The pathophysiology of renal and cardiac changes in canine babesiosis

By

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DECLARATION

I declare that the thesis herewith submitted by me to the University of Pretoria the degree PhD, has not been submitted previously by me for a degree at any other university.

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ABSTRACT

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This thesis showed that dogs with natural infection with *B. canis* had both renal and cardiac dysfunction, both of which can be classified as complications of babesiosis and would thus necessitate supportive therapy.

This thesis demonstrated that RTE celluria, proteinuria, and variable enzymuria and azotaemia occur in dogs with babesiosis. However, these were all minimal changes and all could be consistent with hypoxia, reduced GFR, or reduced cardiac output. This thesis showed that dogs with naturally occurring babesiosis had significant urine met-haemoglobin with no evidence of blood met-haemoglobin. The possibility would be that the urinary met-haemoglobin was either produced in the kidney or possibly by oxidation of haemoglobin to met-haemoglobin in the bladder. It has been shown experimentally that met-haemoglobin can be toxic. The combination of reduced GFR, anaemic hypoxia, and met-haemoglobin can all act synergistically to cause renal damage. Renal haemodynamics are also much more likely to be abnormal when cardiac dysfunction is present. Reduced renal blood flow and glomerular filtration rate are evidence of redistribution of blood flow that commonly occurs in early heart failure. An important finding in this thesis was that dogs with babesiosis had lower serum sodium than control dogs but there was no difference between mild, severe, or complicated cases of babesiosis. In addition, dogs with babesiosis had a lower fractional clearance of sodium than clinically healthy control dogs, which can be interpreted as sodium retention by the kidneys. This sodium retention would also result in water retention, which will result in an expansion of the plasma volume.

In the past heart lesions in canine babesiosis were regarded as rare complications, with the majority of lesions being reported as incidental findings at post-mortem examination of complicated babesiosis cases. This thesis has demonstrated that cardiac lesions to be common in canine babesiosis.

This thesis showed that that ECG changes in babesiosis were similar to the pattern described for myocarditis and myocardial ischaemia, and together with the histopathological findings indicated that the heart suffers from the same pathological processes described in other organs in canine babesiosis, namely inflammation and hypoxia. As the clinical application of the ECG changes found in this thesis was limited, cardiovascular assessment should be based on functional monitoring rather than ECG. Using cardiac troponin as a marker of myocardial injury, this thesis showed that myocardial cell injury occurs with canine babesiosis. Cardiac troponins, especially troponin I, are sensitive markers of myocardial injury in canine babesiosis, and the magnitude of elevation of plasma troponin I concentrations appears to be proportional to the severity of the disease. ECG changes and serum cardiac troponin were correlated with histopathology. On cardiac histopathology from dogs that succumbed to babesiosis, haemorrhage, necrosis, inflammation and fibrin microthrombi in the myocardium were documented, all of which would have resulted in ECG changes and elevations in cardiac troponin. Myocardial infarction causes left ventricular failure, which will result in hypotension and an expansion of the plasma volume due to homeostatic mechanisms.

This thesis showed that dogs with babesiosis had hypoalbuminaemia, which may be because of intravascular volume dilution due to fluid retention. In disease hypoalbuminaemia can occur as a negative acute-phase protein. In the light of the cardiac changes, hyponatraemia, and hypotension, a probable cause would be fluid retention due to myocardial disease. This thesis showed that dogs with babesiosis had left ventricular lesions, which can result in systolic heart failure.

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CHAPTER 1

AN INTRODUCTION TO CANINE BABESIOSIS

Canine babesiosis is a tick-borne disease caused by haemoprotozoan parasites of the genus *Babesia*[128], named after Dr Victor Babes, who in 1887 established the aetiology of the cattle disease in Romania. The first report of canine babesiosis was in South Africa in 1885 by Hutcheon[86]. The parasites were only recognised by Purvis and Koch, in 1896 and 1897, respectively, and Spreull successfully transmitted the disease in 1899[25,195]. Babesiosis primarily affects erythrocytes but can involve multiple organs and can range from a relatively mild to a fatal peracute disease[26,202]. Although haemolytic anaemia is the hallmark of infection, numerous variations and complications can occur[125].

CAUSATIVE AGENT

The parasites *Babesia canis* and *B. gibsoni* (phylum Apicomplexa) are responsible for canine babesiosis throughout the world. *Babesia canis* was previously described as a group of three biologically different subspecies, namely *B. c. canis*, *B. c. vogeli*, and *B. c. rossi*[216], which has been confirmed with PCR using restriction fragment length polymorphism analysis[33,55]. Using a phylogenetic approach the three subspecies of *B. canis* belong to the clade of *Babesia* species *sensu stricto* where *B. c. canis* clusters with *B. c. rossi* whereas *B. c. vogeli* may form a monophyletic group with *B. divergens* and *B. odocoilei*[33]. The proposed name for this group is the Babesids[45]. Recently a *B. canis* was identified from a cat, which on PCR sequences of the internal transcribed and 5.8S rRNA regions of the ribosomal operon used for sub-speciation of *B. canis* was markedly different from the three recognized subspecies of *B. canis*[12]. This novel feline genotype of *B. canis* has been proposed to be a new subspecies, named *B. canis* subspp. *presentii*[12].

The small babesias in dogs are generally considered to be *Babesia gibsoni*[26,202] and using PCR, this group has been further subdivided into 2 subspecies: the North American and Asian subspecies[230]. However, in one dog naturally infected with a small *Babesia*, it was shown that it was distantly related to *B. gibsoni*, but more closely related to *B. microti*, *B. rodhaini*, and *Theileria equi*[229]. It is therefore likely that the small *Babesia* fauna occurring in dogs is more diverse than has been assumed so far.

Babesia c. canis is found in Europe, *B. c. vogeli* in northern Africa, North America and South Africa[131] and *B. c. rossi* in southern Africa[131,202,216]. The pear-shaped trophozoite of *B. canis* measures 4-5 µm long and is usually found in pairs within the erythrocyte, but up to eight or more may be present[26,202]. In comparison, *B. gibsoni* is much smaller; is round to oval in shape; measures 3 µm long; and is found in Asia, Australia, North America and northern and eastern Africa[26,96,150,202].

Schizogony occurs in the gut epithelial cells of the adult tick and results in the formation of large merozoites. The merozoites then undergo successive cycles of schizogony within various cell types, including oocytes. In the salivary glands, schizogony results in the formation of small, infective sporozoites. After the tick has attached to a canine host and feeds, sporozoites in the tick's saliva enter canine erythrocytes with the aid of a specialized apical complex. Once inside the erythrocyte, the merozoite transforms into a trophozoite, from which additional merozoites develop by a process of merogony. These merozoites leave the cell to enter other erythrocytes. Trans-ovarial transmission can occur[30], and it is believed that ticks can remain infective for several generations[195].

EPIDEMIOLOGY

Babesiosis is solely a tick-borne disease, with *B. c. canis* being transmitted by *Dermacentor reticulatus*, *B. c. vogeli* and *B. gibsoni* by *Rhipicephalus sanguineus*, and *B. c. rossi* by

Haemaphysalis leachi[26,202,216]. In experimental studies, *Hyalomma marginatum* and *Dermacentor andersoni* have been shown to transmit *B. canis*[216].

Babesia canis can infect dogs of all ages, although in a survey from the Onderstepoort Veterinary Academic Hospital (OVAH), 77% of infected dogs were younger than 3 years of age[220]. Other canids (e.g., wild dogs, jackals, and wolves) are also susceptible[26]. A seasonal variation in the number of cases has been reported at the OVAH[186], with a higher incidence in the summer months (September to April in South Africa) and peaking in November. This seasonal variation has also been described in North America, with most cases of babesiosis occurring between March and October[113]. The source of infection is either carrier ticks or ticks feeding on dogs that are sick or are incubating the disease and then feeding on a susceptible dog[26]. Other possible sources of infection are carrier dogs and blood transfusions[26,113,202].

According to reports, seroprevalence of *B. canis* in greyhounds from the southeastern United States is higher than that of the general pet population[203]. In California, up to 13% of dogs in animal shelters are seropositive to *B. canis* and 3% positive to *B. gibsoni*[226]. These dogs could act as reservoirs for the disease[203].

PATHOGENESIS

The incubation period following tick exposure is 10-21 days[26]. Dogs experimentally inoculated with *B. canis*-infected blood showed transient parasitaemia on Day 1 after inoculation. Organisms then disappeared from peripheral blood for about 10 days. A second more intensive parasitaemia developed after 2 weeks and peaked around Day 20 after inoculation[26].

Intra-erythrocytic parasitaemia causes both intravascular and extravascular haemolysis, which can result in regenerative anaemia, haemoglobinaemia, haemoglobinuria, and bilirubinuria[26,122]. By the parasites invading and replicating in the erythrocyte, babesiosis results in destruction of the erythrocyte with the development of anaemia and consequently hypoxia. The destruction of the erythrocyte is multi-factorial, including direct parasite damage to the erythrocyte membrane, splenic removal of damaged and parasitized erythrocytes, complement activation, and the presence of anti-erythrocyte antibodies, which result in a secondary immune-mediated haemolytic anaemia.

Pyrexia also can develop and is attributed to the release of endogenous pyrogens from erythrolysis, parasite destruction and activation of inflammatory mediators[26,88]. Splenomegaly can occur from hyperplasia of the mononuclear phagocytic system[26]. The haemolytic crisis that develops results in anaemic hypoxia, anaerobic metabolism and metabolic acidosis[29]. In addition, the remaining haemoglobin does not function optimally, especially at the tissue level, which exacerbates the anaemic hypoxia[206]. The reason for suboptimal functioning of the haemoglobin is that the haemoglobin dissociation curve in dogs with babesiosis is displaced to the right and the carboxyhaemoglobin fraction increases[206].

The varied clinical manifestations of canine babesiosis are difficult to relate to an organism restricted to the erythrocyte. The various complications probably result from an unfocused and excessive inflammatory response rather than from the parasite itself[88]. Although the clinical manifestations are diverse, the mechanisms promoting them are more uniform.

The systemic inflammatory response syndrome (SIRS) is caused by excessive release of inflammatory mediators[36,170]. This syndrome is broadly defined and considered to be present if two or more of the following clinical findings occur: tachycardia, tachypnoea or

As previously stated, babesiosis is associated with haemolysis, damage to vascular endothelium, acidosis, hypoxia, vascular stasis, shock and possibly an endotoxaemic-like state. All of these conditions predispose patients to disseminated intravascular coagulation (DIC)[198].

Based on underlying mechanisms and poor response to diuretic therapy, the pathophysiology for acute respiratory distress syndrome (ARDS) is probably increased alveolar capillary permeability because of a SIRS reaction[88]. Increased pulmonary hydrostatic pressure could also play a role because myocardial damage may be present[148]. Acidosis, which is common in severe cases of babesiosis[28,29,108], results in increased pulmonary vascular resistance and reduced pulmonary function and may play a role in respiratory dysfunction[219].

CLINICAL MANIFESTATIONS

Canine babesiosis can be clinically classified as uncomplicated or complicated[88]. Typical signs of uncomplicated babesiosis are acute haemolysis, fever, anorexia, depression, pale mucous membranes, splenomegaly, and a waterhammer pulse[26,123,202]. Uncomplicated babesiosis is further divided into mild, moderate, or severe disease, depending on the severity of anaemia[88]. Mild uncomplicated babesiosis can progress to severe uncomplicated disease, in which anaemia can become life–threatening (a haematocrit below 15% and sometimes as low as 5%). Complicated babesiosis involves clinical manifestations that are unrelated to haemolytic disease. The more commonly encountered complications are ARF, cerebral babesiosis, coagulopathy, icterus and hepatopathy, immune-mediated haemolytic anaemia (IMHA), peracute babesiosis, ARDS, haemoconcentration, hypotension, myocardial pathology, pancreatitis, and shock. Rare complications include gastrointestinal disturbances[123], myalgia[123,124], ocular involvement[160], upper respiratory signs[124], cardiac

involvement[148], necrosis of the extremities[32], fluid accumulation[124], and chronic disease[124]. Different complications can overlap.

Acute renal failure

Dogs with ARF typically have anuria or oliguria; however, ARF is an uncommon complication of canine babesiosis[19,28,88]. In some dogs, babesiosis may precipitate acute decompensation of underlying, pre-existing renal disease[127]. Evidence of renal damage is reflected on urine analysis by the presence of proteinuria, casts, and renal tubular epithelial cells. Renal damage is common in both complicated and uncomplicated babesiosis but does not necessarily cause renal failure[127,148]. An elevated serum urea alone is an unreliable indicator of renal insufficiency because a disproportionate rise in urea (compared with creatinine) has been related to catabolism of lysed erythrocytes[173]. Renal failure is diagnosed by ongoing evaluation of urine volume, urine analysis and degree of azotaemia[19].

Cerebral babesiosis

Cerebral babesiosis signals the concurrent presence of various neurologic signs. Onset is typically peracute with clinical signs including a combination of incoordination, hindquarter paresis, muscle tremors, nystagmus, anisocoria, intermittent loss of consciousness, seizures, stupor, coma, aggression, paddling or vocalisation[88,123]. Pathological changes in the brain include congestion, macroscopic and microscopic haemorrhaging, sequestration of parasitized erythrocytes in capillary beds[158], and pavementing of parasitized cells against the endothelium. The latter implies that parasitized cells are adherent to the endothelium, although a mechanism for adherence has not been identified[88]. It is speculated that there are receptors on the endothelial surface that facilitate the adherence.

