by the trial fund, and that I will only be liable for costs that pertain to the treatment that would in any event be required by my dog, including any complications that may arise as a result of canine babesiosis.

Signed at Onderstepoort on the ……. day of …… 20.....

Signature Owner/Agent

……...

Home Tel: …… Work Tel: .....

Cell No: ……

Appendix B Client information sheet

PROJECT INFORMATION SHEET

From the clinical examination and laboratory tests so far performed on your dog, it is clear that your dog is suffering from a blood parasite infection called babesiosis or “tick fever”, transmitted via a tick bite. This blood parasite causes a blood loss as red blood cells are broken down. Dogs suffering from babesiosis may die if they are not treated.

There are indications that cases infected with this blood-borne parasite suffer from a severe inflammatory condition as a result a very abnormal and overreactive immune response. We know, for instance, that some dogs develop severe complications, such as brain failure, lung failure and kidney failure. We are planning a research project in an attempt to try and better understand these immune responses. This information will also provide us with information important to developing better cures and possibly even a vaccine. This will require the collection of 3 blood samples taken at presentation, as well as 24 hours after presentation, and at 24 – 48 hours after presentation. These blood samples are normally collected anyway and so no unusual samples will be collected and no experiments will be performed on your dog.

The collection of these samples will in no way be detrimental to your pet and at no time will the study change the treatment your pet would normally receive. No additional costs will be charged to you for the collection of the samples or the blood tests required for this study. The treatment of your dog will be at the discretion of the attending clinician and will not be changed for the purpose of this trial.

The Animal Use and Care Committee of the University of Pretoria have approved this study.

Sincerely

Dr. Y. Rautenbach

Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, 0110

Tel: 012 529 8458

Appendix C Data capture sheet

DATA CAPTURE SHEET

Date:

Day x of study:

Sticker or:	
Owner:	
Owner no:	
Species: **Sex:** **Age:**	
Breed:	
Patient:	
Patient number:	

Signalment

Age:	
Breed:	
Sex:	
Weight:	

History

Chief complaint:	
Duration of illness:	
When did the dog last eat:	
Treatment received after Dx and collection of samples:	

Physical examination

Mentation • **Can the dog stand unaided?** • **If collapsed – response to painful stimuli?**	

T P R	**T:**	**P:**	**R:**
Mucous membranes		**CRT:**	
Peripheral Lnn			
Abdominal palpation **GIT** **Spleen** **Bladder**			
Faecal examination	**Faecal Colour and consistency**		
	Ova count		
MAP (Oscillometric, 3 readings)	**1.**	**2.**	**3.**
Photo/Video collected			

Blood smears from the ear (one right one left) fixed and archived		
Hct and ISA	**Hct:**	**ISA:**

Urine Analysis

Perform urinalysis within 30 minutes of collection	**Method of collection:**			
	Colour:			
	Appearance:			
	SG:		**pH:**	
	Glu:		**Ketones:**	
	Bili:		**Blood:**	
	Prot:		**UBG:**	
	Hb:			
	Sediment:			

	3 x aliquots of urine at -80°C	

Haematology results

Perform CBC within 30 minutes	**Haematocrit (Hct):**		**Lymphocytes:**	
	White cell count (WCC):		**Monocytes:**	
	Mature neutrophils:		**Eosinophil's:**	
	Immature neutrophils:		**Thrombocytes:**	
Classify the anaemia:				

Biochemistry results

Store at -80°C as	**TSP:**		**Albumin:**	
	Creatinine:		**TotBil:**	

0.5 ml aliquotes	**Urea:**		**ALT:**	
	Glucose:		**ALP:**	
	SIP:		**Cl:**	
	Bile acids		**COP:**	
	Arterial blood gas:			

Patient Outcome

Died / Recovered / Euthanised	
Date died / recovered / euthanised	
Time died / euthanised	
Days to recovery / death?	
Post-mortem performed?	
If PM performed, describe findings	

- **Store EDTA plasma ☐ ; Store red cell pellet ☐ Store plasma ☐ ;Store urine ☐**

