C-REACTIVE PROTEIN IN CANINE BABESIOSIS CAUSED BY *BABESIA ROSSI* AND ITS ASSOCIATION WITH OUTCOME

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Submitted to the Faculty of Veterinary Science, University of Pretoria, in partial fulfilment of the requirements for the degree MMedVet(Med)

Pretoria, April 2009

TABLE OF CONTENTS

Acknowledgements **iv**

List of Tables **vi**

List of Figures **vii**

List of Appendices **ix**

List of Abbreviations **x**

Summary **xii**

Chapter 1 Literature Review

1.1 Canine babesiosis 1

1.2 Host inflammatory response to the parasite 2

1.3 Prognosis and outcome in canine babesiosis 3

1.4 The history and biology of acute phase proteins 5

1.5 CRP as a biomarker for disease in humans and dogs 10

Chapter 2 Study Objectives

2.1 Hypothesis and statement of the problem 16

2.2 Objectives of this study 17

2.3 Benefits arising from this project 17

Chapter 3 Materials and Methods

3.1 Model system 18

3.2 Experimental design 18

3.3 Experimental procedures 19

3.4 Observations 21

3.5 Routine procedures 22

3.6 Statistical analysis 23

Chapter 4 Results

4.1 Inclusion and exclusion of patients 25

4.2 CRP concentration and outcome 25

4.3 Univariable association of measured parameters at admission with outcome 25

4.4 Receiver operator characteristic curve for the final model 26

4.5 Determination of CRP concentration associated with 100% survival 26

4.6 Examination of the association between admission CRP and number of days of hospitalisation 27

Chapter 5 Discussion 35

Chapter 6 Conclusion 38

References 39

Appendices 51

Acknowledgements

I would like to acknowledge the following people for their help and assistance with this project:

Dr Mirinda van Schoor, the project leader, whose constant support and encouragement made this project possible.

Dr Amelia Goddard, the co-supervisor, whose insight and guidance has been invaluable to the project.

Professor Mads Kjelgaard-Hansen, who agreed to give input into the project, whose experience and insight into the field has been invaluable to the project.

Professor Peter Thompson for statistical analysis.

Mr Tshepo Matjila for PCR and RLB analysis.

Mrs Elsbe Myburgh, the head laboratory technologist, for assistance and support during the trial.

Staff and final year students working in the outpatient department, for alerting me to the presence of the canine babesiosis cases.

And lastly, but most importantly, my family who always believed in my abilities and gave me their support.

List of Tables

Table 1. Normal ranges of C-reactive protein (CRP) as measured in dogs with a variety of different methods.

Table 2. Results of a multiple exact logistic regression model to estimate effects of serum variables on risk of mortality in canine babesiosis patients.

Table 3. Comparison of sensitivity and specificity of exact logistic regression models to predict mortality in canine babesiosis patients, at optimal cut-off points.

List of Figures

Fig 1. The acute phase response, a schematic representation. The local and general effects of systemic inflammation mediated through the pro-inflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α). Modified from Kjelgaard-Hansen (2004).

Figure 2. Flow diagram depicting the exclusion criteria for the study. Of the dogs diagnosed to be suffering from canine babesiosis based on blood smear examination, 1 dog was eliminated as it was infected with Distemper virus. PCR and RLB revealed that 5 dogs had babesiosis other than *B. rossi* or concurrent *E. canis*. One dog was eliminated as the reason for euthanasia was due to cost of treatment rather than poor prognosis.

Figure 3. Box plot (representing the interquartile range) showing admission CRP concentrations in dogs with severe canine babesiosis, grouped according to outcome i.e. survivors or non-survivors. The box incorporates the middle 50% of the observations with the line inside the box as the median. The whiskers extend to the smallest (25^{th} percentile) and largest (75^{th} percentile) observations, indicating the range of the data. Outliers, values that are 1.5 times removed from the interquartile range, are plotted separately as dots.

Figure 4. Receiver operator characteristic (ROC) curves for the two logistic regression models examining predictors of mortality in canine babesiosis. The area

under the curve (AUC) of the ROC curves for the two models (0.851 and 0.819 respectively) did not differ significantly (p = 0.52). The optimal probability cut-off points (where Se+Sp is maximized) were 0.16 for the model with glucose and CRP, and 0.23 for the model with glucose only.

Figure 5. Box plot depicting admission CRP concentrations (mg/l) in dogs with different durations of hospitalization. See Fig 2 legend for explanation of interpretation. Although there is an apparent linear correlation between the two variables, there is no significant relationship when examined with a multiple regression analysis (p = 0.64), when adjusted for sex and age.

Figure 6. Box plot representing the magnitude of decline (hence the negative value) of CRP concentration (mg/l), in the first 24-hours, for each number of days until discharge. See Fig 2 legend for explanation of interpretation. A multiple regression analysis failed to show a significant relationship between the magnitude of CRP decline and days hospitalised (p = 0.34).

List of Appendices

Appendix A: Client consent form.

Appendix B: Data capture form.

Appendix C: Raw data.

List of Abbreviations

α	Significance (aka p-value)
αGP	Alpha-1 acid glycoprotein
ACTH	Adrenocorticotrophic Hormone
ALT	Alanine Transferase
APP	Acute Phase Proteins
ARDS	Acute Respiratory Distress Syndrome
ARF	Acute Renal Failure
AUC	Area Under the Curve
CRP	C-Reactive Protein
CVD	Chronic Valve Disease
FT4	Free Thyroxin
Ht	Microhaematocrit
IL	Interleukin
IMHA	Immune Mediated Haemolytic Anaemia
IQR	Interquartile Range
ISA	In saline agglutination
MODS	Multiple Organ Dysfunction Syndrome
n	Number of animals
NHL	Non-Hodgkin's Lymphoma
OVAH	Onderstepoort Veterinary Academic Hospital
PCR	Polymerase Chain Reaction
PCR-RLFP	PCR-restriction length fragment polymorphism
P value	Probability value

RLB	Reverse Line Blotting
RNI	Reactive Nitrogen Intermediates
ROC	Receiver Operating Characteristic
Se	Sensitivity
SIRS	Systemic Inflammatory Response Syndrome
Sp	Specificity
T4	Thyroxin
TIA	Turbidometric Immunoassay
TNF	Tumour Necrosis Factor
UK	United Kingdom
WCC	White Cell Count

Summary

C-reactive protein in canine babesiosis caused by *Babesia rossi* and its association with outcome

Köster, L.S. University of Pretoria, 2009

Acute phase proteins (APP) are ideal biomarkers for inflammation due to their stability, relative ease of assay and apparent relation between their concentration and the extent of the insult to tissue. C-reactive protein (CRP) is a positive major APP in dogs and can be used as a predictive marker for risk of disease and to monitor the response to treatment. Increased concentrations in certain diseases are associated with poor outcome. This cross-sectional, observational study of 75 dogs naturally infected with *Babesia rossi*, a cause of virulent canine babesiosis, was designed to examine the association of CRP concentration at admission and the magnitude of CRP change 24 hours after admission with outcome. Dogs were excluded if there was evidence of concurrent inflammatory diseases at the time of admission, infection with subtypes other than *B. rossi*, concurrent *Ehrlichia canis* infections or euthanasia for reasons other than poor prognosis. Diagnosis was confirmed by polymerase chain reaction and reverse line blot. CRP concentrations were determined by an automated human CRP Turbidometric Immunoassay (TIA), previously validated for use in dogs (Bayer CRP TIA, Newbury, UK), on serum samples collected by jugular venipuncture on admission, prior to any therapy, and thereafter daily until discharge or death. There was no significant difference in admission CRP concentration between survivors (n = 57; median = 97.4 mg/l; mean ± SD = 107.5 ± 49.5), and non-survivors (n = 11; median = 101.4 mg/l; mean ± SD = 122.1 ± 64.6) (p = 0.39). After elimination of non-significant predictors, a multiple exact logistic regression model for predicting

mortality contained glucose and CRP. Mortality was associated with decreased glucose levels ($p = 0.0002$) and increased CRP levels ($p = 0.045$) on admission. Multiple regression analysis failed to show a significant relationship between admission CRP concentration and number of days of hospitalization in the survivors, adjusting for age and sex ($p = 0.65$). No significance was found in the relationship between the magnitude of change in CRP concentration 24 hours after admission, and the number of days of hospitalization in survivors, ($p = 0.34$). Using an admission CRP concentration cut-off of 60 mg/l, survival proportions between the two groups were no different ($p = 0.34$) and when applied to the group of dogs that survived, it was not associated with length of hospitalization ($p = 0.25$). In corroboration with previous reports glucose was identified as a major prognostic marker for mortality, but additionally the pro-inflammatory marker CRP was identified as a significant co-prognosticator.

Chapter 1 Literature Review

1.1 Canine babesiosis

Canine babesiosis in South Africa is caused by the intra-erythrocytic protozoan parasites, *Babesia rossi* and *Babesia vogeli* (Matjila, et al., 2008). The disease is characterized by haemolytic anaemia with the course ranging from mild to peracutely fatal (Jacobson and Clark, 1994). The two species differ in their pathogenecity: *B. rossi* frequently causes a fatal infection despite intensive treatment, whereas *B. vogeli* causes a mild clinically unapparent infection (Uilenberg, et al., 1989; Uilenberg, 2006). Canine babesiosis classification is loosely based on the World Health Organisation (WHO) classification for malaria as follows: uncomplicated if the clinical changes are solely attributed to anaemia and then subclassified according to severity, and complicated if the clinical signs are not only attributable to haemolytic anaemia (Jacobson and Clark, 1994). Anaemia may be mild (microhaematocrit [Ht] 15-30%) to severe (Ht< 15%) due to erythrocyte destruction (Jacobson, 2006).

This is a common disease accounting for 10-12 % of the total caseload of sick patients seen in South African veterinary practice, of which 26% are considered complicated (Shakespeare, 1995). Mild, uncomplicated forms of the disease are effectively treated with antibabesial drugs (Jacobson and Swan, 1995). Complicated forms of the disease are difficult to treat with a mortality rate of 45% in one study (Welzl, et al., 2001). Even in cases with low parasitaemia, anaemia can be profound, suggesting that non-parasite factors play a role which includes peripheral sludging in capillaries, erythrophagocytosis by the spleen and liver and possibly immunoglobulin and complement-mediated destruction of erythrocytes (Maegraith, et al., 1957).

1.2 Host inflammatory response to the parasite

The clinical manifestations and pathophysiology of complicated babesiosis parallels falciparum malaria in juvenile humans and *Babesia bovis* in cattle (Clark and Jacobson, 1998; Jacobson and Clark, 1994; Welzl, et al., 2001). This may be ascribable to the similar sensitivities these species have toward endotoxins, and a result of the host inflammatory response. In canine babesiosis there is an association between tumour necrosis factor (TNF) concentration, clinical severity as well as parasitaemia (Vaughan-Scott, 2001). Thus TNF probably plays a role as an intermediary in the production of other harmful substances such as nitric oxide or free oxygen radicals or the interaction between parasitised erythrocytes and the blood vessel wall, much like the pathophysiology of falciparum malaria. Although nitric oxide is proposed to be a mediator of a Multiple Organ Dysfunction Syndrome (MODS), which is seen in complicated canine babesiosis, concentrations of reactive nitrogen intermediates (RNI) did not correlate to severity nor was it predictive of outcome (Jacobson, et al., 2002). A marked increase in α1-acid glycoprotein (αGP) (a positive acute phase protein), concentration in dogs infected with *B. rossi* was three to four fold higher than healthy controls, but there was no correlation with severity of disease or association with outcome (Lobetti, et al., 2000).