Coagulopathy

The most consistent haemostatic abnormality is profound thrombocytopenia, which is a routine finding in both complicated and uncomplicated babesiosis; clinically apparent haemorrhages are rare[1,31,148,159,174]. Despite reports of babesiosis cases with DIC[148], definitive clinical investigations of the presence or absence of DIC in cases of complicated babesiosis have not been done. Confirmation of DIC is difficult because of the nature of the underlying disease process and the reported unreliability of the human fibrin degradation products test[88]. Clinical signs of DIC are difficult to recognize until haemorrhaging develops in the hypocoagulable phase[148,198]. In the hypocoagulable phase, signs are related to microthrombi-induced organ dysfunction, particularly the kidneys, lungs, liver, gastrointestinal tract and central nervous system[148,198].

Icterus and hepatopathy

Icterus can occur in advanced cases[123,125]. Recovery may be delayed in dogs with concomitant icterus[88]. In addition, hypokalaemia is often present. The possibility of hepatic impairment should, however, always be considered. The degree of functional impairment, hepatocelluar damage, and bile stasis all correlate with the degree of icterus observed[125,126]. Histological studies of hepatic specimens may reveal changes from centrilobar congestion to necrosis[74].

In some cases of babesiosis, icterus, elevated liver enzymes, and elevated bile acids occur, which are indicative of a liver insult[139]. Whether the insult is due to inflammatory cytokines, hypoxic damage, or a combination of these is not known. The icterus seen does not solely appear to be due to haemolysis or post-hepatic obstruction. Therefore, liver dysfunction appears to be, at least, contributory. Histopathological changes usually associated with icterus include both diffuse and peri-portal lesions, whereas icteric dogs with babesiosis show a centrilobular lesion. It is possible, however, that the liver has a diffuse mild to

moderate lesion that is insufficient to cause histopathological changes but severe enough to cause a functional change. Hypoxic insults are known to cause diffuse hepatocellular swelling and thus the hypoxia in severe babesiosis may be severe enough to cause a transient hepatopathy.

Immune-mediated haemolytic anaemia

Immune-mediated haemolysis is an increased destruction of erythrocytes caused by erythrocyte membrane-associated antibodies, which can be either primary (in which the membrane is normal) or secondary (in which the membrane is altered and recognized as foreign)[209]. The cardinal feature of babesiosis-associated IMHA is ongoing haemolysis despite successful antibabesial treatment[43]. Diagnosis is by in-saline agglutination, detection of spherocytosis, or both[43]. The Coombs test (direct antiglobulin test) is not diagnostic. Uncomplicated cases as well as cases complicated with IMHA are Coombs positive as the erythrocyte membrane becomes tagged with complement and immunoglobulins (Unpublished data, Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria).

Acute respiratory distress syndrome

ARDS is a severe, frequently catastrophic complication of babesiosis[88,122]. Clinical indications are a sudden increase in respiratory rate, dyspnoea, moist cough, and blood-tinged frothy nasal discharge[88]. Blood-gas analysis can confirm hypoxaemia. Diagnosis of ARDS is based on the presence of diffuse pulmonary infiltrates in thoracic radiographs, hypoxaemia from ventilation-perfusion mismatch, and normal pulmonary capillary wedge. As blood–gas analysis and compliance cannot be measured in most clinical situations, diagnosis depends on recognizing the risk factors for ARDS, taking thoracic radiographs, and excluding other causes of pulmonary oedema. Fluid loads that can be tolerated by normal dogs may exacerbate fatal pulmonary oedema in dogs with ARDS[71].

Haemoconcentration

The paradoxical phenomenon of severe intravascular haemolysis combined with haemoconcentration constitutes a syndrome dubbed the "red biliary syndrome"[88]. Clinical features are congested mucous membranes, visible haemoglobinaemia or haemoglobinuria, and high-normal or elevated haematocrit[88,122,174]. Haemoconcentration has been associated with other complications, such as cerebral babesiosis, DIC, ARF and ARDS[148,174]. In dogs with babesiosis, haemoconcentration may be caused by reduction in blood volume because of fluid shifting from the vascular compartment to the extravascular compartment[88]. Because plasma protein concentrations remain normal, plasma rather than a filtrate of plasma is shifted from the vasculature[88]. The widespread increase in capillary permeability that occurs with SIRS[19] may play an important role in the pathogenesis[88].

Shock

Dogs with severe and complicated babesiosis are frequently presented in a state of collapse and clinical shock. Shock in these animals can resemble the hyperdynamic phase of septic shock. Collapsed dogs with babesiosis may not display classic signs of shock syndrome[19,29], partially because of the haemodynamics of haemolytic anaemia[88]. The pulse may be bounding or weak; temperature elevated or subnormal; and mucous membranes pale, icteric or congested (haemoconcentration)[19,29,88]. Babesial shock, like endotoxic shock, may pass through a hyperdynamic stage followed by a hypotensive stage[88]. Hypotension was initially reported in one case[69]. In a recent study[91], it was shown that hypotension occurs frequently in babesiosis and that the presence and severity of hypotension increases with increased disease severity. The presence of hypotension in a large proportion of dogs with complicated babesiosis is consistent with the hypothesis that inflammatory mechanisms play a major role in this disease, and can result in a sepsis-like state. It is likely that hypotension in babesiosis is a combination of vasodilation, reduced vascular volume due to increased vascular permeability and/or dehydration, and myocardial depression. Hypotension can play a role in

the pathophysiology of the disease as it has been hypothesized to facilitate parasite sequestration.

Acute pancreatitis

Gastrointestinal disturbances have generally been considered a rare complication of babesiosis; however, the aetiology of the reported gastrointestinal disturbances may have been acute pancreatitis. Pancreatic inflammation can extend to the adjacent stomach, duodenum, and ascending and transverse colon, accounting for the gastrointestinal clinical signs seen in acute pancreatitis. Digestive system abnormalities reported as a complication of canine babesiosis have included vomiting, diarrhoea, abdominal pain, gastritis, enteritis, and enterorrhagia. A recent study[144] documented 23 dogs that developed pancreatitis as a complication of babesiosis. This was an incidence of 1.8% amongst hospitalised babesiosis cases. Median time of diagnosis based on serum amylase and lipase activities was 3 days post-admission. No sex predilection was identified, with primarily young, sexually intact dogs being affected. The severity of anaemia did not correlate with the severity or incidence of pancreatitis. The most common clinical signs of acute pancreatitis were anorexia, vomiting, melaena, and abdominal pain. Hypokalaemia was commonly identified, especially in icteric dogs. This newly recognized development of acute pancreatitis as a sequel to canine babesiosis influences the treatment regimens of the affected dogs, incurs additional treatment costs, results in prolonged hospitalisation, and worsens patient prognosis. Early identification of these clinical signs in a dog with babesiosis should increase the clinician's index of suspicion for acute pancreatitis, with timeous diagnostic procedures and therapeutic intervention instituted.

CLINICAL PATHOLOGY

The primary haematological abnormalities in canine babesiosis are regenerative anaemia, thrombocytopenia, and leukocytosis[202]. The haematological profile evidently varies by

geographic location as different subspecies of *B. canis* may be involved. In a report from France[159], mild anaemia and thrombocytopenia were the only abnormalities. In 291 canine babesiosis cases in the Phillippines[31], the only abnormality was mild anaemia. In one Nigerian study[1], 70 cases were classified into four groups: peracute, acute, chronic and subclinical. The peracute group showed severe anaemia, neutrophilia, lymphocytosis, and eosinopenia; the acute and chronic groups had moderate anaemia; but only mild anaemia was present in the chronic group. In a survey of 364 cases at the Onderstepoort Veterinary Academic Hospital (OVAH)[174], severe anaemia, neutrophilia, monocytosis, eosinopenia, and thrombocytopenia were recorded. Thus the most remarkable differences observed in the three surveys were degree of anaemia, macrocytosis (representing reticulocytosis), and leukocytosis in general but particularly neutrophilia. These surveys apparently supported the contention that the parasite strains in France and the Philippines are either the same or very similar. Furthermore, the strains in these two countries are considerably less virulent than those found in South Africa[174]. Data in the Nigerian report[1] may represent two distinct parasite strains; one causing very similar haematological changes to those reported at the OVAH (cases classified as peracute and acute) and one producing little haematological response (cases classified as chronic and subclinical). In the data from the Nigerian study both *B. c. rossi* and *B. c. vogeli* may have been represented in the data[1]. A leukemoid response has also been described[114].

Urine analysis may show bilirubinuria, haemoglobinuria, proteinuria, granular casts, and renal tubular epithelial cells[202]. Alterations in biochemical parameters vary and depend on the severity of the case[26]. Typically, uncomplicated cases have no biochemical changes. Elevated liver enzymes may, however, be evident in the presence of anaemic hypoxia[126]. The hypokalaemia reported in more severely affected animals could be attributed to decreased potassium intake[202], excess renal excretion, gastrointestinal loss, or cellular shifts[35]. Elevated serum urea with normal serum creatinine is a common finding because of

the catabolism of lysed erythrocytes[173]. Gastrointestinal haemorrhaging can also give a similar finding but is uncommon in both uncomplicated and complicated cases. In complicated cases, biochemical changes can reflect the underlying complication (e.g., azotaemia in cases of ARF).

Severe canine babesiosis has been reported to result in metabolic acidosis[28,29] and severe acidaemia[28]. Recent research has shown that acidaemia was not present, however. Instead, either compensated metabolic acidosis or mixed acid-base derangement occurred[108], the latter being defined as the presence of two or more acid-base abnormalities in the same patient[11].

DIAGNOSIS

The diagnosis of babesiosis is based on demonstration of *Babesia* organisms within infected erythrocytes. Large (2 x 5 µm) piriform organisms (usually present in pairs) indicate *B. canis* infection, whereas smaller (1 x 3 µm) singular organisms are *B. gibsoni*.[26,202] The parasite can be detected from blood smears made from peripheral blood or central blood, although parasitaemia apparently is higher in peripheral blood[216].

The parasites can easily be demonstrated with Romanowsky stains (Wright's, Giemsa, or Diff-Quik®). Serologic testing has been used to diagnose babesiosis[110]. Serologic testing is more reliable in detecting occult parasitaemia in endemically unstable areas[202]. Because *B. canis* and *B. gibsoni* are cross-reactive on serologic tests, it is necessary to identify which species is causing the babesiosis[26,202].

THERAPY

The primary therapeutic goals are eliminating or suppressing the parasite and reversing life-threatening anaemia. Mild to moderate cases of uncomplicated babesiosis require only

antibabesial therapy, severe cases of uncomplicated babesiosis require antibabesial therapy and blood transfusions, and all cases of complicated babesiosis require additional therapy. In mild to moderate cases of uncomplicated babesiosis, noticeable signs of recovery can be expected within 24 hours of specific treatment, even though the haematocrit can drop further from the baseline reading[90]. This decreased reading is similar to what occurs in cases of malaria and may be related to treatment, parasite death, or simply a lag phase before erythrocyte destruction stops[90].

Antibabesial therapy

Three drugs are recognized and recommended; diminazene aceturate, imidocarb, and trypan blue[147]. Other drugs that have been used include amicarbalide, euflavine, quinoronium sulphate and chloroquine; however, because of poor efficacy and severe adverse effects, they are no longer recommended and are used infrequently[200]. The antiparasitic activity of diminazene is not well understood but is evidently related to interference with aerobic glycolysis and DNA synthesis in the parasite[135]. Diminazene binds to and inhibits DNA, causing dilation of membrane-bound organelles and perinuclear space, dissolution of cytoplasm, and destruction of the nucleus of the parasite[135,200]. Although resistance to diminazene is suspected, it has not been documented[200]. One dose of intramuscular diminazene (3.5mg/kg) is recommended[135,200]. Because the drug is fast acting and has a very short-lived protective effect[140], it is unsuitable for chemoprophylaxis. Diminazene is the drug of choice for uncomplicated *B. canis* infections but should be avoided in severe cases because of its possible hypotensive and anticholinergic effects[90,147,200], although the latter effect has been disputed[142]. Diminazene has a low therapeutic index[135], with toxicity resulting in depression or stupor, continuous vocalization, ataxia, opisthotonos, extensor rigidity, nystagmus, and seizures[154]. Nervous signs occur 24 to 48 hours after an overdose, are irreversible, and potentially fatal[202]. Diminazene can increase transaminase levels, which can affect interpretation of laboratory results[135,200].

Imidocarb has direct action on the parasite, altering both nuclear number and size and cytoplasmic morphology[135]. One dose of intramuscular or subcutaneous imidocarb (6mg/kg) is recommended[135]. Two doses administered at an interval of 14 days can be effective in eliminating concomitant canine ehrlichiosis[3]. Imidocarb is painful on injection and can cause transient salivation, diarrhoea, and depression within 10 minutes of administration[3,200]. If necessary, these side effects can be controlled with atropine[200]. Delayed reactions that can occur, 10 to 12 hours following injection, include shivering, depression, periorbital oedema and pyrexia. Toxicity can cause severe renal tubular and hepatic necrosis. Associated clinical signs include depression, tachycardia, diarrhoea, lacrimation, tremors, and cardiac arrhythmias[135,200].

Trypan blue blocks the C3b receptor on both the erythrocyte membrane and parasite itself and probably prevents the parasite from entering the erythrocyte[200]. Trypan blue suppresses parasitaemia and alleviates clinical signs but does not eliminate infection. Parasites can reappear within 9 – 12 days, and recurrence of clinical signs is common[200]. Therefore, administration of diminazene or imidocarb often follows the dose of trypan blue in an attempt to sterilise the infection[142]. Because it has no side effects but does have an extremely high therapeutic index, trypan blue was most frequently used in the initial treatment of severe cases[200]. The rationale for this was that it was thought that trypan blue had less of an affect on the haematocrit following treatment. This has been shown not to be true, however, as treatment with either trypan blue or diminazene had a similar effect on the haemocrit[90]. Trypan blue is also used in repeat relapses after treatment with either diminazene or imidocarb[200]. Trypan blue is a tissue irritant and thus is administered strictly as a 1% intravenous solution (10mg/kg)[135]. If necessary, treatment can be repeated. Preimmunity may develop in some dogs, but stress can cause relapses at any stage[200]. Severe bluish discoloration of the mucosa and urine occurs after administration, which can affect patient monitoring[135,200].