Appendix D Signalment results datasheet for *Babesia*-infected dogs

Case Number	Breed	Sex	Age (months)	Weight (kg)	Study Number
BLSC1 T0	Mixed breed dog	MI	23	19.3	C1
BLSC2 T0	Boerboel cross breed	MI	11	28.6	C2
BLSC4 T0	Mixed breed dog	FI	9	11.8	UC1
BLSC5 T0	Jack Russell terrier	MI	9	4.7	C4
BLSC6 T0	Basset hound	FS	48	18	UC2
BLSC7 T0	Jack Russell terrier	MI	43	9.8	UC3
BLSC8 T0	Dachshund	MC	60	7.4	UC4
BLSC9 T0	Mixed breed dog	MC	24	11.6	UC5
BLSC10 T0	Labrador retriever	MI	36	32	UC6
BLSC11 T0	Labrador retriever	FI	6	23	UC7
BLSC12 T0	Siberian husky	MI	9	18.6	C5
BLSC13 T0	Boerboel cross breed	FI	4	8.8	C6
BLSC14 T0	Jack Russell terrier	MI	18	6.8	UC8
BLSC15 T0	German shepherd dog	MI	19	23.8	C7
BLSC16 T0	Jack Russell terrier	FI	5	5.8	C8
BLSC17 T0	Chow chow	MI	6	12.7	C9
BLSC18 T0	Mixed breed dog	MI	36	14.6	C10
BLSC19 T0	Mixed breed dog	MI	24	14.7	C11

BLSC20 T0	German shepherd dog	MC	48	31.6	C12
BLSC21 T0	Wired-haired fox terrier	MI	72	10.6	UC9
BLSC22 T0	Fox terrier cross breed	FI	10	3.4	C13
BLSC23 T0	Mixed breed dog	FI	10	7.8	UC10
BLSC25 T0	Labrador retriever	FI	4	3.6	UC12
BLSC26 T0	Mixed breed dog	MC	18	6.4	C14
BLSC27 T0	Fox terrier	MC	72	6.2	C15
BLSC28 T0	Boerboel	MI	30	30.6	C16
BLSC29 T0	Boerboel	MI	9	45.6	C17
BLSC30 T0	American pit bull terrier	FI	23	20	C21
BLSC31 T0	Mixed breed dog	MI	29	21	UC14
BLSC32 T0	Jack Russell terrier	FI	36	6.5	C18
BLSC34 T0	German shepherd dog	MI	60	34	C20
BLSC35 T0	Rottweiler	MI	10	26.7	UC15
BLSC36 T0	Labrador retriever cross	FS	30	29	UC16
BLSC37 T0	Mixed breed dog	FI	18	6	UC17
BLSC38 T0	Mixed breed dog	FI	12	10.6	UC18
BLSC39 T0	Husky mixed breed	MI	4	11.5	C22
BLSC40 T0	Jack Russell terrier	MI	42	5.6	C23
BLSC41 T0	Jack Russell terrier	MC	91	9.8	C24
BLSC42 T0	Yorkshire terrier	MI	10	2.6	UC19
BLSC43 T0	Jack Russell terrier	MC	84	8.6	UC20

BLSC44 T0	Cockerspaniel	MI	11	11.6	UC21
BLSC45 T0	Mixed breed dog	FI	8	12.6	UC22
BLSC47 T0	Jack Russell terrier	FI	24	6.8	C26
BLSC48 T0	Jack Russell terrier	MC	20	8.8	UC23

MI: Male intact; FS: Female spayed; MN: Male neutered; FI: Female intact

T0: At presentation

Appendix E Signalment results datasheet for healthy control dogs

Case Number	Breed	Sex	Age (months)	Weight (Kg)	Study Number
Cont1 T0	Standard French poodle	MI	76	30.9	CONT1
Cont2 T0	Golden retriever	FS	72	35	CONT2
Cont3 T0	German shepherd dog	FS	44	30	CONT3
Cont4 T0	Mixed breed dog	MN	24	20	CONT4
Cont5 T0	German shepherd dog	FS	42	25	CONT5

MI: Male intact; FS: Female spayed; MN: Male neutered

T0: At presentation

Appendix F Haematological and lymphocyte phenotype datasheet for healthy control dogs and *Babesia*-infected dogs

Case Number	WCC (x10^9/L)	Lymph(x10^9/L)	$CD3^+/CD4^+$ (%)	$CD3^+/CD8^+$ (%)	$CD3^+$ (%)	B cells (%)
CONT1 T0	7.68	1.69	39.7	14.8	63.6	10.2
CONT2 T0	10.84	2.82	28.6	17.8	39.4	15.7
CONT3 T0	14.43	4.18	22.6	52	75.4	5.6
CONT4 T0	9.21	3.22	54.2	14.4	76.8	6.8
CONT5 T0	8.25	1.65	57.7	9.1	71	7.8
BLSC1 T0	6.27	1	14.7	5	0.8	10.1
BLSC1 T1	25.05	4.01	25	10.2	33.6	23.4
BLSC1 T3	14.12	1.55	20.5	7.3	27.7	28.2
BLSC2 T0	5.03	1.46	30.3	10.2	53	7.4
BLSC2 T1	20.83	3.75				
BLSC4 T0	6.38	1.08	23.7	5.5	38.4	8.6