Complications of canine babesiosis include acute renal failure (ARF), cerebral babesiosis, coagulopathy including disseminated intravascular coagulation, icterus and hepatopathy, immune-mediated haemolytic anaemia (IMHA), acute respiratory distress syndrome (ARDS), haemoconcentration, shock and pancreatitis (Jacobson, 2006). These complications are proposed to be the result of a Systemic Inflammatory Response Syndrome (SIRS) present in most cases of canine babesiosis, mediated by

cytokines, nitric oxide and free oxygen radicals. SIRS can eventually progresses to MODS (Jacobson and Clark, 1994; Welzl, et al., 2001). Using the criteria for SIRS in a study by Welzl *et al,* 87% of complicated canine babesiosis cases, presented to the Onderstepoort Veterinary Academic Hospital, South Africa, were positive for this syndrome (Welzl, et al., 2001). Fifty two percent diagnosed with organ dysfunction/damage had single organ involvement and the remaining 48% had MODS.

Two additional processes, besides parasite induced haemolysis, occur in canine babesiosis representing two disease entities: a haemolytic disease characterized by severe anaemia caused by a secondary IMHA with leukocytosis, hypoxic hepatic disease and elevated urea, and a non-anaemic disease due to a severe inflammatory reaction characterized by severe azotaemia, marked electrolyte disturbance and often leukopaenia (Reyers, et al., 1998). The absence of anaemia in the non-anaemic disease group is thought to be due to a rapid acting and overwhelming inflammation with little time to exhibit signs of the slower haemolytic state. The death rate for the non-anaemic group is higher (29%) than for the severely anaemic group (8%) (Reyers, et al., 1998).

1.3 Prognosis and outcome in canine babesiosis

Prognosis can be predicted by hypoglycaemia at admission, serial blood lactate measurements, admission adrenocorticotrophic hormone (ACTH), thyroxine (T4) and free thyroxin (fT4), and specific organ involvement, but not by the severity of anaemia or the presence of SIRS, MODS, and IMHA (Nel, et al., 2004; Schoeman, et al., 2007; Welzl, et al., 2001). Hypoglycaemia is a common complication of canine

babesiosis, and is associated with collapsed state, severe anaemia, age (< 6 months) and icterus, and may have been misdiagnosed as cerebral babesiosis in the past (Keller, et al., 2004). Babesia infected dogs with hypoglycaemia have very high TNF values, which correlates with parasitaemia (Vaughn-Scott, 2001). Lactate has been established as a prognosticator in that mean lactate in non-survivors (145 mg/dL) was higher than in survivors (13.8 mg/dL) (Nel, et al., 2004). Pre-treatment hyperlactataemia and subsequent serial lactate concentrations that failed to return to normal reference range indicated a poor prognosis. Mortality is significantly associated with high cortisol and high ACTH concentrations and low T4 and fT4 concentrations (Schoeman, et al., 2007). Central nervous system involvement results in a 57 times greater chance of death and pulmonary oedema is associated with the highest proportion of fatalities (Welzl, et al., 2001). Hypotension correlates with disease severity and negatively correlates with white cell counts (WCC) but blood pressure is not a predictor of mortality (Jacobson, et al., 2000). A study looking at parasitaemia, circulatory collapse and outcome in dogs infected with *B. rossi* showed that dogs that died had significantly higher capillary and venous parasitaemias than dogs that survived as well as a high association between circulatory score and outcome (Bohm, et al., 2006). However, the parasitaemias of the different groups in this study (outpatient, hospitalized or died) overlapped considerably, possibly due to the varied host response to infection. Icterus is reported to occur in advanced stages of canine babesiosis (Malherbe, et al., 1951). Bilirubin values exceeding 170 µmol/l correlate positively with mortality. High Alanine amino transferase (ALT) activity is associated with a high risk of mortality (Reyers, et al., 1998). An association exists between a high haematocrit, or a haemaotocrit within reference range, which is

inappropriate for the degree of haemolysis evident, and a fatal outcome, indicating that the presence of haemoconcentration has a poor prognosis (Reyers, et al., 1998).

1.4 The history and biology of acute phase proteins

The acute phase response, thought to be an innate host defence mechanism, occurs during the early stages of infection, tissue injury or immunological disorders and is responsible for accumulation and activation of granulocytes and mononuclear cells, which in turn release acute phase cytokines (Eckersall, 2000). The pro-inflammatory cytokines include, interleukin (IL)-1, IL-6 and TNF-α (Eckersall, 2000). During an acute phase response the serum concentration of acute phase proteins (APP) change in response to these cytokines (Ceron, et al., 2005). Some APP will decrease (negative APP), while others will increase in concentration (positive APP). The negative acute phase proteins are albumin and transferrin while the positive acute phase proteins include haptoglobin, C-reactive protein (CRP), serum amyloid A (SAA), ceruloplasmin, fibrinogen and αGP (Fig 1). The acute phase response is rapid, and usually the onset is prior to clinical signs, but lasting only a few days (Yamamoto, et al., 1992, Yamamoto, et al., 1993; Kjelgaard-Hansen, et al., 2003a).

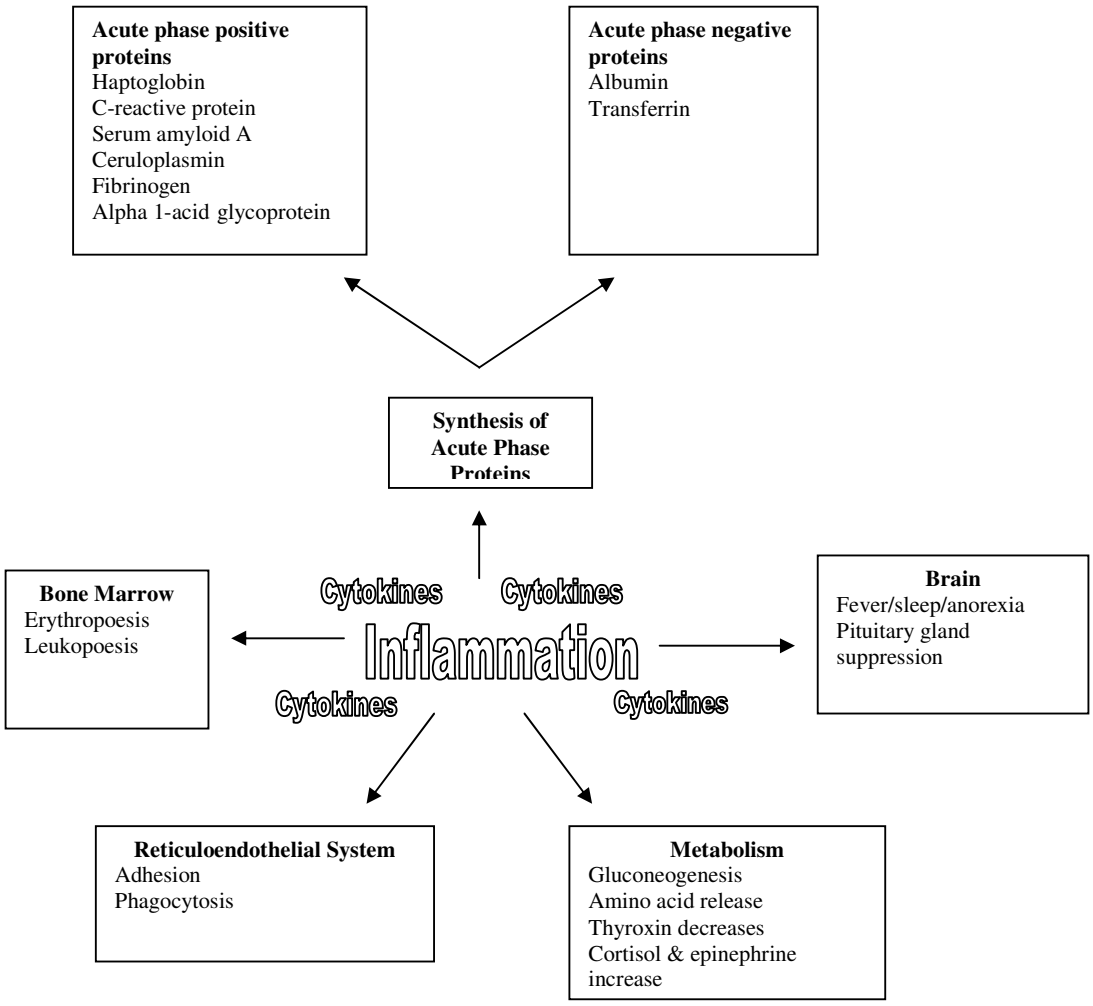

Fig 1. The acute phase response, a schematic representation. The local and general effects of systemic inflammation mediated through the pro-inflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α). Modified from Kjelgaard-Hansen (2004).

Acute phase proteins are biomarkers for inflammation and correlate with extent and activity of disease (Caspi, et al., 1987). In dogs, CRP has been isolated from the acute phase serum and classified as a major positive APP due to the magnitude of its response (Caspi, et al., 1984; Yamamoto, et al., 1992). In addition to hepatocytes, other cells including Kupffer cells, lymphocyte subsets, blood monocytes and alveolar

macrophages have been shown to synthesize this protein (Dong and Wright, 1996). The main biological function of CRP is to recruit the complement system and mononuclear cells by chemotaxis to inflamed tissues during an acute-phase response (Fujimoto, et al., 2003). Human CRP binds with high affinity to phosphocholine residues, but also to a variety of autologous (phospholipids, ribonucleoproteins, and apoptotic cells), and extrinsic (glycans, phospholipid, capsular and somatic components of micro-organisms) ligands, aggregating or precipitating the cellular, particulate or molecular structures bearing these ligands (Pepys and Hirshfield, 2003). When aggregated to ligands, CRP is recognized by C1q and potently activates the complement pathway (Pepys and Hirschfield, 2003). Reports indicate that CRP is a pathological inflammatory mediator and plays a major role in the pathogenesis of ischaemic myocardial inflammation in humans (Griselli, et al., 1999). Impaired CRP activity in human Systemic Lupus Erythrematosus indicates that it may have a role in preventing auto-immunity (Pepys and Hirschfield, 2003). C-Reactive Protein was shown to be responsible for anaemia in humans infected with malaria (*Plasmodium falciparum)* by triggering complement-mediated haemolysis after binding with red blood cells (Ansar, et al., 2006). This may be due to CRP recognizing erythrocytes as foreign antigens due to membrane damage by the parasites.