Blood transfusions

The magnitude of anaemia is the most important consideration when assessing the need for transfusion therapy. In cases of babesiosis, however, factors such as acuteness of onset, clinical signs, degree of erythrocyte regeneration, and presence of concurrent cardiac or respiratory disease must be considered[89].

At the OVAH, dogs with babesiosis are considered candidates for transfusion when the haematocrit is 15% or lower. Blood is almost invariably administered at a haematocrit below 10%, which is in agreement with previous observations[147]. Blood transfusions are also given when a clinical need such as dyspnoea or tachypnoea is apparent. Contrary to earlier suggestions[107], the degree of parasitaemia is not an important factor because it often bears little relation to the degree of anaemia[122].

Packed erythrocytes are the component of choice for treating haemolytic anaemia[42]. Administering the plasma component of whole blood is usually unnecessary and can place the patient at risk of volume overload[89]. If rehydration is required, crystalloid replacement solutions are preferable[10]. Fresh whole blood has a favourable effect on oxygen status and acid-base balance[108] and replaces sub-functional haemoglobin with functional haemoglobin[206].

Supportive therapy

Supportive therapy should be based on thorough patient assessment and ongoing monitoring; appropriate laboratory testing; and accepted therapeutic principles for resolving ARF[105,118], IMHA[19,43,222], DIC[42,137,198,199], hepatopathy[97], shock[28,81,141], and pulmonary oedema[19,44,46,47].

In cases of haemoconcentrated babesiosis, resuscitative fluid therapy with crystalloids (120ml/kg/24hr) is given to produce haemodilution. Because fluid therapy can precipitate or exacerbate ARDS, the patient's respiratory rate and pulmonary sounds should be monitored closely. Hetastarch (10 to 20 ml/kg) causes greater plasma volumes of crystalloids and therefore is beneficial for resuscitative fluid therapy[14].

Cerebral babesiosis is treated empirically. The therapeutic goal is to maintain cerebral perfusion, reduce consumption of cerebral oxygen, and block the effects of inflammatory mediators[14]. Although corticosteroids are usually administered at shock doses, their efficacy is not proven. Dimethyl sulfoxide has been given as a superoxide radical scavenger; however, like corticosteroids, its efficacy is not proven.

PREVENTION

The primary preventative measure is controlling the vector tick by routinely dipping or spraying pets, using tick collars or spot-on preparations, such as fipronil, and spraying the premises[202].

A vaccine against a specific, less virulent canine babesiosis strain is available in France[149]; however, cross-immunity between the different *Babesia* strains apparently does not occur with this vaccine[216]. A recent study has shown that protective immunity to a virulent strain can occur if the infection is not sterilized; therefore, vaccines from different strains of the parasite are feasible[111].

Pre-immunity has been recognized as important in controlling clinical signs of the virulent form of babesiosis in endemic areas[17,165]. Complete eradication of parasites from infected animals therefore may not be advantageous in these areas, and the use of drugs to sterilize

the infection may be undesirable[165]. The role of preimmunity in areas with less virulent strains is unknown[17].

As *Babesia* organisms can be transmitted through blood transfusions, all blood donors must test negative for babesiosis[26]. In endemic areas, regular evaluation of blood smears is recommended. In other areas, donors should be serologically tested[203].

RESEARCH FOCUS OF THIS THESIS

Canine babesiosis is an important tick-borne disease in South Africa. Over the past ten years the research focus of the Small Animal Section of the Department of Companion Animal Clinical Studies has been on elucidating the pathophysiology of canine babesiosis. This has resulted in the description of new complications (acute pancreatitis, cerebellar ataxia, hypoglycaemia), ruling out that haemoglobulin is nephrotoxic, showing that hypotension and cardiac changes occur, and that lactate can be used as a prognostic indicator.

An important reason that the research focus of the Department has been canine babesiosis is that the results of a survey done in 1993[38] showed that an average veterinary practice in South Africa treated between 100 and 500 canine babesiosis cases each year, at a cost to the dog-owning public more than R20 million. In addition acute renal failure and pulmonary babesiosis or 'shock lung', was reported as prevalent forms of complicated babesiosis.

The essence of the work reported in this thesis is to elucidate renal and cardiac pathophysiological changes in canine babesiosis. The thesis describes renal changes in canine babesiosis, looks at the role of met-haemoglobin as a potential nephrotoxin, and highlights cardiac changes. The latter are characterised by ECG changes, the use of a serum marker for myocardial necrosis, as well as pathological changes.

CHAPTER 2

RENAL CHANGES

INTRODUCTION

Acute renal failure (ARF) is an uncommon complication of babesiosis and typically presents as anuria or oliguria despite adequate rehydration[88]. Evidence of renal damage, reflected on urinalysis by the presence of proteinuria, casts and renal tubular epithelial (RTE) cells, is common in both complicated and uncomplicated cases, but does not necessarily reflect or predict renal failure[127]. In babesiosis, elevated serum urea alone is an unreliable indicator of renal insufficiency, as a disproportionate rise in urea, compared with creatinine, occurs, possibly due to catabolism of lysed erythrocytes[173]. Renal failure is diagnosed on the basis of ongoing evaluation of urine volume, urinalysis and degree of azotaemia. In humans, falciparum malaria, a disease clinically similar to canine babesiosis[122], can result in ARF, which resembles sepsis-related acute tubular necrosis[52]. Glomerulonephritis may also be evident[52].

Primary or intrinsic ARF is a syndrome characterized by the sudden onset of impaired renal function, resulting in azotaemia, fractional clearance of sodium (Fc_{Na}) that is greater than 1%, the presence of RTE cells and/or casts in the urine sediment, and characteristic histological changes[5,13,67,76]. Any toxic or ischaemic renal insult may result in cellular degeneration and/or necrosis, with consequent RTE cell loss into the urine. In humans, overt necrosis is not evident in all cases but tubular dysfunction is a uniform hallmark of this form of ARF[5]. Ischaemic injury occurs when renal blood flow is attenuated by decreased blood pressure or renal vasoconstriction[224]. Glomerular afferent arteriolar vasoconstriction caused by the effects of angiotensin II and antidiuretic hormone (ADH), in response to increased renin release, is a proposed mechanism of decreased glomerular filtration rate (GFR) in

ARF[145]. This decreased renal blood flow results in reduced amounts of oxygen and metabolic substrates presented to tubular cells, and this "cellular starvation" initiates the development of acute tubular necrosis with consequent ARF[27]. Acute renal failure associated with malaria has been attributed to hypovolaemia and/or hypotension, intravascular haemolysis, hyperparasitaemia, cholestatic jaundice, catecholamines, and endotoxaemia[168,191].

In canine babesiosis, the morphologic lesions in the kidney have been attributed to anaemic hypoxia resulting from erythrocyte destruction[83]. However, recent unpublished data from 84 dogs with complicated babesiosis have shown that the mean haematocrit of dogs with elevated creatinine was significantly higher (mean 36.5%, SD 20.19) than of those with normal creatinine (mean 22%, SD 16.38), making hypovolaemia a more likely cause than anaemia for the renal failure described in this disease. Babesiosis can result in a kidney that is swollen and dark in colour, with red-brown urine in the bladder. Microscopically the RTE cells are swollen and contain haemoglobin (Hb) droplets and small vacuoles. In severe cases, necrosis of the RTE cells is evident. The lumen of the nephron contains multiple Hb casts[83]. The net effect of babesiosis on the kidney can be ARF, which has been attributed to haemoglobinuric nephropathy[127]. However, ARF is uncommon in babesiosis[83,202,204], and recent work has demonstrated that severe haemoglobinuria, of the magnitude seen in canine babesiosis, did not induce significant nephropathy, regardless of whether or not concomitant anaemia was present[115].

The true pathogenesis of renal lesions in babesiosis is still obscure. Maegraith[122] noted the development of oliguria or anuria in dogs without concomitant haemoglobinuria. Several authors have reported that anoxia, reduction in renal blood flow, and possibly hypotension with intra-renal vasoconstriction and renal ischaemia must be considered of major importance in the pathogenesis, as opposed to mechanical obstruction of tubules by Hb and

the toxic effects of Hb[122,127,232]. It has also been demonstrated that hypoxia results in more injury to renal tubules than haemoglobinuria and that the nephrotoxic effect of Hb appears to be very individual[115]. Malherbe[127] also suggested that the renal damage in babesiosis is usually reversible.

The purpose of this study was to investigate the presence and degree of renal damage in naturally occurring canine babesiosis. Renal function and integrity were evaluated using urinalysis, serum urea and creatinine, serum electrolytes (sodium and potassium), fractional clearance of sodium (Fc_{Na}) and potassium (Fc_K), urine enzyme activity of gamma-glutamyl transpeptidase (*GGT)* and alkaline phosphatase (*ALP),* and quantification of proteinuria.

MATERIALS AND METHODS

Study design

The Animal Use and Care Committee of the Faculty of Veterinary Science, University of Pretoria, approved this study and written consent by the dogs' owners was obtained. Thirty dogs with babesiosis, presented to the Onderstepoort Veterinary Academic Hospital (OVAH), were sequentially enrolled. The diagnosis of babesiosis was based on finding *B. canis* parasites on a thin capillary blood smear, stained with Cams Quick stain (CA Milsch, Krugersdorp, South Africa). These dogs were categorised into three groups: mild uncomplicated (Group 1), severe uncomplicated (Group 2), and complicated (Group 3). Mild cases had mild-to-moderate anaemia (haematocrit 20-30%) with no clinical or biochemical signs of complicated disease. Severe cases had severe anaemia (haematocrit < 20%) with no clinical or biochemical signs of complicated disease. Complicated cases had one or more of the following complications: cerebral signs, ARF, acute respiratory distress syndrome, hypotensive shock, or haemoconcentration. Clinically healthy, aparasitaemic dogs, presented to the OVAH for routine ovariohysterectomy, were used as controls (Group 4).

Groups 1 and 4 were comprised of 10 dogs each and groups 2 and 3, 11 and 9 dogs respectively.

Data collection

Blood was collected from the cephalic vein, using a 22G venoject needle, a holder and a serum vacuum tube (Vacutainer System, Becton Dickinson, Industrial Estate, United Kingdom). A cystocentesis urine sample was collected aseptically using a 23G needle and a 10-ml syringe. All samples were collected prior to any treatment.

Analytical methods

Urea and creatinine were determined on a Technicon RA 1000 system (Technicon Instruments Corporation, Tarrytown, USA) using the Technicon modification of the kinetic method for urea[212] and the alkaline picrate reaction for creatinine, modified as a first order rate reaction[94]. Urine and serum sodium and potassium were determined using an ion selective analyser (Nova 1, Nova Biomedical, Waltham, Massachusetts, USA). Urine ALP and GGT were determined on a Technicon RA 1000 system using the Technicon modification of the p-nitrophenyl phosphate substrate method in AMP buffer for ALP[211] and glutamyl-p-nitroanilide substrate with glycylglycine peptide acceptor for GGT[201]. Urine Hb was determined on a Technicon RA 1000 system using the Drabkins method (CA Milsch, Krugersdorp, South Africa). Total urine protein was determined using a spectrophotometer (Lange LP6 Photometer) based on the Richterich technique using perchloric acid and biuret reagent[85]. Urine Hb was subtracted from the total urine protein and the corrected protein value was expressed as a ratio with the urine creatinine.

The fractional clearance of sodium and potassium was calculated, using the following formula:

$$\frac{\text{Urine electrolyte}}{\text{Serum electrolyte}} \times \frac{\text{Serum creatinine}}{\text{Urine creatinine}} \times 100$$

The physiochemical evaluation of the urine was performed using a urine dipstick (Lenstrip 8 Dipsticks, Benmore Diagnostics, Sandton, South Africa) and an AO veterinary refractometer (American Optical, Scientific Instrument Division, Buffalo, USA). Microscopic evaluation was done on urine sediment stained with Sternheimer-Malbin stain (Kyron Laboratories, Benrose, Johannesburg, South Africa), which enabled the differentiation of RTE cells from other urinary epithelial cells[9]. The presence of RTE cells in the urine was subjectively scored on a scale of 1-4 as follows:

1 represented 1 RTE cell per 2-3 high power fields (HPF),

2 represented 1-2 RTE cells per HPF,

3 represented 2-4 RTE cells per HPF,

and 4 represented more than 5 RTE cells per HPF.

Data analysis

Statistical analysis of the data was performed using a commercial statistical software package (Sigma Stat software, Jandel Scientific Software, USA). Parameters that were statistically analysed were urine specific gravity (SG), serum urea and creatinine, serum sodium and potassium, Fc_{Na}, Fc_{K}, urine ALP and GGT activity (expressed as a ratio to urine creatinine), proteinuria (expressed as urine protein: creatinine ratio), and urine analysis findings (haemoglobinuria and RTE cells in sediment). Data were compared between the groups using analysis of variance (ANOVA). The urine analysis findings were compared using Friedman repeated measures ANOVA on ranks. The Tukey correction was used for group comparisons. The Pearson test was used to check for correlation between haemoglobinuria and serum creatinine concentrations and urine RTE cell score. In all analyses, a value of $p < 0.05$ was considered significant.