BLSC4 T1	9.83	2.46	30.5	7.2	43	12.3
BLSC4 T3	9.22	3.5	41.9	12.7	58.3	14.6
BLSC5 T0	5.3	1.86	20.9	4.7	48.3	4.8
BLSC5 T1	10.6	2.44	20.6	4.9	34.2	2.2
BLSC5 T3	8.95	3.58	32.4	6.9	47.9	
BLSC6 T0	7.14	0.86	6.3	1.3	4.4	0.2
BLSC6 T1	10.49	4.09	41.3	10.7	51.8	2.6
BLSC6 T3	13.31	2.8	22.3	7.3	32.6	3.9
BLSC7 T0	5.27	1.16	49.9	13	75.4	9.4
BLSC7 T1	11.98	4.07	33.3	21.4	40.5	7.7
BLSC7 T3	11.9	4.76	23.8	32.4	18.8	10.1
BLSC8 T0	2.55	0.15	3.7	1.5	6.5	0.5
BLSC8 T1	9.59	0.58	8.7	3.1	13	1.7
BLSC8 T3	35.2	1.41	4.2	2.9	5.2	0.9
BLSC9 T0	11.63	1.16	47.5	9.8	57.5	3.5

BLSC9 T1	21.1	3.17	52.7	11	56.8	7.7
BLSC9 T3	42.45	0.85	37.4	8.7	46.7	8.1
BLSC10 T0	6.01	0.96	27.3	12.4	34.1	0.7
BLSC10 T1	14.15	2.69	19.4	7.7	22.7	1.3
BLSC10 T3	14.31	3.86	28.6	12.4	48.2	6.5
BLSC11 T0	6.53	1.96	42.8	8.6	59.7	20
BLSC11 T1	13.9	4.87	26.7	26.8	59.6	2.4
BLSC12 T0	5.58	0.73	0.7	0.2	0.4	0.2
BLSC12 T1	8.53	0.94	1	0.1	1.3	1.4
BLSC12 T3	8.6	2.24	25.2	11.9	53.9	4.4
BLSC13 T0	23.6	1.89	23.7	6.7	34.8	7.7
BLSC13 T1	26.32	2.11	41.7	8.5	47.9	14
BLSC13 T3	11.77	2.59	17	4.2	58.9	17.4
BLSC14 T0	1.99	0.64	34.3	10.8	68.5	4.5
BLSC14 T1	4.37	2.62	32.3	10.1	62.3	5.6

BLSC14 T3	14.56	4.8	21.5	11.4	70.1	7.2
BLSC15 T0	4.75	0.95	1.2	0.4	32.8	3.4
BLSC15 T1	12.94	2.46	22.6	1.7	34.1	4
BLSC15 T3	27.44	4.66	35.8	6.5	57.2	8.1
BLSC16 T0	3.2	0.64	6	2.1	19.3	2.1
BLSC16 T1	7.27	1.53	10.9	6.3	25.7	2.8
BLSC17 T3	17.39	6.11	13.2	5.8	31.7	2.3
BLSC17 T0	6.36	1.91	30.6	17.8	56.6	3.5
BLSC17 T1	7.55	1.81	40.4	20.5	73.4	3
BLSC18 T0	10.21	1.02	10.7	1.6	9.8	0.9
BLSC18 T1	9.62	1.15	24.5	8.2	44.1	2.1
BLSC19 T0	11.5	2.88	20.7	5.2	27.6	7.1
BLSC19 T1	17.89	3.94	16	4.5	19.4	3.7
BLSC19 T3	12.9	2.45	33.4	6.2	46.5	12.1
BLSC20 T0	5.02	1	41.5	9.3	65.6	6.3