Dillman and Coles (1966) were first to demonstrate a non-specific serum protein fraction in the serum of dogs with experimentally induced inflammation, that had properties analogous to the serum CRP fraction of man (Dillman and Coles, 1966). Twenty-one dogs were subjected to inoculation of foreign antigen known to be associated with an inflammatory response. In some dogs, CRP–positive serum was the only indication of an inflammatory reaction, as many failed to show a simultaneous neutrophilic response. A positive CRP reaction was shown as early as

24 hours post-inoculation, which is one of its more valuable properties as a biomarker. More recently, CRP was characterized by calcium dependant affinity chromatography and later by ion-exchange chromatography (Caspi, et al., 1984; Yamamoto, et al., 1992). The molecular weight was estimated to be 100 000 and 155 000 to 157 000 Dalton in the respective studies, and is composed of five subunits, each estimated to be approximately 20 kiloDalton, of which two subunits are glycosylated. The structure had a cyclic pentameric disc-like morphology typical of the pentraxin family (Caspi, et al., 1984). The protein was found to be thermolabile and loses its antigenecity when heated at 70°C for 15 minutes (Yamamoto, et al., 1992). Canine CRP concentration has been estimated by a variety of tests that have been developed and validated for use in dogs, including electroimmunoassay, immunodiffussion, enzyme-linked immunosorbent assay (ELISA), turbidometric immunoassay (TIA), and a time-resolved immunofluorometric assay (TR-IFMA) (Caspi, et al., 1984; Conner, et al., 1988; Kjelgaard-Hansen, et al., 2003a; Kjelgaard-Hansen, et al., 2003b; Parra, et al., 2005, Parra, et al., 2005, Parra, et al., 2006, Yamamoto, et al., 1992).

CRP can be measured in serum, whole blood, and more recently in saliva and effusions (Parra, et al., 2005; Parra, et al., 2006). Although saliva samples have the advantage of non-invasive harvesting, there are many factors that can influence CRP concentrations i.e. increased rate of saliva flow causing dilution of CRP, periodontitis or gingivitis causing an increase in CRP levels (Parra, et al., 2005).

Normal ranges of CRP concentrations in healthy animals have been determined by different researchers and are tabulated (Table 1).

Researcher	**Sample**	**Method**	**Concentration (mg/L)**
Caspi *et al*. 1987	Serum	Calcium-dependant affinity chromatography	< 5
Yamamoto *et al*. 1992	Serum	Ion and affinity exchange chromatography with agar block electrophoresis	0.198-0.826
Kjelgaard-Hansen *et al*. 2003a	Serum	Turbidometric Immunoassay	1.1-6.3
Parra *et al*. 2005	Saliva	Time-resolved Immunofluorescent Assay	0.0021-0.0061
Parra *et al*. 2006b	Serum	Time-resolved Immunofluorescent Assay	0-7.1

Table 1. Normal ranges of C-reactive protein (CRP) as measured in dogs with a variety of different methods.

There are no statistically significant differences in CRP concentrations between arterial and venous samples (Palsgaard-Van Lue, et al., 2007). Haemolysis, lipaemia and hyperbilirubinaemia cause a statistically significant change in the CRP concentrations measured but differences observed are unlikely to be clinically

relevant (Martinez-Subiela and Ceron, 2005). Corticosteroids may participate as a co-factor in the regulation of APP, but no statistically significant changes occurred in the concentrations of CRP in dogs dosed with exogenous glucocorticoids (Martinez-Subiela, et al., 2004), and dogs with uncomplicated hyperadrenocorticism did not have CRP concentrations that were any different to healthy controls (Caldin, et al., 2008). Although non-steroidal anti-inflammatory drugs (NSAID) do not directly inhibit IL-6, dogs with experimentally induced acute synovitis and treated with a NSAID had a smaller percentage increase in CRP concentrations as compared to those dogs treated with an opiod agonist-antagonist as an analgesic (Borer, et al., 2003).

1.5 CRP as a biomarker for disease in humans and dogs

In human medicine, CRP has gained popularity after it was recognized in the 1990s to be a strong predictor of atherothrombotic events (Pepys and Hirschfield, 2003). Elevated CRP concentration predicts risk of heart failure after myocardial ischaemia or infarctions and may play a direct role in augmenting microvascular inflammatory response after an ischaemic insult (Berton, et al., 2003). Raised CRP also predicts the development of type-2 diabetes independently from traditional risk factors in humans and supports the notion that inflammation is related to insulin resistance (Freeman, et al., 2002). Due to the lack of specificity of this test, patients are screened by serial sample collections at weekly intervals. If concentrations are persistently raised then the search for an inflammatory focus e.g. rheumatoid arthritis, ensues. Increased CRP concentrations at admission into the intensive care unit (ICU) are associated with organ failure, prolonged ICU stay, and high infection and

mortality rates, with persistently raised CRP concentrations indicating a very poor prognosis (Lobo, et al., 2003). Interleukin-6 plays a central role in some B-cell malignancies, namely non-Hodgkin's lymphoma (NHL) and multiple myeloma. A significant relationship exists between CRP and IL-6 levels in patients diagnosed with NHL (Legouffe, et al., 1998). Evaluation of CRP serum concentrations constitutes a prognosticator in patients suffering from NHL. Patients with elevated CRP concentrations had a median survival of 8.5 months in contrast to the group of patients with CRP levels < 10 mg/L, which did not reach a median survival time, as 75% were still alive at 32 months (Legouffe, et al., 1998). Semi-immune and non-immune patients with uncomplicated *Plasmodium falciparum* infection had elevated serum CRP concentrations at onset of symptoms (Graninger, et al., 1992). Severity of falciparum malaria in humans has been determined using CRP concentrations rather than clinical assessment of patients at admission, which often fails to predict the development of complications (Gillespie, et al., 1991). Finger-prick CRP tests are used to estimate malaria-specific morbidity in high infection-density areas, as serum CRP concentrations are correlated to degree of parasitaemia (Hurt, et al., 1994). Kala-azar (visceral leishmaniasis) in children requires repeated bone marrow/splenic aspiration to monitor response and duration of therapy required as well as to detect resistance, which necessitates a change in medication. C-reactive protein concentrations have been shown to be predictive of the presence or absence of infection after treatment without the need for these invasive diagnostic tests, and is recommended as a monitoring assay every 5-10 days during therapy (Singh, et al., 1999).

Studies in dogs show that the serum level of CRP is acutely increased by surgical stimulation and inflammation (Caspi, et al., 1987; Conner, et al., 1988). The increased

concentration of CRP can first be detected at 4 hours and is dramatically increased within 24 hours after surgical stimulation (Caspi, et al., 1987; Yamamoto, et al., 1992). The average peak in concentration is 2 days after inoculating dogs with turpentine oil with peak CRP concentrations being significantly lower in 1-month-old dogs as compared to 3 and 18-month-old dogs and higher concentrations are found in pregnant bitches peaking at 30 or 45 days after ovulation (Hayashi, et al., 2001; Kuribayashi, et al., 2003). CRP concentrations will be elevated in many diseases with inflammation and tissue damage, particularly in neoplastic and immune-mediated diseases (Nakamura, et al., 2008). CRP is less likely to be elevated in neurological disease (spinal/brain) and upper respiratory tract disease and localised tumours making it a valuable tool in aiding distinguishing these diseases from differential diagnoses (Nakamura, et al., 2008). It has been shown that CRP concentrations will increase in pathological conditions, such as infection with *Ehrlichia canis*, *Leishmania infantum*, acute gastric mucosal injury, and neoplasia (Martinez-Subiela, et al., 2003; Otabe, et al., 2000; Shimada, et al., 2002; Tecles, et al., 2005). In dogs with mammary tumours, CRP values are higher when disseminated disease and complications are present (Caspi, et al., 1987).

Several researchers have explored the acute phase response and specifically CRP in canine babesiosis. A preliminary retrospective study examined CRP, ceruloplasmin and haptoglobin concentrations in dogs with naturally acquired *B. canis* infections (Ulutas, et al., 2005). Serum CRP and ceruloplasmin concentrations were significantly higher in dogs infected with babesiosis, however haptoglobin concentrations were lower as compared to control dogs proposing that measuring the acute phase response could be helpful in determining the severity of babesiosis in dogs. A retrospective study examined the clinical pathology results of dogs living in Italy that had a clinical

history consistent with tick-borne disease and later confirmed to be infected with *B. canis* and *B. vogelli* by PCR-restriction fragment length polymorphism (RLFP) (Solano-Gallego, et al., 2008). In addition to other parameters CRP, albumin and fibrinogen concentrations were assessed and showed hyperfibrinogenaemia, elevated CRP concentrations and hypoalbuminaemia in dogs infected with *B. canis* but no discernable pattern was noted in the *B. vogelli* infected dogs. Dogs with babesiosis caused by *B. canis* in Croatia had higher concentrations of serum CRP in dogs classified as 'complicated' as compared to those with the 'uncomplicated' form of the disease and was proposed to be able to predict severity of canine babesiosis (Matijatko, et al., 2002). Matijatko *et al*, monitored the serial decline in CRP, haptoglobin and SAA concentrations, as well as erythrocyte sedimentation rate (ESR), in dogs naturally infected with *B. canis* after receiving antibabesial treatment and found that of the APP measured, CRP and SAA had a higher diagnostic sensitivity than haptoglobin, which increased in concentration daily in contrast to the CRP and SAA values and that these tests (CRP and SAA) correlated with traditional markers of inflammation including ESR, and WCC (Matijatko, et al., 2007). Schetters *et al*., found that plasma CRP was triggered by *B. canis* organisms in experimentally infected dogs prior to the onset of clinical signs (Schetters, et al., 2009). The time period from infection until the detection of CRP was dependant on the infectious dose, developing 2 days after infection with a high dose, 3 days after an intermediate dose and 4 days after the low dose was injected. Interestingly, the CRP concentration was comparable between different groups (divided according to the inoculated dose of parasitized red blood cells), and there was little change in plasma CRP concentration during the experimental period even after chemotherapeutic cure. This study did show that of all the systemic inflammatory response parameters measured (fever,

thrombocyte and WCC, Ht, creatinin, fibrinogen, coagulation and CRP), the level of plasma CRP was the first to be affected.

Concentration of CRP has been a useful criteria in the classification of effusions in dogs, in differentiating pyometra from cystic endometrial hyperplasia/mucometra, and correlated well with the canine Inflammatory Bowel Disease (IBD) activity index (CIBDAI) and is thus suitable as a laboratory test to assess the effect of therapy in patients with IBD (Parra, et al., 2006; Fransson, et al., 2004; Jergens, et al., 2003). Serum CRP concentrations are elevated in dogs with naturally occurring acute pancreatitis with concentrations decreasing by day 3 (Holm, et al., 2004). C-reactive protein was useful in predicting complete remission status in dogs with multicentric lymphoma treated with cytotoxic drugs, but concentrations were not able to detect or predict relapse (Nielsen, et al., 2007). In diseases with resistance to treatment or prone to relapse, particularly if the diagnosis is invasive, CRP can be used to monitor response to treatment. In a study comparing two different treatment protocols for *Leishmania infantum* infection by measuring serial serum APP concentrations over the treatment course, relapses are predicted by rising concentrations of APP (Martinez-Subiela, et al., 2003). Similarly, relapses with *Trypanasoma brucei* can be detected when CRP concentrations are used to monitor response to treatment in experimentally infected dogs (Ndung'u, et al., 1991). Elevated CRP concentrations are detected as long as the parasite persists, decrease when the parasite is eliminated with chemotherapy, and quickly return to high levels when a relapse infection occurs. Despite being a highly predictive marker for risk of heart failure in humans, CRP lacks sensitivity and specificity for detecting chronic valvular disease in dogs due to the large overlap in CRP concentration ranges of dogs with chronic valvular disease (CVD) and controls. Although dogs with CVD do have increased CRP

concentrations, concentrations are not associated with the presence of heart failure or degree of the murmur (Rush, et al., 2006).