RESULTS

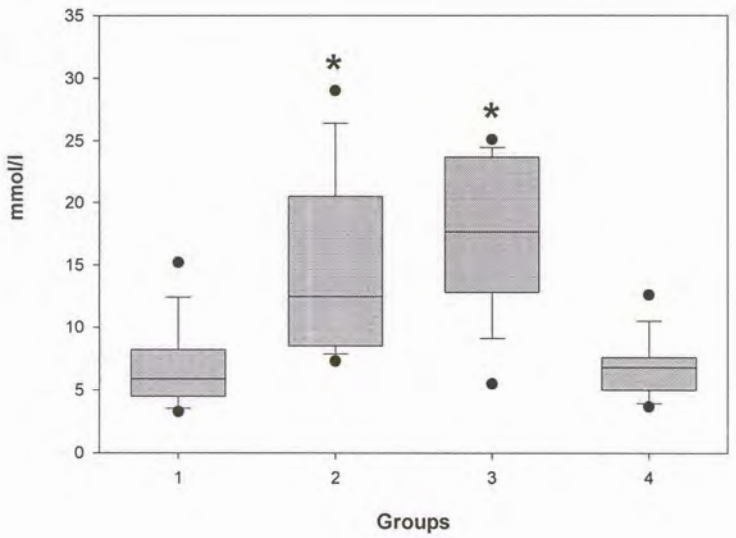
Clinicopathological findings are summarised in Table 2.1.

Table 2.1: Clinicopathological parameters in dogs with mild, severe, and complicated babesiosis and normal control dogs

	Mild (n = 10)		Severe (n = 11)		Complicated (n = 9)		Control (n = 10)	
Parameter	Median	Range	Median	Range	Median	Range	Median	Range
Serum urea (mmol/l)	5.9	3.3 - 15.3	12.8	7.3 – 29	19.3	5.5 – 25.1	6.8	3.7 – 12.6
Serum creatinine (μmol/l)	98	74 - 168	93	38 – 130	125	59 – 281	119.5	105 – 155
Serum sodium(mmol/l)	141.5	138 – 145	142	139 – 150	142	133 – 149	147.5	141 – 152
Serum potassium (mmol/l)	3.85	3.6 - 4	3.9	2.8 – 5	4.3	2.4 – 6.6	4.4	4.2 – 4.7
Urine ALP: creatinine ratio	0.27	0.2 – 3.41	0.51	0.16 –2.42	0.14	0.07 – 46.91	0.5	0.25 –1.17
Urine GGT: creatinine ratio	0.57	0.28 – 2.05	1.27	0.24 – 4.03	1.11	0.22 – 6.7	0.86	0.48 – 1.78
Fc_{Na} (%)	0.17	0 – 0.67	0.06	0 – 0.27	0.05	0 –0.22	0.49	0 – 1.4
Fc_{K} (%)	11.69	4.33 – 29.88	20.42	2.88 – 43.88	28.09	8.98 – 43.05	16.26	7.84 – 27.76
Urine haemoglobin (g/l)	1.25	0.2 – 2.5	1.6	0.3 – 11.6	1.95	0.92 –3.8	0.65	0.3 – 3.4
Urine protein: creatinine ratio	0.08	0.01 – 0.98	0.77	0.04 – 2.66	1.24	0.08 – 3.78	0.08	0.01 – 0.40
Specific gravity	1.05	1.04 – 1.06	1.05	1.03 – 1.06	1.05	1.035 –1.06	1.043	1.03 – 1.06
RTE cells	0.5	0 – 1	1	0 – 2	2	0 – 4	0	0 – 0

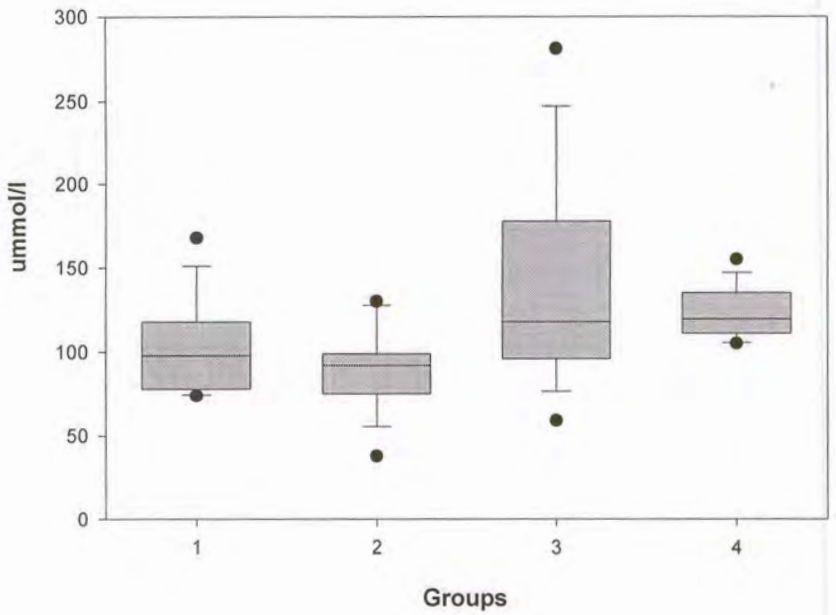
The majority of dogs in the severe and complicated groups (8/11 and 8/9 respectively) had elevated serum urea, compared with only 1/10 in the mild group (Figure 2.1).

Figure 2.1: Serum urea for the 4 groups. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes), and 10th and 90th percentiles (T-bars). An asterisk indicates significant differences between the babesiosis groups and the control group. Black dots represent outliers.



The serum creatinine did not mirror the serum urea in that 1/10 in the mild group, 0/10 in the severe group, and 3/10 in the complicated group had elevated serum creatinine levels (Figure 2.2).

Figure 2.2: Serum creatinine for the 4 groups. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes), and 10th and 90th percentiles (T-bars). Black dots represent outliers.



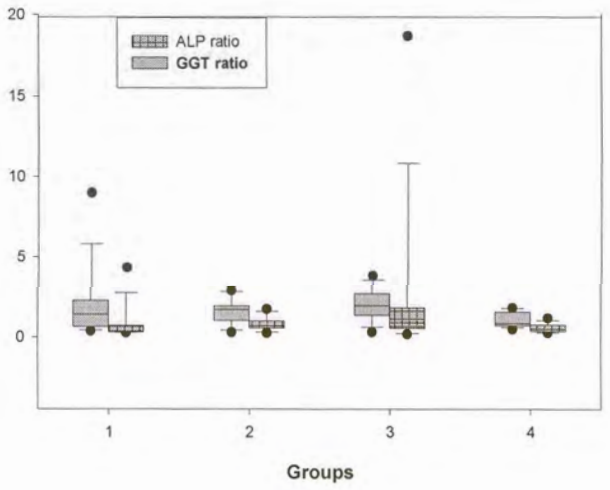
There was a significant positive correlation between serum urea and creatinine in the mild ($r=0.89$, $p<0.05$) and complicated ($r=0.83$, $p<0.05$) groups, but not in the severe group.

One dog in each of the babesiosis groups showed mild hypokalaemia. Severe hypokalaemia was present in one dog in the severe and one in the complicated group. Hyperkalaemia was present in one dog in the complicated group. There was no statistically significant difference between any of the groups for either hypo- or hyperkalaemia ($p = 0.310$). Marginal hyponatraemia was present in 2/10 in the mild and severe groups, and 3/10 in the complicated group. Dogs with babesiosis had statistically significant lower serum sodium than the control group ($p< 0.001$), irrespective of the class of babesiosis (mild, severe, complicated) that the dog had. None of the dogs showed hypernatraemia. Although there was no elevation in the Fc_{Na} that was suggestive of acute tubular dysfunction, there was,

however, a statistically significant difference between all three groups and the control group in that the babesiosis groups had a much lower Fc_{Na} than the control group ($p = 0.007$). There was neither elevation of, nor any statistically significant differences for, Fc_K ($p = 0.160$).

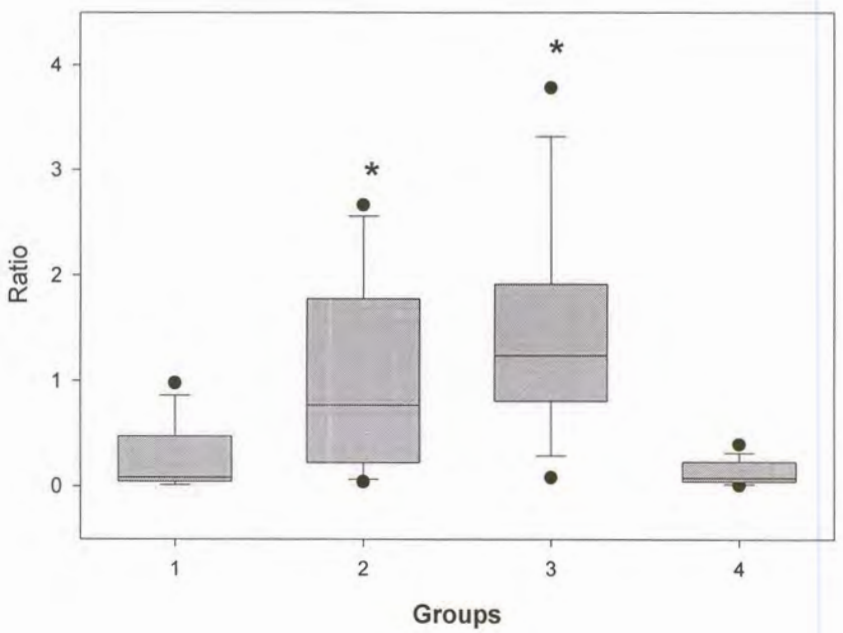
The urine ALP:creatinine ratio was elevated in one dog in the mild group and one in the severe group, and two in the complicated group. The urine GGT:creatinine ratio was elevated in one dog in the mild group, 2/11 in the severe group, and 2/9 in the complicated group. There was, however, no correlation between the ALP and GGT creatinine ratios nor was there any statistical difference between the groups for urine ALP ($p = 0.785$) and GGT ($p = 0.452$) activity (Figure 2.3). Only one dog in the complicated group showed marked enzymuria (both ALP and GGT), but this was not associated with azotaemia.

Figure 2.3: Urine ALP: creatinine and GGT: creatinine ratio for the 4 groups. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes), and 10th and 90th percentiles (T-bars). Black dots represent outliers.



An elevated urine protein: creatinine ratio was evident in 3/10 of the mild group, 6/11 of the severe group, and 8/9 of the complicated group. When compared with the control group, the proteinuria was statistically significant in the severe ($p = 0.002$) and complicated groups ($p = 0.001$) (Figure 2.4).

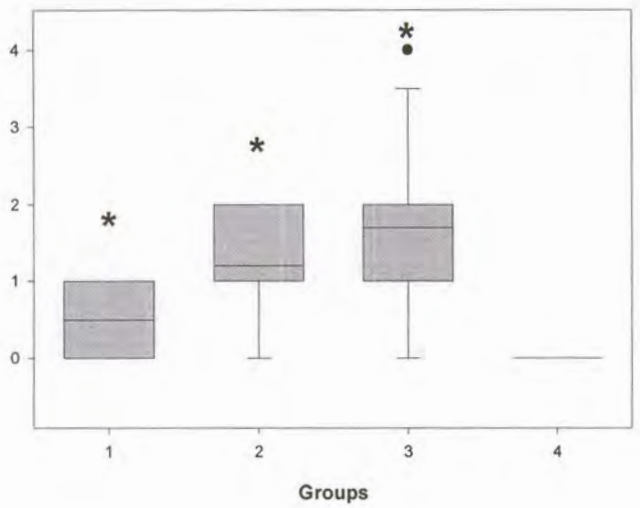
Figure 2.4: Urine protein: creatinine ratio for the 4 groups. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes), and 10th and 90th percentiles (T-bars). An asterisk indicates significant differences between the babesiosis groups and the control group. Black dots represent outliers.



There was no difference in the urine specific gravity (SG) between the groups. All dogs had well-concentrated urine (SG > 1.030). Haemoglobinuria was present in all babesiosis groups with medians of 1.25, 1.65 and 3.95 g/l for the mild, severe and complicated groups, respectively. There was no correlation between the haemoglobinuria and serum creatinine concentrations or urine RTE cell score. Some dogs in all the babesiosis groups had RTE

cells in the sediment, which increased in severity from the mild to the complicated groups (Figure 2.5). Tubular casts (cellular or granular) were not evident in any of the dogs.

Figure 2.5: Renal tubular epithelial cells in urine sediment for the 4 groups. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes), and 10th and 90th percentiles (T-bars). An asterisk indicates significant differences between the babesiosis groups and the control group. Black dots represent outliers.



Four dogs showed a number of clinicopathological changes consistent with acute renal damage (one in the mild and three in the complicated groups). The dog in the mild group had moderate azotaemia, mild elevation in the urine enzymes, and moderate proteinuria. Hyperkalaemia, elevated Fc_{Na}, or RTE celluria were not present. In the complicated group, the three dogs had moderate azotaemia and moderate RTE celluria. One of these had hyperkalaemia and hyponatraemia, and two had moderate proteinuria and enzymuria. None of the dogs showed elevated Fc_{Na}. All four dogs responded well to standard therapy for ARF[14].

DISCUSSION

In this study, the most consistent finding indicative of renal damage was proteinuria and its severity was related to the severity of the babesiosis. Renal and renal-related disorders due to human falciparum malaria, a disease similar to canine babesiosis[122], include both extra-renal and renal manifestations[52]. The former consist of fluid and electrolyte disorders. The latter vary widely, ranging from mild proteinuria and glomerulonephritis to ARF[62,191]. In humans the reported incidence of ARF associated with falciparum malaria ranges from 1-4%[62]. Jacobson and Clark[88] reported that ARF is an uncommon complication in canine babesiosis, being diagnosed in only 3 of 134 cases reviewed, giving an incidence of 2.2%.