BLSC20 T1	14.06	3.52	31.3	13.5	57.4	2.4
BLSC21 T0	7.56	1.21	28.8	16.4	73.8	2.9
BLSC21 T1	9.6	3.17	15.6	23.1	20.5	2.6
BLSC21 T3	10.86	2.17	24.9	21.7	38.2	7
BLSC22 T0	4.2	1.26	0.2	0.1	1.4	0.1
BLSC22 T1	8.48	1.61	16.6	0.8	23.7	1.2
BLSC22 T3	32.98	5.94	19.3	8.9	31.2	2
BLSC23 T0	3.9	0.7	48.3	10	74.8	4.6
BLSC25 T0	3.76	1.2	34	6.2	56.9	20.1
BLSC25 T1	14.92	3.88	24.9	5.6	42.2	20.9
BLSC25 T3	25.45	14.51	16.2	5.5	50	9.9
BLSC26 T0	3.63	0.54	42.9	7.4	53.3	10.7
BLSC26 T1	4.99	0.55	45.5	8.1	58.4	15.8
BLSC26 T3	19.66	2.75	43.1	8.8	54.5	19.8
BLSC27 T0	5.8	1.74	22.5	7.7	47.1	9.8

BLSC27 T1	15.27	3.05	30.7	2.9	43.7	19.3
BLSC27 T3	32.03	2.56	27.9	4.5	33.8	30.7
BLSC28 T0	103.55	1.04	1.1	0	4.1	3.5
BLSC29 T0	21.6	4.97	4.9	2	13.3	2.6
BLSC30 T0	9.94	0.99	32	8.1	56.4	1.5
BLSC30 T1	23.45	3.52	26.7	11.3	49.3	1.8
BLSC30 T3	17.9	3.76	23.4	9.4	44.6	8.2
BLSC31 T0	8.81	2.29	35.1	13.3	55.3	11.7
BLSC31 T1	15.73	5.03	19.5	8.1	47.7	3.7
BLSC32 T0	1.12	0.32	4	13.2	21.1	0.8
BLSC32 T1	2.92	0.47	13.3	26.3	48.6	1.7
BLSC34 T0	14.36	0.86	11.8	3.1	10	4.3
BLSC34 T1	57.15	1.71	28.7	6.7	55.4	3.8
BLSC35 T0	5.2	0.88	25.1	19.2	75.7	5
BLSC36 T0	7.11	1.78	36.6	13.6	60.5	5.2

BLSC36 T1	18.69	4.11	31.2	12.3	56.6	8.5
BLSC37 T0	9.63	1.16	58.4	15.8	82.9	8.4
BLSC37 T1	7.45	1.12	44.8	18.6	67.2	15.5
BLSC37 T3	14.35	8.47	27	7	36	7.9
BLSC38 T0	5.14	2.31	51	10.3	63.8	4.6
BLSC38 T1	5.89	2.06	50.7	15.7	73.5	5.8
BLSC38 T3	20.91	5.85	33.7	13.8	54	18.3
BLSC39 T0	9.27	1.11	57.5	12.1	78	9.7
BLSC40 T0	5.4	1.3	28.5	21.2	37.6	4.5
BLSC40 T1	11.29	4.85	28.1	22.5	53.3	3.1
BLSC41 T0	5.19	0.62	14.6	4	33.1	4.5
BLSC41 T1	13.85	1.66	24.2	9.4	39.5	14
BLSC41 T3	35.7	2.5	16.5	7.8	37	13.8
BLSC42 T0	4.9	1.18	28.9	12.3	48.8	17.3
BLSC43 T0	4.04	1.21	41.4	5.6	69.1	2.4

BLSC43 T1	11.49	5.52	35.1	6.2	73.6	1.2
BLSC43 T3	13.23	5.03	23.9	12.2	70.6	8.7
BLSC44 T0	9.59	1.44	43	5.9	64	7.5
BLSC44 T1	24.7	6.67	27.9	8.9	59.9	6.1
BLSC45 T0	12.81	1.92	25.3	6.5	40.2	16
BLSC45 T3	11.46	2.18	21	8	28.7	25.6
BLSC47 T0	4.47	1.21	21	8.6	41.8	2.7
BLSC47 T1	7.98	1.52	15.1	6	33.6	1.6
BLSC47 T3	19.78	2.57	36.9	18.1	67.5	4.7
BLSC48 T0	4.84	2.27	44	7.8	63.6	9.8
BLSC48 T1	10.74	6.98	34.2	11.6	66	11

T0: At presentation; T1: 24 hours post-presentation; T3: 48 - 72 hours post-presentation

CONT: Healthy control dog; BLSC: *Babesia*-infected dog