Chapter 2 Study Objectives

2.1 Hypothesis and statement of the problem

A quick, objective way of assessing the disease severity in cases of *B. rossi* infection is necessary. Studies have shown that dogs naturally infected with *B. canis* have significantly elevated concentrations of CRP and the CRP concentrations correlate with severity and decrease sequentially after treatment of the infection (Matijatko, et al., 2002; Matijatko, et al., 2007; Solano-Gallego, et al., 2008). These studies support the hypothesis that the outcome of dogs with canine babesiosis is predicted by the host inflammatory response, much like human SIRS models. C-Reactive protein concentrations have never been measured in *B. rossi* infections in South Africa. Further, the association between CRP concentration and outcome has not been examined before.

The null hypotheses are as follows:

i. Admission CRP concentrations in dogs with babesiosis is not predictive of survival outcome.
ii. Rate of change in CRP concentrations in the first 24-hours is not predictive of outcome.
iii. Admission CRP concentrations are not associated with length of time of hospitalisation.

2.2 Objectives of this study

To measure CRP in order to see if it can be used as a biomarker in *B. rossi* infected dogs to predict outcome (survival) and severity of disease. This may help clients make decisions based on prognosis and enable clinicians to better manage the disease and formulate more accurate cost estimates.

Long-term applications could include monitoring response to treatments, comparing therapies, and identifying patients that need immediate intensive therapy and more vigilant monitoring to prevent complications that could arise from severe complicated babesiosis. Because aspects of canine babesiosis have been suggested as a model for aspects of *Plasmodium falciparum* infection in humans, the results may be of benefit in the human medical field.

2.3 Benefits arising from this project

The investigator hopes to demonstrate how an acute phase protein, as a rapid fully automated test assay, can be used as an early biomarker for severity of canine babesiosis. This may have benefits as a prognosticator in a range of other veterinary and medical conditions that result in a systemic inflammatory response syndrome (SIRS). To the author's knowledge a preliminary investigation on CRP concentrations in naturally occurring canine babesiosis in dogs in Turkey has been conducted, as well as an investigation into the acute phase response in dogs in Croatia naturally infected with *B. canis*. This study specifically also measured sequential decline in concentrations of inflammatory markers including CRP following antibabesial therapy (Ulutas, et al., 2005; Matijatko, et al., 2002; Matijatko, et al., 2007).

Chapter 3 Material and Methods

3.1 Model system

We conducted a prospective cross-sectional observational study.

3.2 Experimental design

This study was approved by the Animal Use and Care Committee and the Research Committee of the University of Pretoria (protocol number V010/07). Seventy-five client-owned dogs that were diagnosed with canine babesiosis and admitted to the Intensive Care Unit (ICU) of the Onderstepoort Veterinary Academic Hospital (OVAH), Faculty of Veterinary Science, University of Pretoria, South Africa between August 2007 and June 2008 were included in the study. Clients signed consent to inclusion of their pets in the study (Appendix A). Diagnosis of canine babesiosis was made on the basis of morphological demonstration of the intra-erythrocytic babesia trophozoite on a thin capillary blood film stained with a Romanowsky stain (Kyro-quick, Kyron Laboratories PTY Ltd, South Africa). Dogs that exhibited concurrent infections on clinical examination or had a history of being treated for an infection in the last 7 days were excluded from the study. Confirmation of infection with *B. rossi* was determined by PCR Reverse Line Blot hybridisation assay. Dogs infected with *B. vogeli*, or concurrent *E. canis* or *Theileria* infection, were excluded. Dogs that were euthanased for a reason other than a grave prognosis were excluded from the study (Fig 1).

Patients received standard care for canine babesiosis, which included antibabesial treatment, and blood transfusions as needed. In addition, any complications were treated at the discretion of the attending clinician. The primary investigator was blinded to the results of the CRP concentrations and parasite PCR/RLB results due to the delay in the processing of these samples. Outcome was recorded as survived and discharged, or died.

3.3 Experimental procedures

The blood samples for measuring CRP concentration and PCR/RLB were collected from the jugular vein by needle venipuncture in serum and EDTA Vacutainer® brand tubes (Beckton Dickinson Vacutainer Systems, UK) respectively, prior to any therapy being instituted. Thereafter serum was collected on a daily basis until discharge or death. Serum samples were allowed to clot at room temperature and then centrifuged at 3000g for 10 min. Serum was stored at –80º C until analysis, which took place within 6 months.

PCR and RLB

DNA was extracted from 200 µl of each whole blood specimen. The QIAmp blood and tissue extraction kit (Qiagen, Hilden, Germany) was used for DNA extractions, following the manufacturer's protocols. The *Babesia / Theileria / Hepatozoon* PCR was performed with primers RLB-F2 (5′ -GAC ACA GGG AGG TAG TGA CAA G-3′) and RLB-R2 (biotin-5′-CTA AGA ATT TCA CCT CTG ACA GT-3′) amplifying a fragment of 460 to 540bp from the 18S rRNA gene spanning the

V4 region (Gubbels et al., 1999; Matjila et al., 2004). The *Ehrlichia / Anaplasma* PCR was performed with the forward primer Ehr-F (5'GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') and Ehr-R (5'-Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3') amplifying a fragment of 460 to 520 bp from the V1 hypervariable region of the 16S SSU rRNA gene (Bekker et al., 2002; Nijhof et al., 2005). The conditions for the PCR included an initial step of 3 minutes at 42º C, 10 minutes at 94ºC, 10 cycles of 94ºC (20s)- 67 ºC (30s)- 72º C (30s), with lowering of annealing step after every second cycle with 2º C (touchdown PCR). The reaction was then followed by 40 cycles of denaturation at 94º C for 30s, annealing at 57º C for 30s and extension at 72º C for 30s. Reverse line blot hybridisation was subsequently conducted on amplified products (*Babesia*, *Theileria*, *Hepatozoon*, *Anaplasma* and *Ehrlichia*) as previously described (Matjila et al., 2004).

CRP Assay

CRP measurements were performed in batches to minimize analytic variation. All the samples were assayed in two batches. C-Reactive protein concentrations in serum samples were measured by an automated human C-Reactive Protein Turbidometric Immunoassay (TIA), previously validated for use in dogs (Bayer CRP TIA, Newbury, UK) (Kjelgaard-Hansen et al., 2003a). The analysis was performed using an automated analyser (Nexct, Alfa Wasserman, Bayer, South Africa) according to the manufacturer's instructions. Haemoglobin concentrations were measured in all serum samples at the Clinical Pathology laboratory at the Onderstepoort Veterinary Academic Hospital. None of the samples exceeded the limit of 10 mg/mL set by the manufacturer of the TIA.

3.4 Observations

The following data was collected (Appendix B):

- Signalment: age, sex and breed of the dog,
- Number of days of symptoms noted by the client prior to presentation,
- The total number of days of hospitalisation until discharge or death,
- A microhaematocrit (Ht) % at admission,
- In-saline agglutination (ISA) test at admission,
- Blood glucose at admission,
- The presence of complications as listed below,
- *E. canis* PCR results,
- The species of *Babesia*,
- CRP concentration at admission and on a daily basis in those patients hospitalised, and
- Outcome: survival, death or euthanased.

Dogs admitted for hospitalisation included those with uncomplicated babesiosis with severe anaemia (haematocrit (Ht) < 15%) or complicated babesiosis. The classification criteria for *B. rossi* infected cases with severe disease included:

- severe anaemia (Ht < 15%);
- acute renal failure (ARF) (urine output < 1ml/kg/hr as measured by indwelling urinary catheterization, persistently elevated serum creatinine concentration despite appropriate fluid therapy);
- cerebral babesiosis (neurological signs in normoglycaemic dogs including inco-ordination that is not weakness, hindquarter paresis, muscle tremors,

nystagmus, anisocoria, intermittent loss of consciousness, seizures, stupor, coma, aggression, paddling and crying);

- icterus indicating hepatopathy with cholestasis (as detected on mucous membrane examination and/or icteric plasma);
- immune-mediated haemolytic anaemia (IMHA) (in saline agglutination positive and/or marked spherocytosis);
- acute respiratory distress syndrome (ARDS) (dyspnoea, adventitious lung sounds, radiological evidence of lung consolidation or edema, and blood gas evidence of ventilation-perfusion mismatch);
- haemoconcentration (haemoglobinuria or haemoglobinemia with high normal or high Ht and normal or low total serum plasma protein);
- pancreatitis (icterus, melena, diarrhea and or vomiting with elevated amylase and lipase concentrations and/or ultrasonographic evidence of acute pancreatitis) and
- coagulopathy (clinically apparent haemorrhages, with prolonged prothrombin time and activated partial thromboplastin time).

3.5 Routine procedures

The following procedures were performed at the outpatient clinic at admission as part of a baseline assessment of all dogs diagnosed with babesiosis: blood smear and parasitaemia score, microhaematocrit, glucose concentration, potassium concentration, in-saline agglutination, and total plasma protein. The results of the microhaematocrit percentage, in saline agglutination test and glucose concentration at admission were recorded for this study. The microhaematocrit value (%) was obtained

by centrifugation of microhaematocrit tubes (Haematospin 1400, Hawksley & Sons Ltd, UK) and reading the value using a microhaematocrit reader (Hawksley & Sons Limited, USA). The ISA test was performed by mixing 1 drop of whole blood with 6 drops of saline and then placing 1 drop of the mixture on a glass slide, covered with a cover slip, and assessing for agglutination both macroscopically and microscopically. Patients were considered positive if red blood cells were seen to agglutinate. Blood glucose concentration was estimated from a venous blood sample using a hand-held glucometer and reagent strips according to the manufacturers recommendation (Ascensia Elite$_{TM}$XL, Bayer Corporation, USA).

3.6 Statistical analysis

Data were analyzed using Stata 10.1 statistical software (StataCorp, College Station, TX, USA). Significance was set at $\alpha = 0.05$. Admission CRP data were assessed for normality using the Shapiro-Wilk test, and median admission CRP concentrations were compared between survivors and non-survivors using the Wilcoxon rank-sum test. The univariable association of admission CRP concentration, sex, age, rectal temperature, glucose, haematocrit and ISA status with outcome were tested using logistic regression. Any of the above predictors showing an association with outcome at $p < 0.25$ was selected for inclusion along with admission CRP concentration in a multiple exact logistic regression model. Exact logistic regression was used because of the small number of mortalities ($n = 11$) in the dataset. The model was then developed by backward elimination, by dropping the least significant predictor and re-running the model until all remaining predictors were significant with $p < 0.05$. A receiver operating characteristic (ROC) curve for the model was

generated and the area under the curve (AUC) was calculated. A ROC curve was also generated for a model with glucose only (the strongest predictor), and AUC was compared between the two curves using the algorithm of DeLong *et al.* (1988). Estimates of sensitivity and specificity at the point where (sensitivity + specificity) was maximized were compared between the curves using Fisher's exact test. A Fisher's exact test was used to compare the survival rates between two groups defined by a cut-off CRP concentration below which 100% survival occurred. Among survivors, multiple regression analysis was used to estimate the association between CRP concentration at admission and length of stay in ICU, adjusting for age and sex. A Wilcoxon rank-sum test was used to compare the median length of stay in ICU between those dogs with an admission CRP concentration below the selected cut-off that predicted 100% survival and those dogs that exceeded this cut-off. Multiple linear regression was again used in the survivors to estimate the association between the magnitude of CRP concentration change over the first 24 hours and length of stay in ICU, adjusting for age and sex.