The elevated serum urea in the severe uncomplicated group in this study, without a corresponding increase in the serum creatinine, confirms previous findings[173]. The phenomenon has been attributed to catabolism of lysed erythrocytes, resulting in an increased ammonia load on the liver and consequent increased urea production[173], but could also be associated with more generalized protein catabolism resulting from a febrile, inflammatory illness. Other possible causes would include gastro-intestinal haemorrhage and ingestion of a high protein meal. These latter causes are unlikely in canine babesiosis. Thus elevated serum urea alone is an unreliable indicator of renal insufficiency in babesiosis. The elevated serum urea in the complicated group in this study was associated with a concomitant increase in the serum creatinine, which could reflect decreased renal blood flow, possibly because of decreased blood pressure and/or hypovolaemia. A recent study showed that hypotension occurred frequently in canine babesiosis and the presence and severity of hypotension increased with increased disease severity[91]. As the elevated creatinine, evident in some of the cases, was not correlated with any of the other parameters of renal function or integrity, it may have been pre-renal in origin. Another study has shown that elevated creatinine is associated with increased risk of death in canine babesiosis[221], indicating that it might nonetheless be a useful measure of renal insufficiency. In babesiosis,

a higher cut-off value and/or serial creatinine determinations would assist in distinguishing pre-renal from renal causes.

Urine enzyme activity is both an early and persistent indicator of renal tubular damage[178]. Both GGT and ALP are brush border enzymes present in the proximal convoluted tubule of the kidney[77]. Although 24-hour urine enzyme activity is more accurate, evaluation of the ratio of urine enzyme: creatinine in a spot urine sample is technically simpler and has been shown to correlate well with a 24-hour sample[178]. Urine ALP and GGT activity > 10 U/l and a ratio > 2 (calculated using SI units) can be considered to be elevated[75,178]. Only one dog in the complicated group showed severe changes in the urine enzyme activity; however, this was not accompanied by abnormal urine SG or Fc_{Na}.

The Fc_{Na} can be used as an indicator of acute tubular dysfunction, with an increase in the Fc_{Na} over 1% reportedly indicating acute tubular dysfunction[10]. Two of the control dogs had a Fc_{Na} greater than 1%, raising the possibility that a Fc_{Na} greater than 1% is not always indicative of acute tubular dysfunction. In this study an unexpected finding was that the median Fc_{Na} in the dogs with babesiosis was lower than that of the control dogs. The majority of sodium is actively re-absorbed from the proximal convoluted tubules of the kidneys, resulting in passive water re-absorption. Further sodium re-absorption occurs in the distal convoluted tubules (secondary to the active re-absorption of chloride ions) and collecting ducts (controlled by aldosterone)[178]. In this study, the lower Fc_{Na} can be interpreted as either renal retention of sodium secondary to aldosterone secretion or inhibition of prostaglandins, or because of activation of the renin-angiotensin-aldosterone system, in response to renal arterial hypotension[79,130,167]. The well-concentrated urine in all dogs with babesiosis can also be attributed to sodium and water retention. However, the sodium retention may have been inadequate as some of the dogs showed hyponatraemia, which could be indicative of early renal tubular dysfunction.

Hyponatraemia, usually asymptomatic, can occur in up to 67% of patients with malaria without renal failure and is often associated with severe disease[191]. In children, hyponatraemia occurred during the acute phase of the disease with increased fractional sodium excretion and continuing sodium wastage in 17% after recovery[61,196]. In the current study, hyponatraemia was present in only 17% of the cases and Fc_{Na} was not increased in any of the dogs. In patients with falciparum malaria it has been shown that there is a decreased response to water load, attributed to peripheral vasodilatation which results in a decreased effective blood volume leading to the release of vasopressin and norepinephrine, increased renin activity and decreased renal perfusion[191]. It has also been hypothesised that water retention occurs in babesiosis[183]. Hyponatraemia in malaria has been ascribed to multiple factors: increased secretion of ADH, hypervolaemia, and accumulation of sodium in both parasitized and non-parasitized red blood cells as a result of decreased sodium-potassium ATP-ase activity[191].

Hypokalaemia is an uncommon finding in malaria, which is similar to the findings in this study. In malaria, hypokalaemia has been ascribed to decreased potassium intake and respiratory alkalosis[52], both of which can occur in babesiosis[109]. The five dogs that showed hypokalaemia had a normal to increased Fc_K, which is an abnormal response and could thus be indicative of early renal tubular dysfunction or as a response to the alkalosis.

Hyperkalaemia occurs in malaria patients with either ARF or intravascular haemolysis[191]. In this study, two dogs showed hyperkalaemia, of which one had ARF. In a study in children, plasma potassium was significantly higher and the Fc_K significantly lower during the acute illness than after recovery[196]. As canine red blood cells are much lower in potassium than human red blood cells, hyperkalaemia is unlikely to occur because of haemolysis in the dog.

In this study, a number of dogs had RTE cells in the urine sediment. In malaria, abnormal urinary sediment, consisting of red and white blood cells and occasional granular casts,

commonly occurs in patients without renal failure[191]. Other urinary sediment abnormalities, such as the presence of RTE cells, are not commonly reported[156]. The presence of RTE cells in the urine sediment can indicate renal damage due to hypoxia, hypoperfusion, or toxic damage. In this study, the number of RTE cells in the urine sediment increased with increased disease severity. Renal hypoxia results in rounding and retraction of RTE cells with a disruption of actin microfilaments, because of which a large number of viable RTE cells are sloughed into the urine[171]. Acute renal hypoxia has also been shown to induce apoptotic changes[95]. This may explain the lack of correlation between urine enzyme activity and the presence of RTE cells.

Proteinuria was a common finding in this study. Proteinuria was demonstrated by Maegraith[122] in experimental babesiosis, and occurred within 24 hours of the first appearance of parasites in the blood. In later stages of the infection, there was evidence of tubular damage in the form of granular and hyaline casts. Cast formation was not present in this study. In children with malaria, mild proteinuria occurred in 40% during the acute illness but was not related to creatinine clearance, body temperature at presentation, or peripheral parasite density. Proteinuria was also absent after recovery[196]. *Babesia rodhaini*-infected mice developed immune-complex-induced mesangiopathic glomerulonephropathy and moderate renal tubular necrosis[6]. These mice had elevated serum urea and proteinuria. *Babesia microti*-infected mice showed relatively mild immune-complex-induced mesangiopathic glomerulonephropathy and mild renal tubular necrosis, with no increase in serum urea and no proteinuria[6]. The degree of proteinuria in this study was consistent with tubulo-interstitial disease[57,66]. Canine babesiosis has not been associated with glomerulonephropathy[83].

Acute renal failure in malaria is commonly associated with blackwater fever, characterized by fever, massive intravascular haemolysis and haemoglobinuria[52]. The condition is associated with quinine administration in patients with glucose-6-phosphate dehydrogenase

(G6PD) deficiency[191]. The presence or absence of G6PD deficiency in dogs with babesiosis has never been evaluated, but severe haemoglobinuria is unlikely to mimic the human situation, since the common antibabesial drugs are dissimilar to quinine and haemoglobinuria generally occurs prior to treatment. In our experience, dogs with babesiosis that die as a result of ARF show severe haemoglobinuria and oliguria, and frequently produce a small volume of urine that is almost black in colour. In this study there was no correlation between the haemoglobinuria and serum creatinine and RTE cells in the urine. This supports a previous study that showed that haemoglobinuria does not induce renal failure[115].

CHAPTER 3

HAEMOGLOBIN AND MET-HAEMOGLOBIN AS ENDOGENOUS TOXINS

INTRODUCTION

Babesia canis is an intra-erythrocytic protozoal parasite that causes erythrocyte destruction, which may result in haemoglobinaemia and haemoglobinuria[25,202]. It is widely recognised that in both man and animals, haemoglobinuria can be associated with the development of acute renal failure (ARF).

The pathogenic mechanism(s) for ARF associated with haemoglobinuria are controversial. One school of thought suggests that haemoglobin is not directly toxic[18,53,92,93,104,115] but that other factors (inflammatory mediators, hypotension) result in ARF. A second school of thought holds that haemoglobin *per se* in not nephrotoxic but after release from the red cell it is converted into met-haemoglobin, which has been identified as a nephrotoxin[80,122,228].

Renal pathology associated with haemoglobinaemia resulting from *B. canis* infection is ascribed to haemoglobinuria, with or without a contribution from anaemic hypoxia. In an experimental study[115] the relative roles of haemoglobinaemia and hypoxia on renal function and pathology in healthy dogs was investigated. Three groups of 6 dogs each were used over a 4-day period. The dogs in the 1st group were infused with homologous canine haemoglobin, anaemic hypoxia was induced in the 2nd group, and both treatments were applied in the 3rd group. Full urinalyses, serum urea and creatinine concentrations, fractional clearance of sodium and the activity of urine enzymes, were assessed daily. At the end of the trial period, the glomerular filtration rate (GFR) was determined and kidney specimens collected for light and electron microscopy. In the group with hypoxia only, the urine sediment contained more casts and a greater number of renal tubular epithelial (RTE) cells than in either of the other groups. Hypoxia resulted in greater enzymuria, suggestive of RTE

cell pathology, whereas haemoglobinuria did not appear to have any effect on urine enzyme activity. Hypoxia resulted in a decreased GFR. Histological examination revealed mild, single-cell tubular necrosis in the majority of the animals (all 3 groups), with granular casts in the hypoxic groups. There appeared to be a large individual variation in the ability of the kidney to handle infused haemoglobin. It was concluded that severe haemoglobinaemia did not induce significant nephropathy, anaemic hypoxia appeared to cause a very mild nephropathy, and the combination of haemoglobinaemia and anaemic hypoxia did not exacerbate this change. These lesions were very different from those described in canine babesiosis.

Experimentally, the infusion of met-haemoglobin into dogs has resulted in ARF[18,80], whereas in rats, met-haemoglobin infusions have resulted in ARF only if there was concurrent aciduria[228]. In the latter study, renal hypoxia also appeared to predispose to the toxic effects of met-haemoglobin. The conversion of haemoglobin to met-haemoglobin may occur either in the blood or after filtration through the glomeruli of the kidneys. Studies in dogs with naturally occurring *B. canis* infection have been unable to document met-haemoglobin in the blood[122,205]. It has been shown experimentally that met-haemoglobinuria can occur[122], however, although the samples in the study were not collected anaerobically. In one experiment[122], intravenous infusion of haemoglobin solutions into dogs resulted in the appearance of met-haemoglobin in the urine. The urine samples were collected directly from the renal pelvic ureters. This suggests that conversion of haemoglobin to met-haemoglobin may occur in the kidney. Another factor that may predispose the dog to the toxic effects of met-haemoglobin is the fact that canine urine is generally acidic[59].

In natural *B. canis* infection there is often haemoglobinuria[122,202]. Met-haemoglobin fractions in the blood have been shown to be negligible[205], however, and met-haemoglobin fractions in the urine have never been reported. It is possible that in *B. canis* infection, haemoglobin is converted to met-haemoglobin in or by the kidney.

The purpose of this investigation was to compare whole blood and urine met-haemoglobin fractions from dogs with naturally occurring *B. canis* infection and visible haemoglobinuria, with that of clinically healthy dogs.

MATERIALS AND METHODS

Urine samples were collected by cystocentesis and heparinised blood samples were collected from the cephalic vein of control dogs (n = 5) as well as dogs with naturally occurring *B. canis* infection and visible haemoglobinuria (n =6). Both the urine and blood samples were collected aseptically and anaerobically. The control group comprised clinically healthy dogs. The 6 dogs with *B. canis* infection were all presented to the Outpatients Clinic of the Onderstepoort Veterinary Academic Hospital. For inclusion in the trial, these dogs had to have confirmed *B. canis* infection, diagnosed on a stained, thin capillary blood smear (Quick stain, CA Milsch); be in-saline agglutination negative, and had to have visible haemoglobinuria. The colour varied from red-brown to purple black. Urine and blood samples were collected before any therapeutic agents were administered and both samples were assayed within 30 minutes after collection. A haemoxymeter (Osm 3, Radiometer, Copenhagen, Denmark) was used to determine the met-haemoglobin fractions in the blood and urine. A blood gas machine was used to determine the urine pH (ABL 3, Radiometer, Copenhagen, Denmark). The instruments were calibrated using standard calibration procedures and standard solutions. The data from the control and *B. canis* infection groups were compared using analysis of variance (Sigma Stat for Windows, Version 1.0, Jandel Corporation). Significance was set at $p<0.05$.

RESULTS

The results are summarised in Tables 3.1 and 3.2. The means of the urine haemoglobin, blood haemoglobin, urine met-haemoglobin and urine pH form the dogs with *B. canis*

infection differed significantly from those of the control group. The urine pH and blood Hb levels were significantly lower in infected than in control animals. The means of the blood met-haemoglobin did not differ significantly between the two groups.

The urine haemoglobin concentration ranged from 1 to 4 g/l, which was related neither to the blood haemoglobin concentration nor the haematocrit. Only 2 cases showed detectable blood met-haemoglobin fractions, although the amounts were negligible (0,9 and 1,3%). Urine met-haemoglobin fractions ranged from 28 – 59%. The urine pH in the *B. canis* infected group was lower than that of the control group, which was a statistically significant difference.

Table 3.1: Haematocrit (Ht), serum and urine haemoglobin (Hb) and met-haemoglobin (met-Hb) fractions, and urine pH from healthy dogs and dogs with babesiosis and haemoglobinuria.