Chapter 4 Results

4.1 Inclusion and exclusion of patients

The results of 75 dogs were available, 69 of these met the inclusion criteria; 1 dog was euthanased due to other reasons and excluded, 11 dogs died and 57 dogs survived (Fig. 2 and Appendix C).

4.2 CRP concentration and outcome

The CRP concentration on admission in survivors (n = 57) ranged from 3.5 to 220.9 mg/l (median = 97.4; mean ± SD = 107.5 ± 49.5) and in non-survivors (n = 11) from 63.2 to 254.4 mg/l (median = 101.4; mean ± SD = 122.1 ± 64.6) (Fig. 3). The distribution of CRP values amongst the non-survivors only was inconsistent with normality (p = 0.02). At the univariable level, median admission CRP concentration did not differ significantly between survivors and non-survivors (p = 0.71).

4.3 Univariable association of measured parameters at admission with outcome

Measured parameters were investigated regarding their univariable association with outcome in order to include the most significant parameters in a multiple logistic regression model. Using simple logistic regression, CRP concentration was not

significantly associated with mortality (p = 0.39). However, since it was the predictor of interest, it was included in the multiple exact logistic regression model. The other predictors selected for inclusion in the model (and the p-values for their univariable association with outcome) were glucose (p = 0.005), rectal temperature (p = 0.22) and age (p = 0.24). After elimination of non-significant predictors, the final model contained glucose and CRP (Table 2). Mortality was associated with decreased glucose levels (p = 0.0002) and increased CRP levels (p = 0.045) on admission.

4.4 Receiver operator characteristic curve for the final model

The ROC curves for the final model, as well as for a model containing only glucose, are shown in Fig. 4. The AUC of the ROC curves for the two models (0.851 and 0.819 respectively) did not differ significantly (p = 0.52). The optimal probability cut-off points (where Se+Sp is maximized) were 0.16 for the model with glucose and CRP, and 0.23 for the model with glucose only. Sensitivity and specificity at these cut-offs are compared between the two models in Table 3. They did not differ significantly.

4.5 Determination of CRP concentration associated with 100% survival

None of the non-survivors had an admission CRP concentration less than 63.2 mg/l. When an admission CRP concentration cut-off of 60 mg/l was selected, the survival rate for dogs with an admission CRP concentration < 60 mg/l was 100%

(9/9) and those dogs with an admission CRP concentration ≥ 60 mg/l was 81.4 % (48/59). However, these two proportions did not differ significantly (p = 0.34).

4.6 Examination of the association between admission CRP and number of days of hospitalisation

Although there appeared to be some association between admission CRP concentration among the survivors and eventual number of days the dogs were hospitalized (Fig. 5), the multiple regression analysis failed to show a significant relationship between these two variables, adjusting for age and sex (p = 0.65). Similarly, there was no significant association between the magnitude of change in CRP concentration over the first 24 hours from admission, and the number of days of hospitalisation (p = 0.34) (Fig.6). There was also no significant difference in the median number of days hospitalised in those dogs that survived with an admission CRP concentration < 60 mg/l (3 days, range 2 – 4 days; n = 9) compared to those survivors with an admission CRP concentration ≥ 60 mg/l (2 days, range 1 – 6 days; n = 48) (p = 0.25). As expected, the median number of days hospitalised was significantly greater in those dogs with complicated babesiosis (3 days, range 1 – 6 days, n = 24) compared to those dogs with uncomplicated babesiosis (2 days, range 1 – 6 days, n = 33) (p = 0.006).

Predictor	*b**	Odds ratio	95% C.I. (OR)**	*P*
CRP on admission	0.015	1.02	1.00; 1.03	0.045
Glucose on admission	–1.273	0.28	0.11; 0.61	0.0002
Intercept	0.218	–	–	–

* Estimated regression coefficient

** 95% confidence interval for the odds ratio

Table 2. Results of a multiple exact logistic regression model to estimate effects of serum variables on risk of mortality in canine babesiosis patients

	Sensitivity (n = 11)	95% C.I. (Se)	Specificity (n = 57)	95% C.I. (Sp)
Glucose and CRP	0.909	0.587; 0.998	0.764	0.630; 0.868
Glucose only	0.727	0.390; 0.940	0.818	0.691; 0.909
p-value	0.59		0.64	

Table 3. Comparison of sensitivity and specificity of exact logistic regression models to predict mortality in canine babesiosis patients, at optimal cut-off points.

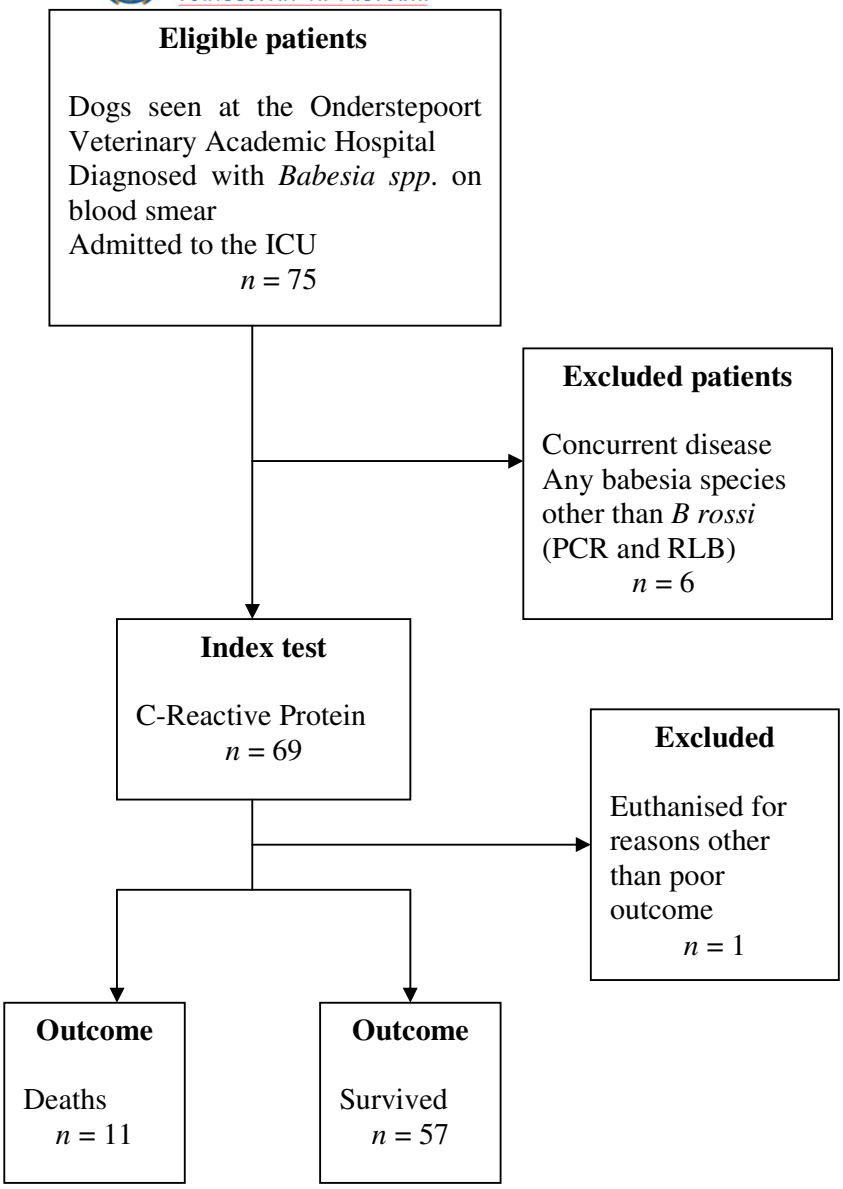

Fig 2. Flow diagram depicting the exclusion criteria for the study. Of the dogs diagnosed to be suffering from canine babesiosis based on blood smear examination, 1 dog was eliminated as it was infected with Distemper virus. PCR and RLB revealed that 5 dogs had babesiosis other than *B. rossi* or concurrent *E. canis*. One dog was eliminated as the reason for euthanasia was due to cost of treatment rather than poor prognosis.

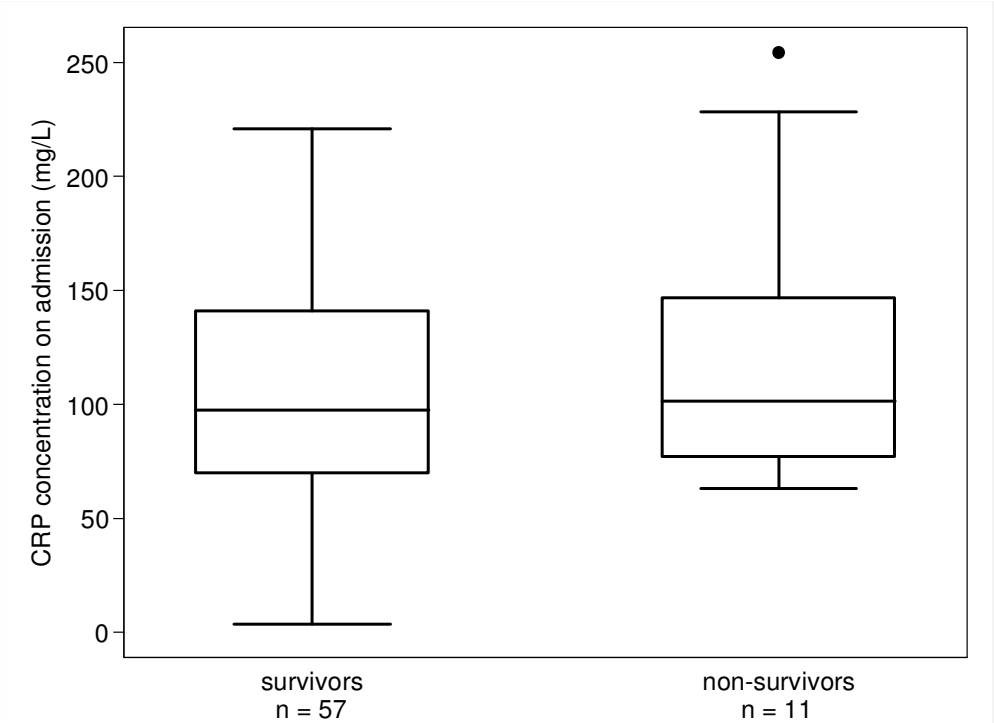

Fig 3. Box plot (representing the interquartile range) showing admission CRP concentrations in dogs with severe canine babesiosis, grouped according to outcome i.e. survivors or non-survivors. The box incorporates the middle 50% of the observations with the line inside the box as the median. The whiskers extend to the smallest (25^{th} percentile) and largest (75^{th} percentile) observations, indicating the range of the data. Outliers, values that are 1.5 times removed from the interquartile range, are plotted separately as dots.

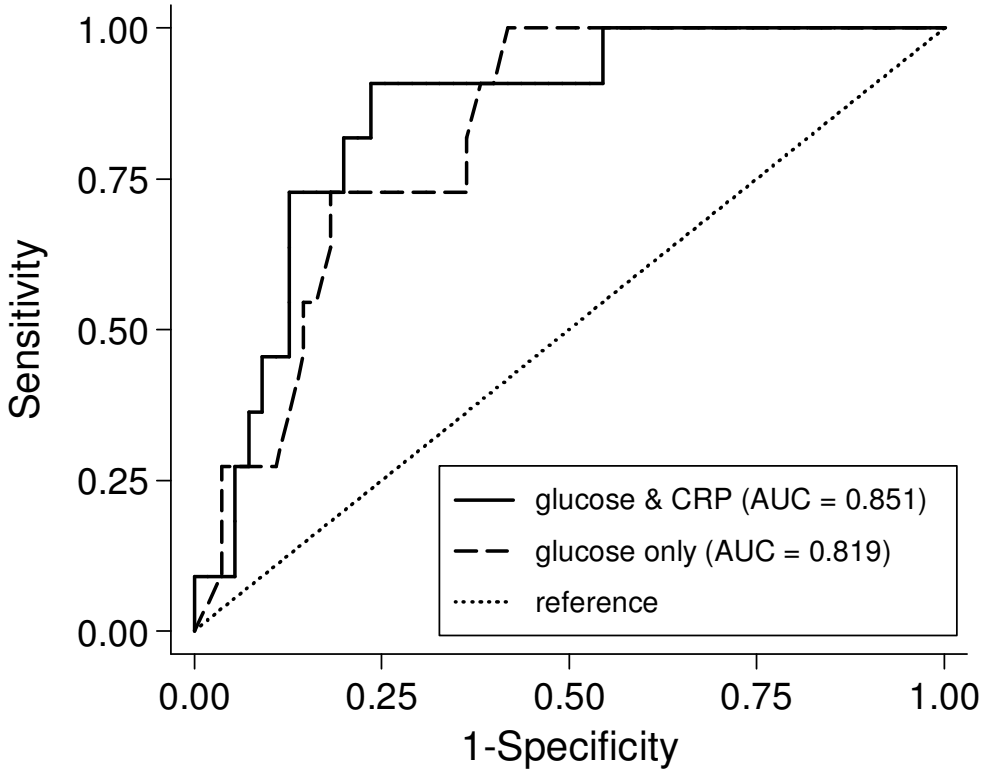

Fig 4. Receiver operator characteristic (ROC) curves for the two logistic regression models examining predictors of mortality in canine babesiosis. The area under the curve (AUC) of the ROC curves for the two models (0.851 and 0.819 respectively) did not differ significantly (p = 0.52). The optimal probability cut-off points (where Se+Sp is maximized) were 0.16 for the model with glucose and CRP, and 0.23 for the model with glucose only.

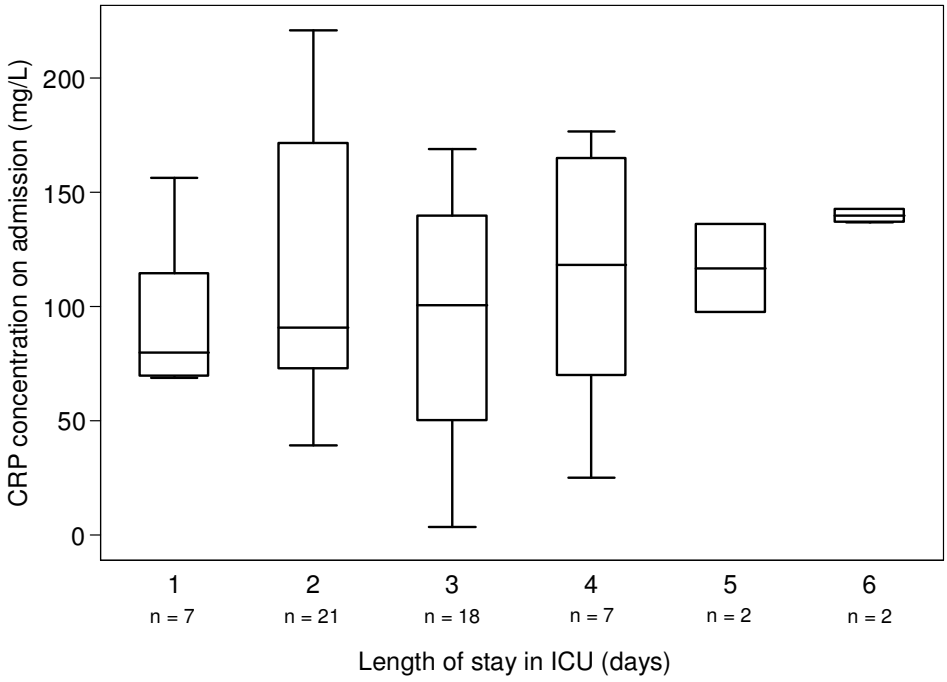

Fig 5. Box plot depicting admission CRP concentrations (mg/l) in dogs with different durations of hospitalisation. See Fig 2 legend for an explanation of interpretation. Although there is an apparent linear correlation between the two variables, there is no significant relationship when examined with a multiple regression analysis ($p = 0.64$), when adjusted for sex and age.

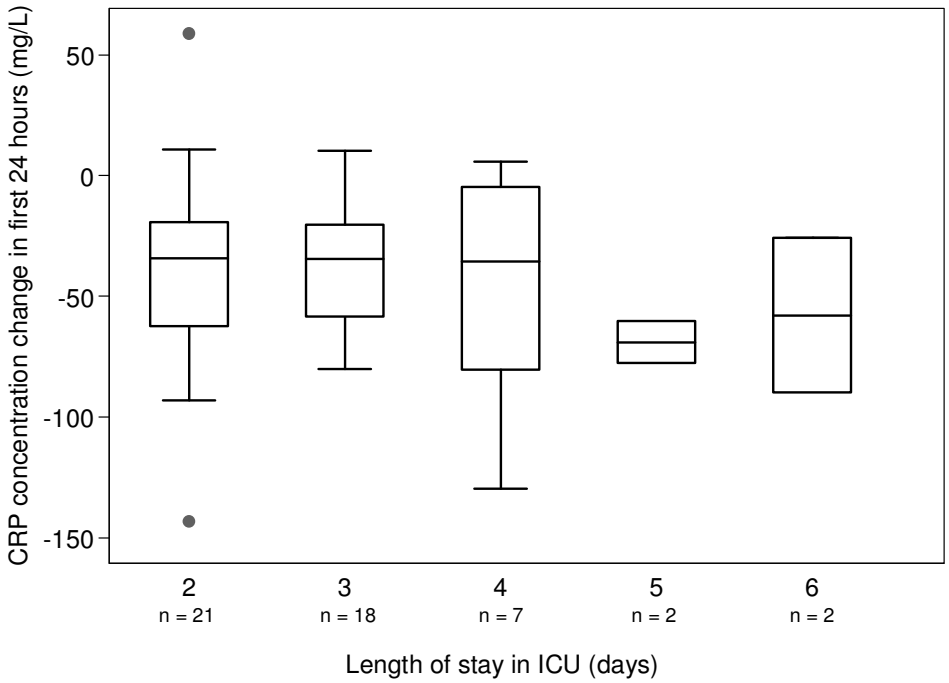

Fig 6. Box plot representing the magnitude of decline (hence the negative values) of CRP concentration (mg/l), in the first 24-hours, for each number of days until discharge. See Fig 2 legend for an explanation of interpretation. A multiple regression analysis failed to show a significant relationship between the magnitude of CRP decline and days hospitalised ($p = 0.34$).

Chapter 5 Discussion

Previous categorisation of canine babesiosis, based on the World Health Organisation classification for malaria (Jacobson and Clark, 1994) is considered artificial and probably unnecessary (Jacobson, 2006), and should be replaced by simple clinical measures such as state of collapse (Böhm et al., 2006), or biomarkers such as lactate and glucose (Nel et al., 2004) and endocrine markers (Schoeman et al., 2007), as these parameters are predictive of outcome. Conditions/diseases where serum CRP has been shown to be a predictor of mortality or morbidity in humans include: CRP was correlated with an increased risk of organ failure and death in a heterogenous human ICU population (Lobo et al., 2003); CRP accurately predicted severity of malaria (Gillespie et al., 1991); CRP accurately predicted survival in humans suffering from non-Hodgkin's lymphoma (Legouffe et al., 1998); and CRP predicted heart-failure associated mortality in the year following acute myocardial infarction (Berton et al., 2003), to name a few. In dogs serum CRP concentration has previously been correlated to disease severity and outcome in several inflammatory conditions, as in the case of canine IBD, where CRP was shown to be positively correlated to disease severity (Jergens et al., 2003), as well as infectious diseases, including dogs in Croatia naturally infected with canine babesiosis (Matijatko et al., 2002). This *B. rossi* study in dogs, examined a well-established predictor of outcome, glucose, together with a previously untested biomarker, CRP. Supporting previous findings, glucose was a good stand-alone predictor of outcome. In this study, no statistically significant difference in admission CRP concentration between survivors and non-survivors could be demonstrated. However, when included in a regression model together with glucose, CRP was significantly associated with outcome. CRP

also improved the predictive ability of the model, albeit not significantly. The reason for the variation in outcome amongst dogs from the same geographic area, naturally infected with the same species of parasite as demonstrated by PCR and RLB hybridization technique, and treated identically with interventional therapy as dictated by the complications that developed, was therefore likely not primarily due to differences in the acute phase response. However a contributory factor for lack of significant predictive value of serum CRP concentrations may be the heterogeneity in terms of breed, sex, level of immunity and clinical presentation in the population of infected dogs in this study. In dogs infected with *B. rossi*, CRP plays the role of an acute phase reactant and an indicator of inflammation rather than a biomarker of disease severity or potential for recovery. This supports the hypothesis that, although inflammatory mechanisms are important in the pathogenesis of babesiosis, tissue hypoxia (Jacobson, 2006; Böhm et al., 2006) and metabolic dysfunction (Nel et al., 2004; Schoeman et al., 2007) play a major role in the disease process and outcome. In this study all non-survivors died within 24 hours of admission, possibly due to the peracute development of the fulminant form of the disease, thus the changes in CRP concentration in non-survivors and survivors could not be compared, as planned.

In diseases with low mortality rates, biomarkers can be used to predict morbidity-based outcome including length of hospitalisation. In dogs diagnosed with immune-mediated haemolytic anaemia, CRP concentration returning to baseline within 30 days of discharge was associated with a favourable outcome (Mitchell et al., 2007). In dogs with neoplastic diseases, a favourable response to therapy was associated with a decrease in CRP concentration (Tecles et al., 2005). C-Reactive protein was associated with SIRS status in dogs diagnosed with pyometra and positively associated with prolonged hospitalisation (Fransson et al., 2007). In this study CRP

concentration at admission or a measure of the decline in concentration in the first 24 hours after admission was not helpful in predicting length of hospitalisation.