	Ht (%)	Blood Hb (g/l)	Urine Hb (g/l)	Blood met-Hb (%)	Urine met-Hb (%)	Urine pH
Control 1	45	128	0	0	0	7.694
Control 2	43	164	0	0	0	7.917
Control 3	48	145	0	0	0	6.274
Control 4	58	176	0	0	0	7.654
Control 5	50	160	0	0	0	6.807
Case 1	16	50	1.2	0.9	59.7	6.039
Case 2	18	32	1	1.3	45.3	6.181
Case 3	13	29	2	1.3	33.8	5.918
Case 4	10	62	3	0	28.7	6.334
Case 5	12	42	1	0	46.4	5.946
Case 6	15	48	4	0	29.2	6.242

Table 3.2: Statistical comparison of urine and serum haemoglobin (Hb) and met-haemoglobin (Met-Hb) fractions from healthy dogs and dogs with babesiosis and haemoglobinuria.

	Control group (n = 5)				Babesia dogs (n = 5)				p-value
	Mean	Median	SD	Range	Mean	Median	SD	Range	
Urine Hb (g/l)	0	0	0	0	2	1.6	1	1-4	0.004
Blood Hb (g/l)	154.6	160	18.5	128-176	43.8	45	12.2	29-62	< 0.0001
Urine Met-Hb	0	0	0	0	40.52	39.55	12.15	28.7-59.7	< 0.0001
Blood Met-Hb	0	0	0	0	0.58	0.45	0.9	0-1.3	0.428
Urine pH	7.27	7.65	0.699	6.274-7.917	6.11	6.11	0.168	5.918-6.334	0.003

DISCUSSION

This study revealed that significant urine met-haemoglobin is found in dogs with naturally occurring babesiosis. To date it has been shown that in experimental *B. canis* cases met-haemoglobinuria is present and that experimental infusion of haemoglobin solution will produce met-haemoglobinuria[122]. It appears that in natural *B. canis* infection a similar process occurs.

As no significant blood met-haemoglobin was found, urinary met-haemoglobin was probably produced by the kidney or possibly by oxidation of haemoglobin to met-haemoglobin in the bladder. It has, however, been shown that haemoglobin infusions result in met-haemoglobinuria in the renal pelvis or ureters[122], suggesting that the oxidation occurs in the kidney.

It has been shown experimentally that met-haemoglobin can be toxic, especially if it occurs concurrently with aciduria[228]. In the present study the *B. canis*-infected group had a statistically significant lower urine pH than the control group. The urine met-haemoglobin fractions were statistically significant. Blood met-haemoglobin fractions were very low, as reported in previous studies in dogs[122,205].

Whether met-haemoglobinuria is toxic in *B. canis* infection was not demonstrated in this study, as none of the animals succumbed to the infection and renal function tests were not performed. All the animals made an uneventful recovery and were discharged with no obvious renal dysfunction.

CHAPTER 4

CARDIAC CHANGES

INTRODUCTION

Heart lesions appear to be a rare complication of babesiosis[88,116]. However, to date, the prevalence has been poorly described. Heart lesions have been reported as incidental findings at post-mortem examination of complicated babesiosis cases[148]. Lesions that have been observed are foci of myocardial necrosis with macrophage and neutrophil infiltration (unpublished data, Pathology Section, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria), subepicardial and subendocardial haemorrhages, micro-thrombi of the myocardium[148,204], hydropericardium, and haemopericardium[148,177,204]. Dilated cardiac blood vessels containing considerable numbers of parasitized erythrocytes and free parasites have been reported[73,122]. In bovine and equine babesiosis, ecchymotic haemorrhages in the epicardium, endocardium and myocardium have been described[83]. These cardiac lesions probably develop because of one or both of the mechanisms postulated for tissue damage in babesiosis, namely an overwhelming inflammatory response and anaemic hypoxia[175].

Electrocardiographic (ECG) abnormalities have not been previously reported in canine babesiosis but ECG abnormalities consistent with ischaemia and myocarditis are well described in man[172]. Ischaemia can result in alterations of the T wave. Myocardial injury can also cause alteration in the ST segment (elevation, or less commonly, depression), and myocardial necrosis can cause alteration in the QRS wave (widening, high voltage Q-amplitude, slurring and notching)[172]. Myocarditis can cause slow conduction resulting in widened QRS and/or complete atrioventricular (AV) block[99]. In dogs with myocarditis, ventricular premature complexes (VPC), and paroxysmal ventricular tachycardia are reported[164].

Myocardial infarction in the dog and cat is associated with ventricular tachycardia, atrial fibrillation, and presence of VPCs[58,134]. On histopathological examination myocardial necrosis and inflammatory infiltrate are present. The cause of some of the infarcts is thrombo-embolism resulting from immune-mediated haemolytic anaemia (IMHA), which is a relatively common complication of babesiosis[88,116].

In babesiosis a number of extracardiac conditions that could induce ECG abnormalities include anaemia, hypoxia, hypokalaemia, metabolic acidosis, hyperkalaemia, and uraemia[87,88,99,108,116,122]. The presence of these extra-cardiac conditions would reduce the specificity of the ECG to detect myocardial lesions *per se*.

Clinical history and examination are imperfect tools for the diagnosis of acute myocardial injury in humans. Although the ECG is often used, comparisons, based on post mortem diagnosis of acute myocardial infarction, show that its diagnostic sensitivity is only 40-60%[136,231]. The test of choice for the diagnosis of myocardial infarction in humans is measurement of serum cardiac troponin I (cTnI) and T (cTnT) concentrations[40]. Cardiac troponins are thin, filament-associated proteins of cardiac muscle that act as the regulatory subunit of the troponin complex associated with the actin thin filament within muscle cells. Troponins are integral in the regulation of muscle contraction[166]. The diagnostic time window of these markers in humans is wide, being up to 72 hours[40]. The markers have 100% sensitivity for diagnosing acute myocardial infarction 12 hours after presentation to hospital, and concentrations remain raised for as long as 10 days[40].

Serum concentrations of cTnT are elevated in some dogs with congestive heart failure, following extensive soft tissue trauma, and after chemotherapy with doxorubicin for lymphoma[54]. The diagnostic sensitivity of serum cTnT to detect myocardial cell injury in dogs with blunt thoracic trauma is less than that of cTnI[185]. The use of serum cTnI concentrations was recently shown to be useful in the evaluation of a horse with suspected myocardial

disease[41]. Cardiac troponin I and cTnT have also been used as easily measurable target variables for detection of cardiotoxic and/or cardio-degenerative effects in experimental rats[15].

MATERIALS AND METHODS

Study design

Thirty-four dogs of any breed or sex and weighing more than 3 kg with babesiosis were used in this study. All the dogs were presented to the Onderstepoort Veterinary Academic Hospital (OVAH). Babesiosis was diagnosed on a thin capillary blood smear, stained with Cams Quick stain (CA Milsch, Krugersdorp, South Africa.) Dogs with known or suspected concurrent cardiac disease of other causes were excluded from the study. Other exclusion criteria were treatment that was started prior to presentation and any clinical signs that could not be ascribed to babesiosis.

Four groups of babesiosis cases were identified: mild uncomplicated (Group 1, 8 dogs), severe uncomplicated (Group 2, 9 dogs), complicated (Group 3, 8 dogs), and concurrent IMHA (Group 4, 9 dogs). Mild cases had mild to moderate anaemia (haematocrit ≥ 15%) with no clinical or biochemical signs of complicated disease. Severe cases had severe anaemia (haematocrit < 15%) with no clinical or biochemical signs of complicated disease. Complicated cases showed one or more of the following complications: cerebral signs; acute renal failure (ARF) characterized by oliguria (< 1ml/kg/hour) that did not resolve with rehydration; bleeding tendency characterized by petechiation and/or other haemorrhage; severe icterus; or acute respiratory distress syndrome (ARDS) characterized by dyspnoea that did not resolve with blood transfusion; and/or the presence of abnormal lung sounds. These complications were diagnosed clinically to aid classification at presentation. This classification was, however, aided by further diagnostic procedures in most of the hospitalized cases. Immune-mediated haemolytic anaemia was diagnosed by the presence

of autoagglutination, determined on a slide agglutination (in-saline agglutination) test. Immune-mediated haemolytic anaemia with or without icterus was the only complication allowable in Group 4. Dogs, from the Onderstepoort area, that were presented to the OVAH for routine ovariohysterectomy or castration, were used as controls (Group 5, 9 dogs). The control group showed no abnormalities on clinical examination, were negative for *B. canis* on peripheral blood smear examination, had normal haematocrit, and were monitored for 48 hours after surgery without developing any abnormal clinical signs.

Data collection

A Nihon Kohden ECG monitor using a paper speed of 50 mm/sec, amplitude calibration of 1mv = 1cm, and manual Hum filter (notch filter at 50 Hz) and EMG filter (cutoff frequency of –3dB at 35 Hz) was used for the ECG recordings. A lead II ECG tracing was collected from all dogs prior to anti-babesial therapy. A second ECG was recorded 24 hours after admission from dogs that were hospitalized (Groups 2-4) and had survived 24 hours. No ECG recordings were performed on the control group. The ECG recording was done for 1 minute with the dogs in right lateral recumbency and according to standard protocol[84]. Arrhythmias were classified according to their origin, named in the standard way and counted[213]. P-amplitude, P-duration, QRS-duration and ST depression or elevation were noted as normal or high, and PR-interval, R-amplitude and QT-duration were noted as low, normal or high. Presence of a notch on the descending slope of the R-wave and ST coving were noted.

A venous blood sample was collected in a heparinised syringe (Lithium heparin, Instrumentation Laboratory, Italy). After collection the blood was centrifuged and the plasma separated and stored at -20° C. Cardiac troponin I concentrations were determined with a microparticle enzyme immunoassay (Abbot Axsym system, Abbott Laboratories, Abbott Park, Illinois, USA) using cTnI-specific purified, polyclonal murine and caprine antibodies with independent immunologic epitopes for cTnI. The lower detection limit of the assay is 0.3

ng/ml. Cardiac troponin T concentrations were determined with an electro-chemiluminiscence immunoassay (Elecsys 1010 Troponin Stat, Boehringer Mannheim, Mannheim, Germany) using two different monoclonal murine antibodies directed against different epitopes of human cTnT. The lower detection limit of the assay is 0.01 ng/ml. Reference values were taken from results of a study performed in 40 normal dogs[185], as well using the control group in this study. In this study, the actual reference range was not a specific factor as all results were statistically compared to the control group. The upper limit of normality for the cTnI and cTnT used in this study was 2 and 0.09 ng/ml, respectively. This level was based on the cut-off value used in humans and a recent canine study to identify significant myocardial cell injury[185].

Gross and histopathological assessment of the heart was performed in 5 dogs. Of the 5, 3 dogs from the complicated group died naturally (non-survivors) and 2 were euthanased (1 from the complicated group and 1 from the IMHA group, both due to financial constraints). Samples were collected within 60 minutes after death to avoid post mortem changes that could mimic ischaemia and necrosis. The pericardium was examined for fluid and its colour. The heart was opened in standard fashion and the endo- and epicardium evaluated macroscopically for haemorrhage and gross infarcts. Full thickness (from epi- to endocardium) samples were taken for histological examination from the papillary muscles of the left and right ventricles, interventricular septum, left and right atrium, and the apex, and placed in 10% buffered formalin. Samples were wax-embedded, sectioned at 5µm, and stained with haematoxylin and eosin for routine light microscopy and modified Martius-Scarlet blue for the detection of fibrin. Twenty fields were examined at 400x magnification and scored separately for necrosis, cell infiltration, haemorrhage, and fibrin. Necrosis of myocardial fibres was recognized by increased cytoplasmic eosinophilia, loss of striation, fragmentation and nuclear pycnosis. Myocardial necrosis was defined as abnormal if 6 or more necrotic cells were found in any one of the 20 fields. Myocardial infiltration was defined as 6 or more inflammatory cells detected in any one field. Haemorrhages were examined for

their location and whether intercellular or intracellular. Fibrin thrombi were examined for their location. The presence of any haemorrhage or fibrin thrombi was defined as abnormal.

Data analysis

Data were tabulated in a spreadsheet programme (Excel, Microsoft Corporation, USA). Statistical analysis was performed with the aid of a statistical software package (Sigma Stat, Jandal Corporation, USA). The ECG was evaluated and compared to the standard dog's ECG[213]. Descriptive statistics were used to describe the data. The Kruskal-Wallis one-way analysis of variance on ranks was used to test for statistical differences between groups. The Pearson Product correlation was used to determine correlation between variables. The level of significance was set at $p < 0.05$.

RESULTS

Five dogs died, 3 of which died naturally (non-survivors) and were all from the complicated group. The specific complications were as follows: the first dog had ARDS and ARF; the second ARDS and a bleeding tendency; and the third ARF, cerebral signs and icterus.

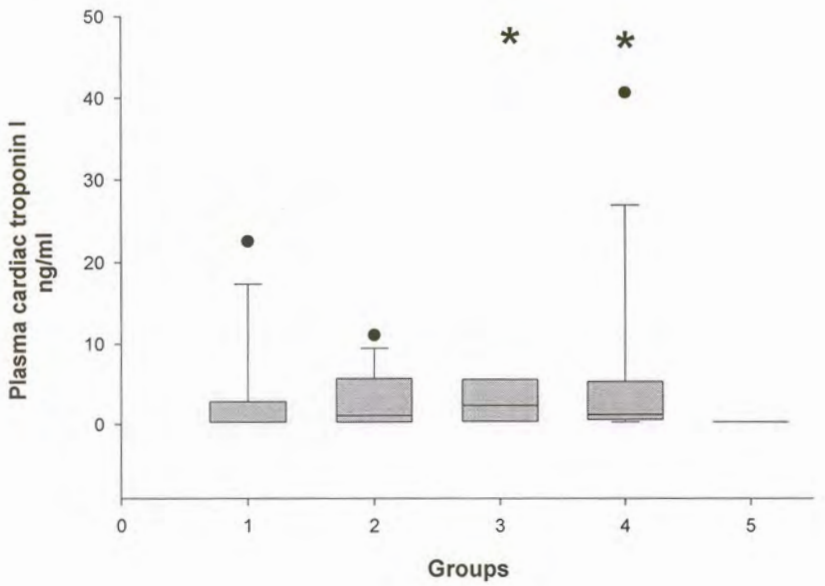
Signalment

The median age in Group 1 was 24 months (range 6-108); Group 2, 6 months (range 1-8); Group 3, 6 months (range 3-48) and Group 4, 15 months (range 3-60). The median weight in Group 1 was 28.5 kg (range 10-34); Group 2, 14.5 kg (range 5.3 – 23.5), Group 3, 10.3 kg (range 3.5 – 15.9) and Group 4, 28.2 kg (range 3.3 – 45). The median age and weight for the control group were 14.5 months (range 11-28) and 27.5 kg (range 25-37), respectively. There were no statistically significant differences in the above variables between the different groups.

Cardiac troponin I

In Group 1, 5/8 (62.5%) dogs had concentrations below the detection limit (0.3 ng/ml), 1/8 (12.5%) showed a concentration below the upper limit of the reference range (2 ng/ml), and 2/8 (25%) had elevated concentrations. In Group 2, 4/9 (44.5%) had concentrations below the detection limit, 1 (11%) showed a concentration below the upper limit of the reference range, and 4/9 (44.5%) had elevated concentrations. In Group 3, 2/8 (25%) had concentrations below the detection limit, 1/8 (12.5%) had concentrations below the upper limit of the reference range, and 5/8 (62.5%) had elevated concentrations. In Group 4, 2/9 (22%) had concentrations below the detection limit, 3/9 (33%) had concentrations below the upper limit of the reference range, and 4/9 (45%) had elevated concentrations. All dogs in the control group had concentrations below the detection limit (Figure 4.1). There was a statistically significant difference in cTnI concentration between the control group and groups 3 ($p = 0.003$) and 4 ($p = 0.006$). There was no statistically significant difference between the babesiosis groups ($p = 0.403$). The 2 dogs that were euthanased had undetectable low concentrations whereas the 3 dogs that died naturally had severely elevated concentrations (4.3, 15.1 and 47.1 ng/ml).

Figure 4.1: Cardiac troponin I concentrations in plasma of 34 dogs with babesiosis and in plasma of 9 unaffected dogs. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes), and 10th and 90th percentiles (T-bars). Black dots represent outliers. An asterisk indicates significant differences between a babesiosis group and the control. The upper limit of normal for cTnI is 2 ng/ml. Group 1 = mild, uncomplicated; Group 2 = severe, uncomplicated; Group 3 = complicated; Group 4 = concurrent IMHA; and Group 5 = control.

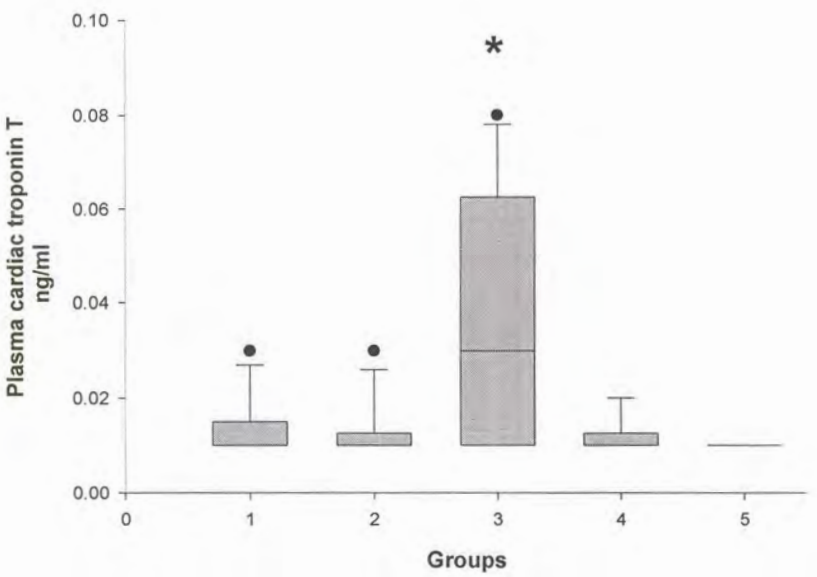


Cardiac troponin T

In the dogs with babesiosis 23 had cTnT concentrations below the detection limit (0.01 ng/ml) and 11 below the upper limit of the reference range (0.09 ng/ml). All dogs in the control group had undetectable concentrations (Figure 4.2). Although still within the upper limit of the reference range, Group 3 was statistically significantly different from the other babesiosis groups (P = 0.002). There was no statistically significant difference between the babesiosis groups (p = 0.093). The 2 dogs that were euthanased had concentrations below

the detection level whereas the 3 dogs that died naturally had concentrations that tended towards the upper limit of the reference range (0.06, 0.07 and 0.08 ng/ml).

Figure 4.2: Cardiac troponin T concentrations in plasma of 34 dogs with babesiosis and in plasma of 9 unaffected dogs. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes), and 10th and 90th percentiles (T-bars). Black dots represent outliers. An asterisk indicates significant differences between a babesiosis group and the control. The upper limit of normal for cTnT is 0.09 ng/ml. Group 1 = mild, uncomplicated; Group 2 = severe, uncomplicated; Group 3 = complicated; Group 4 = concurrent IMHA; and Group 5 = control.



Cardiac lesions

Of the 3 dogs that died naturally, 2 showed sero-haemorrhagic pericardial effusion and macroscopic epi- and endocardial haemorrhages. Histologically these 2 hearts had inflammatory cell infiltrate (round cells and neutrophils) and intercellular haemorrhage and necrosis of myocardial fibres in multiple sites. All 3 dogs had fibrin thrombi in multiple cardiac

blood vessels. Of the 2 dogs that were euthanased, 1 had no cardiac lesions whereas the other had macroscopic epicardial haemorrhage and myocardial necrosis, inflammatory cell infiltrate (round cells and neutrophils) and intercellular haemorrhage in multiple sites. It was not possible to test for correlation between cardiac troponin levels and selected pathological changes as the numbers were too small.

ECG abnormalities

The median heart rates in Groups 1 to 4 at presentation were 134 bpm (range 80-160), 148 bpm (range 116-180), 144 bpm (range 96-172), and 128 bpm (range 84-176), respectively. The median heart rates in Groups 2 to 4, 24 hours after presentation, were 124 bpm (range 60-140), 140 bpm (range 100-144), and 120 bpm (range 80-136), respectively. There were no significant differences in heart rate between the groups or between 0 and 24 hours within the groups. The following changes in the ECG were most commonly found: prolonged QRS duration, ST deviation and coving, and notching of the R-wave. No dogs had abnormalities in P wave amplitude or duration; one mildly affected dog had a prolonged P-P interval; QRS duration was prolonged in 4 mildly affected dogs, one severely affected dog, and 3 dogs with IMHA; QT interval was prolonged in 1 dog with IMHA; ST interval was prolonged in 3 mildly affected dogs, one severely affected and complicated dog, and 4 dogs with IMHA; ST coving was present in 3 mildly affected dogs, one severely affected and complicated dog, and 4 dogs with IMHA; and notching of the R wave was present in 2 mildly affected dogs, one severely affected and complicated dog, and 6 dogs with IMHA. The fewest changes occurred in the complicated group, although this group had the highest number of dogs with elevated cTnI concentrations. Of the 3 non-survivors only 1 dog showed an ECG abnormality, namely notching of the R wave although these 3 dogs had the highest cTnI concentrations and all of them had myocardial lesions on histopathology.

In Group 1 only 1 dog showed an arrhythmia, namely first degree AV block. In Group 2, 2 dogs showed VPCs, and 1 dog had a sinoatrial (SA) block. All 3 dogs showed the arrhythmia

only on the 2nd ECG. In Group 3, 3 dogs showed different arrhythmias on the 1st ECG tracing, namely: SA block with escape rhythm, VPCs, and ventricular tachycardia. The dog with the SA block was euthanased and the other 2 dogs showed a normal ECG tracing on the second evaluation. In Group 4, 2 dogs showed VPCs, one of which developed 24 hours later to ventricular tachycardia, and the other dog had a normal 2nd ECG tracing. The ventricular tachycardia resolved the next day after treatment with mexiletine (Mexitil, Boehringer Ingelheim, South Africa). The 6 dogs showing VPCs had high cTnI concentrations, whereas the dogs with SA block and AV block had undetectable cTnI concentrations. There was a significant correlation between the presence of VPCs and elevated cTnI concentrations.

DISCUSSION

This study demonstrated that myocardial cell injury occurs with canine babesiosis, as dogs that died naturally of disease had myocardial lesions as well as higher concentrations of cardiac troponins in plasma than did those that survived. The study also demonstrated that the dogs from the complicated and the IMHA groups had higher concentrations of cardiac troponins compared with the control group. Thus, cardiac troponins appear to be sensitive markers of myocardial injury in this disease, and the magnitude of elevation of plasma cTnI concentrations appears to be proportional to the severity of the disease.

Cardiac troponins are important cardiac biomarkers in humans and represent a highly sensitive marker for myocardial cell death[103]. In human cardiology, they play an integral and essential role in the diagnosis of myocardial infarction[210]. The cardiac troponin immunoassay developed for diagnosis of cardiac injury in humans is useful across a wide range of species. The amino acid sequence of cardiac troponins in humans and dogs is nearly identical because troponins are phylogenetically highly conserved proteins in mammals and myocardial concentrations of cardiac troponins in man and dogs are similar[157,185].

Cardiac troponins are a more sensitive and persistent indicator of cardiac injury, with high tissue specificity, than other markers in the presence of marked skeletal muscle injury, liver disease, and chronic renal failure[143,157,181]. This is an important consideration in babesiosis, especially the complicated forms, in which renal failure, liver impairment, and rhabdomyolysis can occur[88,116]. Although a number of the plasma samples in this study showed visible icterus or haemoglobinaemia, this has been shown not to cause a clinically significant interference with the test[181]. In this study, the diagnostic sensitivity of cTnT to detect myocardial damage seemed less than that of cTnI, which is in agreement with a previous study in dogs[185].

Although cTnI and cTnT values are useful tools for the diagnosis of acute myocardial infarction in man, they must be interpreted according to the number of hours from the onset of chest pain. The test is particularly useful at ruling out infarction when the concentration is negative at 8 or more hours after the onset of chest pain[60]. With myocardial infarction, concentrations remain elevated for as long as 10 days[40]. In babesiosis, the dog will have been ill for more than 8 hours but usually not for longer than 10 days before presentation[116], which makes the determination of cardiac troponin concentrations ideal for establishing the presence or absence of myocardial injury.

Following myocardial contusion or infarction in the dog, it has been shown that analysis of serum cTnI is more sensitive in detecting myocardial abnormalities than determination of lactate dehydrogenase (LD), creatine kinase (CK), α-hydroxybutyrate dehydrogenase (α-HBDH), MB isoenzyme of CK (CK-MB) or ECG[185]. Aspartate aminotransferase, myoglobin, LD, CK, or α-HBDH are very sensitive markers of myocardial cell damage, but lack cardiac specificity[185]. In babesiosis these variables could also be falsely elevated due to both muscle and liver pathology. A variable of higher specificity for myocardial cell injury in man is the CK-MB. However, the CK-MB isoform in the canine myocardium represents only 4 - 13% of

the total cardiac CK activity, compared to an average of 40% in man[4]. Other sources of CK-MB in dogs are the spleen, muscle, lungs, and intestinal tissue[4], all of which can be affected in canine babesiosis.

Cardiac troponin I has been shown to accurately detect cardiac contusions, with a greater sensitivity than transthoracic echocardiography, and it can detect myocardial injury in the absence of diagnostic ECG abnormalities[2]. A similar finding has been reported in dogs with blunt thoracic trauma[185]. Following cardiac contusions the resultant ischaemia, cytokine release, autonomic dysbalance, reperfusion injury, and/or acid-base and electrolyte derangements may all result in diminished myocardial performance or cardiac arrhythmias. As anaemic hypoxia, and acid-base and electrolyte derangements occur in babesiosis[88,116], it is likely that myocardial integrity is also compromised in this disease.

Conventionally used laboratory variables often fail to show myocyte injury in patients with clinically suspected myocarditis, possibly because of a low extent of myocardial injury in these patients. The measurement of plasma concentrations of cTnT provides more sensitive evidence of cardiac myocyte injury in human patients with clinically suspected myocarditis than does the conventional determination of cardiac enzyme levels[106]. Myocardial cell damage may be present even in the absence of histological signs of myocarditis with additional immunohistologic analysis often showing lymphocytic infiltrates and elevated concentrations of cTnT being highly predictive for myocarditis in these patients[106]. In this study, multifocal myocardial necrosis and secondary inflammation was seen in 3/5 dogs that were necropsied. In man, cardiac troponins (I or T) are the preferred biomarker to detect microscopic zones of myocardial necrosis with almost absolute specificity and sensitivity[210]. Endocardial biopsy is the antemortem standard method to diagnose myocarditis[193]. However, this a very invasive method, and it is not practical in critically ill babesiosis cases. As cTnI has been shown to be elevated in myocarditis[193], it can assist in making this diagnosis in canine babesiosis.