This study failed to demonstrate that CRP could predict outcome in dogs infected with *B. rossi*. The reasons may be due to the peracute fulminant nature of *B. rossi* infection once clinical signs develop, which is capable of stimulating the synthesis of impressively high concentrations of CRP in dogs at the time of diagnosis. The dogs in this study already had an overwhelming acute phase response at the time of diagnosis. Schetters *et al.* (2009) has shown that plasma CRP concentrations are increased within a few days of infection, well before the onset of clinical signs thus the acute phase response is maximally stimulated by the time of diagnosis. Death as a result of canine babesiosis in dogs admitted to the OVAH in most cases occurs within 24-hours of admission, this is too short a time period to utilise the half-life of CRP in detecting change in the acute phase response. Other markers, like lactate, glucose and endocrine parameters are obviously more dynamic within that time frame and thus more helpful in prognosticating and monitoring. It has been demonstrated that the cause of death in the peracute phase of canine babesiosis is metabolic, however in disease caused by other species of Babesia and in other septic conditions, CRP has been demonstrated to be an accurate biomarker, highlighting the role of inflammation in disease outcome. The reason may be not that inflammation is unimportant in disease outcome but rather that other inflammatory markers, such as cytokines, may be more accurate in predicting outcome.

Chapter 6 Conclusion

In conclusion, the findings of this prospective cross-sectional study of outcome prognosticators in canine babesiosis caused by *B. rossi* in South Africa support previous identification of glucose being a major predictor of mortality, but it also indicates that the pro-inflammatory marker CRP is an important co-prognosticator.

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Appendices

Appendix A: Client Consent Form

Case number:

Client number:

Consent Form-Canine babesiosis

Your dog has been diagnosed with *B. rossi* infection (babesiosis/ tick fever/ biliary). This is a tick transmitted disease which causes anaemia, in some cases requiring a blood transfusion. The disease may cause complications requiring hospitalization in the ICU department and occasionally death despite intensive treatment and monitoring.

At present we are conducting studies to evaluate the inflammatory protein marker (C-Reactive Protein) in canine babesiosis. This entails the collection of blood samples (± 5ml of blood/ a teaspoon) taken at the time of diagnosis as well as 2ml daily during the stay in hospital. At no time will the studies interfere with the treatment of your pet.

The costs of these tests will not be added to your account. The project funds will pay for the extra tests. However, you will still be responsible for other routine tests and the treatment of your dog.

This study has been passed by the Ethics Committee of the Faculty of Veterinary Science, University of Pretoria.

Thank you for your willingness to allow your animal to be entered into these studies. We hope that the information we gain will improve our understanding and treatment of babesiosis. Should you require more information please contact:

Dr Liza Kőster

Companion Animal Medicine

Onderstepoort Veterinary Academic Hospital

Tel: 529-8288 or 529-8128 or 529-8096

I,…….hereby give permission that my dog (name)……., a (colour)……., (sex)……., (breed)……., may participate in the clinical studies at the Onderstepoort Veterinary Academic Hospital. I understand that the studies will in no way harm my dog. Furthermore I understand that no additional costs will be incurred by me in respect of the trial for blood sampling and testing.

Signed at Onderstepoort on the …….. day of ……2007.

Signature owner/authorised person ……..

Home tel: ……

Work tel: …….

Cell: ……

Geval nommer:

Klient nommer:

Toestemmings vorm – Honde babesiose

U hond is gediagnoseer met *B. rossi* infeksie(babesiose/ bosluiskoors). Dit is ‘n bosluisoorgedraagde siektetoestand wat bloedarmoede veroorsaak, en in sommige gevalle word ‘n bloedoortapping benodig. Die toestand kan komplikasies veroorsaak wat hospitalisasie in ISE vereis en kan soms ly tot dood ten spyte van intensiewe behandeling en monitering.

Ons is tans besig met studies om die inflammasie merkers (C-Reaktiewe Proteïn) te evalueer in honde met bosluiskoors. Dit vereis die neem van bloedmonsters (+- 5ml/ ‘n teelepel bloed) tydens diagnose en 2ml per dag gedurende verblyf in die hospitaal. Neem van hierdie monsters sal op geen stadium inmeng met die behandeling van u hond nie.

Die kostes van hierdie toetse sal NIE by u rekening gevoeg word nie. Die koste van die toetse sal deur die projekfondse gedek word. U sal wel steeds verantwoordelik wees vir al die ander toetse en behandeling van u hond.

Hierdie studie is goedgekeur deur die Etiese Kommitee van die Fakulteit Veeartsenykunde, Universiteit van Pretoria.

Dankie vir u bereidwilligheid om u dier te laat deelneem aan hierdie studie. Ons hoop dat die inligting wat ons hierdeur sal bekom ons kennis oor en die behandeling van bosluiskoors sal verbeter.

Indien u meer inligting wil bekom, kontak my asseblief :

Dr Liza Kőster

Geselskapsdier Geneeskunde

Onderstepoort Veterinere Akademiese Hospitaal

Tel: 529-8288 of 529-8128 of 529-8096

Ek,……gee hiermee toestemming dat my hond (naam)……, 'n (kleur)……, (geslag)……, (ras)……, mag deelneem aan die kliniese studie by die Onderstepoort Veterinere Akademiese Hospitaal. Ek verstaan dat die studie op geen manier skadelik sal wees vir my hond nie. Ek verstaan ook dat geen ekstra kostes by my rekening gevoeg sal word vir die neem van die monsters of die uitvoer van die ekstra toetse nie.

Geteken te Onderstepoort op die …….. dag van ……2007.

Handtekening van eienaar of gemagtigde persoon……

Huis tel: ……

Werk tel: …….

Sel: ……

Appendix B: Data capture form

Case number:

Owner number:

C-reactive Protein in Canine Babesiosis

Date of admission to the OVAH (day 0):

Time of admission:

Signalment

Age:

Sex:

Breed:

History

The number of days the dog has been showing clinical signs of disease:

Type of clinical signs noted by the owner:

Presentation Results

Microhaematocrit (%)	
In saline agglutination (+ or -)	
Blood glucose (mmol/L)	

Observational Study

Complication	**Yes**	**No**	**Not assessed**
[a]Oliguria/anuria			
[b]Respiratory distress*			
[c]Cerebral			
[d]Icterus			
[e]Congested m/m			
[f]Gastro-intestinal			

Extended Data Base (if done)

Clinical chemistry (Tbil, ALT/ALKP, Creat, TSP, alb, glob):

Blood gas:

Thoracic radiographs:

Coagulation tests (PT, PTT, FDP, D-dimer):

Sample	Time interval	Date/Time
EDTA	**Day 0**	
Citrate	**Day 0**	
Serum	**Day 0**	
Serum	**Day 1**	
Serum	**Day 2**	
Serum	**Day 3**	
Serum	**Day 4**	
Serum	**Day 5**	

Outcome

Discharge Date: …….. Time: ……

Survived	Dead

or

Date of Death: ……. Time: …….

or

Date of euthanasia: …… Time: …….

Reason for euthenasia	Cost	Poor Prognosis	Other

[a]Oliguria or anuria: less than 1ml/kg/hr urine output in the presence of normal hydration for > 8 hrs.

[b]Respiratory distress: as a tachypnoea (>30 breaths/minute), dyspnoea, cyanotic mucous membranes, or increased lung sounds.

[c]Neurological diseases in the absence of hypoglycaemia.

[d]Hyperbilirubinaemia assessed on mucosa of gums, conjunctiva or vulva.

[e]CRT < 1.5 s

[f]Pancreatitis suspected if vomiting, diarrhoea, abdominal pain, or melena

Case Number	Age 1	Age 2	Sex	Breed	Days Showing Disease	Type of Clinical Signs Noted	Haemoconcentration	Presenting Ht	Presenting ISA	Hypoglycaemia	Presenting Blood Glucose	Oliguria/Anuria	Respiratory Distress	Cerebral	Icterus	Congested m/m	Gastro-intestinal
1	5	Years	Male	Pug	1	lethargy, collapse, pigmenturia	no	26	Negative	yes	0	No	Yes	No	No	No	No
2	1	Years	Male	Terrier cross	2	annorexia, lethargy	no	6	Negative	no	4	No	No	No	No	No	No
3	3	Months	Male	Boerboel	2	Annorexia, lethargy, diarrhoea	no	14	Negative	no	4.4	No	No	No	No	No	No
4	6	Months	Male	Dalmatian	2	Collapse, annorexia	no	10	Negative	yes	2.4	No	Yes	No	Yes	No	No
5	4	Months	Female	Terrier	1	Collapse, lethargy, annorexia	no	12	Negative	yes	2.3	No	No	No	No	No	No
6	4	Months	Female	Crossbreed	3	and lethargy	yes	30	Negative	yes	3.1	No	No	No	No	No	Yes
7	2	Months	Male	Boerboel	1	lethargy	no	9	Negative	yes	2.2	No	No	No	Yes	No	No
8	6	Years	Male	Boerboel	3	annorexia, lethargy, vomiting	yes	35	Positive	no	8.1	No	No	Yes	Yes	No	No
9	6	Years	Male	Dachshund	5	Lethargy	no	14	Negative	yes	2.6	No	No	No	No	No	No
10	4	Years	Male	Boerboel	0.5	Collapsed	yes	35	Positive	yes	2	Yes	No	Yes	Yes	Yes	No
11	1	Years	Male	Dog	0.5	diarrhoea	yes	54	Negative	yes	2.8	No	No	No	No	No	Yes
12	1	Years	Male	Fox terrier	0.5	Collapse	no	18	Negative	no	5.3	No	No	No	Yes	No	No
14	8	Months	Female	Shar pei	1	Collapsed	no	8	Negative	no	3.7	No	No	No	Yes	No	No
16	1	Years	Female	Terrier	1	Annorexia	no	16	Positive	no	5.5	No	No	No	No	No	No
17	7	Months	Female	Chow chow	2	Annorexia	no	12	Negative	no	4.9	No	No	No	No	No	No
18	2	Years	Male	Doberman	7	Annorexia	no	15	Negative	yes	2.4	No	No	No	No	No	No
19	9	Years	Male	Pug	1	annorexia, dyspnoea	no	8	Negative	yes	2.36	No	Yes	No	Yes	No	No
20	3	Years	Female	Fox terrier	3	annorexia	no	11	Negative	no	7.6	No	No	No	No	No	No
21	3	Years	Female	Dog	1	Annorexia and weakness	no	8	Negative	no	3.9	No	Yes	No	Yes	No	No
22	3	Years	Male	Siberian husky	1	Lethargic, annorexia	no	19	Positive	no	5.5	No	No	No	Yes	No	No
23	2	Years	Female	doberman	1	Lethargy and annorexia	no	25	Negative	no	3.8	No	No	No	No	No	No
24	5	Years	Female	dog	1	Lethargy	no	16	Positive	yes	3.1	No	Yes	No	No	No	No
25	3	Months	Female	breed	2	Annorexia, lethargy	no	17	Negative	no	4.62	No	No	No	No	No	No
26	4	Years	Female	Yorkshire terrier	1	Annorexia, lethargy	no	22	Positive	no	7.5	No	No	No	No	No	No
27	2	Years	Male	Crossbreed	2	Lethargic	no	10	Negative	no	3.7	No	No	No	No	No	No
28	1	Years	Female	American Pitbull	2	Weight loss, annorexia	no	14	Negative	no	4.6	No	No	No	No	No	No
29	2	Months	Female	St Bernard	2	pigmenturia	no	11	Negative	yes	2	No	No	No	No	No	No
30	1	Years	Male	Crossbreed	3	pigmenturia	no	9	Negative	yes	1.7	No	No	No	Yes	No	No
32	3	Years	Male	breed	2	lethargy	no	16	Negative	no	4.7	No	No	No	Yes	No	No
33	3	Months	Male	Boerboel	1	Pigmenturia	no	14	Negative	yes	2	No	No	No	No	No	No
34	2	Years	Male	Yorkshire terrier	1	Vomiting	yes	50	Negative	yes	1.9	Yes	No	No	No	No	No
35	6	Months	Male	Boerboel	1	Collapsed	no	11	Negative	yes	2	No	No	No	Yes	No	No
36	6	Months	Male	Pekegnese	1.5	weakness	no	14	Negative	no	4.6	No	No	No	No	No	No
37	1	Years	Female	Terrier	1	Depressed	no	9	Negative	yes	2.1	No	Yes	No	No	No	No
38	6	Years	Female	dog	1	Collapse	no	15	Negative	yes	3.1	No	No	No	No	No	No
39	2	Years	Female	Crossbreed	4	Depression	no	11	Negative	yes	2.6	No	No	No	No	No	No
40	7	Years	Male	Dog	1	Annorexia	yes	46	Positive	no	3.7	No	No	No	No	Yes	No
41	3	Months	Female	Rottweiller	1	collapsed	no	17	Negative	no	5.7	No	No	No	No	No	No
42	2	Months	Male	Chow chow	1	Collapse	no	6	Negative	yes	1.5	No	No	Yes	No	No	No
43	3	Years	Female	Boxer	0.5	Collapse	yes	41	Negative	no	3.4	No	Yes	Yes	No	Yes	No
44	4	Years	Female	Labrador retriever	0.5	Trembling	no	14	Negative	no	3.5	No	No	No	No	No	No
45	6	Months	Male	Chow chow	4	Collapse	no	8	Negative	yes	1.5	No	No	Yes	Yes	No	No
46	2	Years	Female	Crossbreed	7	distended abdomen	no	11	Negative	yes	3	No	No	No	No	No	Yes
47	4	Years	Male	Crossbreed	2	annorexia	no	16	Positive	no	5.4	No	No	No	No	No	No
48	5	Years	Male	Boerboel	14	weight loss	no	15	Negative	no	3.7	No	No	No	Yes	Yes	Yes
49	15	Years	Female	Fox terrier	2	abdomen	no	15	Negative	no	4.5	No	No	No	Yes	No	No
50	5	Months	Female	Boerboel	1	lethargy, collapse, pigmenturia	no	14	Negative	no	6.1	No	No	No	Yes	No	No
51	11	Months	Male	Terrier	1	annorexia	no	16	Positive	no	5.3	No	No	No	No	No	No
52	11	Months	Male	Terrier	2	annorexia, lethargia	no	20	Negative	yes	2.7	No	No	No	Yes	No	No
53	10	Years	Female	Spaniel cross	0.5	annorexia, lethargy, depressed	no	25	Negative	no	3.6	No	No	No	Yes	No	No
54	11	Months	Male	Whippet	0.5	collapsed	no	10	Negative	no	4.4	No	No	No	Yes	No	No
58	8	Years	Female	Maltese cross	1	annorexia, weakness	no	11	Negative	no	3.6	No	No	No	No	No	No
59	2	Years	Male	Crossbreed	3	Lethargy	no	10	Positive	yes	1.2	No	No	No	No	No	No
60	7	Months	Female	Dachshund	5	Weight loss, lethargy	no	24	Negative	no	3.9	No	No	No	Yes	No	No
61	11	Months	Male	Dog	3	Collapse and annorexia	no	14	Negative	no	4.8	No	No	No	No	No	No
62	2	Years	Male	Saint Bernard	1	Weakness	no	12	Positive	yes	1.8	No	No	No	No	No	No
63	11	Months	Female	Crossbreed	3	Annorexia, lethargy, icteric	no	8	Positive			No	No	No	Yes	No	No
65	7	Years	Male	Doberman	1	Lethargy, annorexia	no	25	Negative	no	4.4	No	No	No	No	No	No
66	10	Months	Male	Husky	0.5	Collapse, seizures	no	8	Negative	yes	1.8	No	No	No	No	No	No
67	4	Years	Male	Terrier	7	Innapetant	no	15	Negative	no	3.7	No	No	No	No	No	No
68	3	Years	Female	Boerboel	1	Lethargy	no	27	Negative	no	3.3	No	No	No	Yes	No	No
69	6	Months	Female	breed	0	None-picked up incidentally	no	32	Negative	no	4.4	No	No	No	No	No	No
70	10	Years	Male	Crossbreed	2	Lethargy, annorexia	no	16	Negative	no	3.4	No	No	No	Yes	No	No
71	3	Months	Female	Labrador retriever	2	Lethargy, annorexia, collapse	no	6	Negative	yes	1.2	No	No	No	Yes	No	No
72	8	Years	Male	Boerboel	7	depression	no	24	Negative	yes	2.3	No	No	No	Yes	No	No
73	2	Years	Male	Bull terrier	1	Weakness, annorexia	no	28	Positive	no	3.9	No	No	No	No	No	No
74	1	Years	Male	Pekegnese	5	Collapse, annorexia	no	9	Positive	no	3.9	No	No	No	No	No	No
75	2	Years	Female	Terrier	2	Collapse	no	8	Negative	yes	1.2	No	No	No	No	No	No

Case Number	[CRP] Day 0 (mg/l)	[CRP] Day 1 (mg/l)	[CRP] Day 2 (mg/l)	[CRP] Day 3 mg/l	[CRP] Day 4 mg/l	[CRP] Day 5 (mg/l)	Outcome	Reason for Euthanasia
1	136.00	75.80	53.50	43.70	39.90	0.00	Survived	
2	139.90	75.50	54.00	0.00	0.00	0.00	Survived	
3	171.60	109.10	0.00	0.00	0.00	0.00	Survived	
4	94.50	14.30	31.90	41.50	0.00	0.00	Survived	
5	41.30	100.20	0.00	0.00	0.00	0.00	Survived	
6	77.50	0.00	0.00	0.00	0.00	0.00	Survived	
7	97.30	17.30	6.70	0.00	0.00	0.00	Survived	
8	97.40	19.50	53.20	43.50	36.80	0.00	Survived	
9	87.20	51.50	0.00	0.00	0.00	0.00	Survived	
10	101.40	0.00	0.00	0.00	0.00	0.00	Dead	Poor Prognosis
11	194.10	101.20	0.00	0.00	0.00	0.00	Survived	
12	44.50	6.80	32.00	0.00	0.00	0.00	Survived	
14	114.60	0.00	0.00	0.00	0.00	0.00	Survived	
16	176.60	47.00	67.00	40.40	0.00	0.00	Survived	
17	68.70	0.00	0.00	0.00	0.00	0.00	Survived	
18	136.90	46.80	123.20	179.20		169.00	Survived	
19	77.60	0.00	0.00	0.00	0.00	0.00	Dead	
20	186.00	96.70	0.00	0.00	0.00	0.00	Survived	
21	118.10	82.60	97.80	55.10	0.00	0.00	Survived	
22	142.70	84.10	65.00	0.00	0.00	0.00	Survived	
23	138.70	85.00	53.70	0.00	0.00	0.00	Survived	
24	141.00	89.80	48.50	0.00	0.00	0.00	Survived	
25	114.10	124.40	96.50	0.00	0.00	0.00	Survived	
26	72.90	42.60	0.00	0.00	0.00	0.00	Survived	
27	69.40	0.00	0.00	0.00	0.00	0.00	Survived	
28	96.50	0.00	0.00	0.00	0.00	0.00	Survived	
29	38.00	9.30	4.00	0.00	0.00	0.00	Survived	
30	24.90	10.70	12.60	20.90	0.00	0.00	Survived	
32	54.00	33.60	24.20	0.00	0.00	0.00	Survived	
33	94.40	0.00	0.00	0.00	0.00	0.00	Dead	
34	168.90	94.10	52.30	0.00	0.00	0.00	Survived	
35	69.70	75.50	53.10	31.30	0.00	0.00	Survived	
36	220.90	77.70	0.00	0.00	0.00	0.00	Survived	
37	64.90	0.00	0.00	0.00	0.00	0.00	Dead	
38	105.40	71.20	0.00	0.00	0.00	0.00	Survived	
39	64.40	54.80	0.00	0.00	0.00	0.00	Survived	
40	156.40	0.00	0.00	0.00	0.00	0.00	Survived	
41	163.50	110.00	62.20	40.70	0.00	0.00	Survived	
42	76.90	0.00	0.00	0.00	0.00	0.00	Dead	
43	254.40	234.00	0.00	0.00	0.00	0.00	Dead	
44	121.30	92.50	0.00	0.00	0.00	0.00	Survived	
45	105.50	0.00	0.00	0.00	0.00	0.00	Dead	
46	119.20	130.10	0.00	0.00	0.00	0.00	Survived	
47	103.80	69.20	49.40	0.00	0.00	0.00	Survived	
48	82.60	78.90	49.70	0.00	0.00	0.00	Survived	
49	142.70	117.00	98.60	70.10	58.10	52.60	Survived	
50	131.80	66.00	51.80	0.00	0.00	0.00	Survived	
51	66.20	44.90	14.90	0.00	0.00	0.00	Survived	
52	150.60	127.70	40.70	0.00	0.00	0.00	Survived	
53	228.20	164.10	161.50	0.00	0.00	0.00	Dead	
54	44.00	41.00	31.30	0.00	0.00	0.00	Survived	
58	84.70	39.70	0.00	0.00	0.00	0.00	Survived	
59	85.20	8.40	0.00	0.00	0.00	0.00	Survived	
60	60.90	54.30	0.00	0.00	0.00	0.00	Survived	
61	79.80	0.00	0.00	0.00	0.00	0.00	Survived	
62	89.20	56.40	0.00	0.00	0.00	0.00	Survived	
63	188.20	102.20	0.00	0.00	0.00	0.00	Survived	
65	201.10	181.80	0.00	0.00	0.00	0.00	Survived	
66	165.10	160.50	64.40	41.90	0.00	0.00	Survived	
67	90.70	64.00	0.00	0.00	0.00	0.00	Survived	
68	63.20	37.20	0.00	0.00	0.00	0.00	Dead	
69	3.50	0.00	21.50	0.00	0.00	0.00	Survived	
70	50.00	41.00	28.30	0.00	0.00	0.00	Survived	
71	39.30	2.20	0.00	0.00	0.00	0.00	Survived	
72	130.10	0.00	0.00	0.00	0.00	0.00	Dead	
73	135.00	73.70	0.00	0.00	0.00	0.00	Survived	
74	71.70	60.50	0.00	0.00	0.00	0.00	Survived	
75	146.80	0.00	0.00	0.00	0.00	0.00	Dead	Poor Prognosis

Appendix C: Raw Data

Data from 68 dogs included in the study. Of the dogs diagnosed to be suffering from canine babesiosis based on blood smear examination (n = 75), 1 dog was eliminated as it was infected with Distemper virus. PCR and RLB revealed that 5 dogs had babesiosis other than *B. rossi* or concurrent *E. canis*. One dog was eliminated as the reason for euthanasia was due to cost of treatment rather than poor prognosis. Data includes signalment (age, sex, breed), number of days showing disease, clinical signs at admission, haematocrit, presence of hypoglycaemia, glucose concentration, presence of complications (haemoconcentration, IMHA, anuria, respiratory distress, cerebral icterus, congested mucous membranes, gastro-intestinal disease), CRP concentrations, outcome, and reason for euthanasia.