In this study, 3/4 dogs that showed histological myocardial lesions had elevated cardiac troponin concentrations. Although the numbers were small, the results of this study suggest the possibility that an increase in cTnI concentration may be associated with a poor outcome, with all non-survivors having an elevated cTnI concentration. In addition, non-survivors tended to have higher cTnT concentrations than survivors. It has been shown that high and persistently elevated concentrations of cTnI reflect ongoing cardiac damage[215].

This study showed that dogs with babesiosis can develop a variety of ECG changes. However, the incidence of the changes was not higher in either the complicated or the non-survivor groups. Only one non-survivor in this study showed ECG changes, which was notching of the R wave. However, all non-survivors had both myocardial lesions on histopathology and elevated cTnI concentrations. One dog that was euthanased had a SA block and junctional ventricular escape beats, but no myocardial lesions and undetectable cTnI and cTnT concentrations. This illustrates that there is not always a clear-cut relationship between ECG changes and myocardial injury.

The exception was the presence of VPCs, which were associated with elevated cTnI concentrations. Nine dogs in this study had elevated cTnI concentrations but showed no VPCs. Thus in this study, the presence of VPCs as an indicator of myocardial damage on an ECG appears to have high specificity but low sensitivity. The sensitivity and specificity of the ECG to diagnose myocardial damage could have improved by longer and more frequent ECG recordings and/or by the use of 24-hour Holter recordings.

Although ST-segment elevation does not reliably indicate myocardial injury, a significant cTnI increase is seen in human patients with acute pericarditis in correlation with ST elevation[22]. Myocardial ischaemia, infarction or reperfusion may be important causes of myocardial cell injury and are commonly associated with ST segment changes[185,213]. In this study, as ST coving was not correlated with cardiac troponin concentration, it can be

concluded that ST changes on ECG are not specific predictors of increased concentrations of circulating cardiac troponins, which is similar to what has been reported in another study in dogs[185].

Notching of the R wave can be indicative of myocardial infarction[203]. In this study, notching of the R wave was an inconsistent finding in Groups 1-3, but more common in Group 4. Group 4 represented dogs with secondary IMHA, the primary form of which has been shown to predispose dogs to thrombo-embolic disease and myocardial infarction[58]. Only 1 non-survivor showed notching of the R wave and had myocardial haemorrhage and cell infiltration on histopathology.

CHAPTER 5

CARDIAC PATHOLOGY

INTRODUCTION

Heart lesions appear to be rare in canine babesiosis[88,116], but the prevalence has never been reported. Gross lesions that have been identified are subepicardial and subendocardial haemorrhages[139], hydropericardium, and haemopericardium[26,73,148], and, rarely, focal necrosis and infarctions (Unpublished necropsy reports, Pathology Section, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria). Histologically, microthrombi have been described in the myocardium[148], which may lead to infarction and necrosis[26]. Multifocal necrosis with secondary macrophage and neutrophil infiltration are infrequently reported (Unpublished necropsy reports, Pathology Section, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria). Dilated and congested cardiac blood vessels with considerable numbers of infected erythrocytes and free parasites have been also reported[73,122], as well as haemorrhages[26]. In cattle and horses, ecchymotic haemorrhages in the epicardium, endocardium and myocardium have been described, but they are non-specific agonal lesions in these species[83]. In cattle infected with *B. bovis,* blood vessels in the myocardium had distinctly higher parasitaemia than the jugular blood, and degeneration of the myocardium and myocardial haemorrhages have been described[225].

According to the redefinition of sepsis in human literature, which includes systemic response to any infection (bacterial, viral, fungal and protozoal)[20,21], babesiosis is a septic condition. The synergistic combination of tumour necrosis factor-α and interleukin-1 β has been identified as playing an important role in the mechanism of cardiac myocyte depression in septic shock[102]. These mediators play an important role in malaria infection[223] and have been

proposed to play the same role in babesiosis[88]. An older explanation for myocardial depression in septic shock is ischaemia[162], which is part of the proposed pathophysiology of complicated babesiosis[175]. Various electrocardiographic (ECG) changes have been described for sepsis. Ventricular premature contractions are a common ECG change detected in sepsis or endotoxic-like states in the dog[49,120,132,151,152,154] and can develop to ventricular tachycardia (VT), and multifocal ventricular premature contractions (VPCs) that can result in asystole and death. Other changes recorded in dogs and cattle with sepsis are premature atrial depolarization, atrial fibrillation[152], first or third degree AV block, ST segment slurring, and left bundle branch block[135,189]. Tachycardia is a common sign of sepsis[162], and in man, a heart rate below 106 beats per minute (bpm) at presentation and below 95 bpm, 24 hours after the onset of septic shock, have been found to be good prognostic indicators[161].

Light microscopic examination of hearts from dogs with induced sepsis showed minimal myocardial neutrophilic infiltrate and no necrosis, but electron microscopy showed endothelial cell oedema, capillary intraluminal fibrin deposition, focal myofibrillar loss, mitochondrial loss, mitochondrial swelling and degenerative myelin[194]. In a study on dogs with gastric dilatation and volvulus, 9/13 dogs had myocardial degeneration and necrosis and 5 of them had concurrent inflammatory infiltrate. An attempt to correlate these lesions with cardiac arrhythmias was unsuccessful[153].

The acute phase in human malaria has many similarities to canine babesiosis[88,122,177]. Gross cardiac changes that have been described in malaria include general dilatation[138] and pericardial effusion[68]. Histological changes that have been reported include myocarditis, local haemorrhages in the endocardium and epicardium[23,138], fragmentation of myocardial fibres[197], necrosis, and ischaemia[16]. Changes associated with myocarditis, included foci of inflammatory infiltrates consisting of histioid cells, young fibroblasts, round cells and plasma cells[180]. Somewhat similar infiltrate was also noted in the pericardium[180]. Fatty degeneration

of the myocardium following vascular occlusion has also been described[23,138,197]. Myocardial degeneration and necrosis can be extensive and resemble myocardial infarction in rare cases[23,138]. Capillaries within the myocardium showed microthrombi, dilatation, congestion, and sequestration of parasitised erythrocytes with resultant capillary and coronary vascular occlusion[34,180]. The endothelial wall contained parasites, and capillary endothelium showed cytoplasmic changes with hyalinosis of the interstitial collagen tissue especially near the epicardium and beyond the muscular tissue of both ventricles[180]. In a study comparing the prevalence of parasitised erythrocyte sequestration in various organs, the heart had the second highest prevalence after the brain and before the liver, lung and kidneys. However, in this study the vessels did not appear tightly packed, and there were no signs of endothelial damage[121]. The cardiac lesions are most commonly acute, but endomyocardial fibrosis as a chronic process due to malaria has been rarely reported[23,187]. There are no precise data about prevalence of cardiac pathology in malaria; it has been described by some authors as rare[187] and by others as occasional[23].

MATERIALS AND METHODS

Gross pathology and histopathological assessment of the heart was done on 16 dogs that had died of canine babesiosis. All the dogs were presented to the Onderstepoort Veterinary Academic Hospital (OVAH). Dogs that showed lesions typical for pre-existing heart disease, such as moderate and severe endocardiosis and chamber dilatation or hypertrophy, were excluded from the study.

Inclusion criteria were:

- Babesiosis diagnosed on a thin capillary blood smear, stained with Cams Quick stain (CA Milsch, Krugersdorp, South Africa) on light-microscopy.
- Any breed or sex.

- No cardiac disease previously diagnosed or treated, or suspected at presentation on full clinical examination.

Exclusion criteria were:

- Any concurrent medication started before presentation.
- Clinical signs that had not been previously described for babesiosis.
- Evidence of chronic heart problem.
- Concurrent *Ehrlichia canis* or *Anaplasma platys* identified on peripheral blood smear.

Data collection

To avoid post mortal changes, which could mimic ischaemia and necrosis, samples were collected 30 - 60 minutes after death. The pericardium was examined for fluid and, if present, its colour. The heart was opened in standard fashion, and evaluated for changes such as dilatation, gross valvular and jet lesions. Both the endocardium and the epicardium were evaluated macroscopically for haemorrhages (size and location) and gross infarcts. Samples were taken for histological examination from the following sites: papillary muscles of the left and right ventricles, interventricular (IV) septum, left and right auricle of the atrium, and apex. Full thickness (from epi- to endocardium), 5 – 7 mm thick sections were taken and placed in marked containers with 10% buffered formalin. Additional samples were taken from haemorrhages seen macroscopically.

Samples were wax embedded, sectioned at 5 µm, and stained with haematoxylin and eosin and modified Martius Scarlet blue, specifically for fibrin. Sections were evaluated by light microscopy.

Twenty 400x, adjacent but not overlapping fields, 10 from the subendocardium and 10 from the subepicardium of each site, were examined. The following was noted and scored separately for each site: necrosis, inflammatory cell infiltration, haemorrhage, and fibrin.

Necrosis of myocardial fibres was recognised by increased cytoplasmic eosinophilia, loss of striation, fragmentation and nuclear pycnosis. Scoring was done as follows:

0: No necrotic cells found in any 1 of the 20 fields.

1: Five or less necrotic fibres in any 1 of the 20 fields.

2: Six to twenty necrotic fibres in any 1 of the 20 fields.

3: More than 20 necrotic fibres in any 1 of the 20 fields.

Other parameters such as location within the myocardium (subepicardial, mid-myocardial or subendocardial) and the distribution (focal, multifocal or diffuse) were also noted.

Interstitial myocardial infiltration was examined for the cell type, location and distribution, and was scored as follows:

0: No inflammatory cells in any 1 of the 20 fields.

1: Five or less cells in any 1 of the 20 fields.

2: Six to 20 inflammatory cells in any 1 of the 20 fields.

3: More than 20 inflammatory cells in any 1 of the 20 fields.

Haemorrhages and fibrin microthrombi were examined for their location. Both haemorrhage and fibrin were scored 0 if they were not present in any field and 1 if present.

RESULTS

Macroscopic study of cardiac lesions

Twelve of the 16 dogs had macroscopic cardiac lesions including pericardial effusion and pericardial, epicardial and endocardial haemorrhages (Table 5.1). Four dogs had pericardial effusion, defined as more than 5 ml of effusion; three of these had about 10 ml and one had more than 20ml. The fluid was red, clear, and watery, resembling haemolysed serum. One dog had a large pericardial ecchymosis. Ten dogs had macroscopic epicardial haemorrhages. These haemorrhages tended to be superficial and did not extend more than

3 mm into the myocardium. They were multifocal in six dogs, focal in two and diffuse in two. The diffuse haemorrhages extended to all chambers in one dog, and were limited to the ventricles in the other one. The distinct epicardial haemorrhages were ≤ 5mm in diameter in 6/8 dogs, 20mm in one and 30mm in another. The most common site of the epicardial haemorrhages was the left ventricle (n = 8) especially around the coronary arteries, followed by right ventricle (n = 4), right atrium (n = 3) and left atrium (n = 1). Macroscopic endocardial haemorrhages were seen in nine dogs, five multifocal, three focal and one extending diffusely through the left ventricle. All were ≤ 2mm deep. Seven out of eight distinct haemorrhages were ≤ 5mm in diameter and one was 20mm. The most common site of the haemorrhages was the left ventricle (n = 6), especially at the papillary muscles followed by the right ventricle (n = 4), right atrium (n = 3) and left atrium (n = 1).

Histopathological study of the myocardial lesions

Fifteen of the dogs had at least one microscopic lesion. Necrosis (score ≥ 2) was seen in eight dogs, in the left ventricle (n = 2), IV septum (n = 2), left atrium (n = 3), right atrium (n = 1) and apex (n = 2). The necrosis was moderate (score 2) in 80% of the lesions, severe (score 3) in 20%, multifocal in 80% and focal in 20%. The location of the necrotic fibres ranged from subepicardial myocardium to subendocardial myocardium with no predilection site. The foci tended to be concentrated in one area of the cardiac muscle (seen in a particular area in 80% of lesions and scattered in 20%). Cell infiltrate (score ≥ 2) was seen in 10 dogs, in the left ventricle (n = 6), right ventricle (n = 4), IV septum (n = 3), left atrium (n = 3), right atrium (n = 6), and apex (n = 3). The inflammatory infiltrate was moderate in 64% (score 2) and severe (score 3) in 36%. In 96% of the sites with inflammatory infiltrate, it was multifocal and diffuse in 4%. The cell infiltrate was composed of round cells (macrophages and lymphocytes) in 57% of the lesions, round cells and neutrophils in 36% and neutrophils in 7%. In 53% of the lesions the inflammatory foci were scattered in the myocardium, while in

the rest of the lesions they were limited to one area mainly in the subendocardial myocardium (21%), or the subepicardial myocardium (11%).

Microscopic haemorrhages were seen in 11 dogs: in the left ventricle (n = 8), right ventricle (n = 4), IV septum (n = 5), left atrium (n = 2), right atrium (n = 1), and apex (n = 6). The haemorrhages were multifocal in 81% and focal in 19%. The haemorrhages were located from subepicardial myocardium to subendocardial myocardium with no predilection site, and tended to be concentrated in one area of the cardiac muscle (seen in a particular area in 84% of lesions and scattered in 16%).

Fibrin microthrombi were seen in 12 dogs; in the left ventricle (n = 8), right ventricle (n = 3), IV septum (n = 5), left atrium (n = 4), right atrium (n = 4), and apex (n = 6). The fibrin microthrombi were multifocal in 94% and focal in 6%. The fibrin microthrombi tended to be concentrated in one area of the cardiac muscle (seen in one area in 71% of lesions and scattered in 29%), and the location ranged from subepicardium (26%) to mid-myocardium (19%) to subendocardium (13%).

Table 5.1: The distribution of the cardiac macro- and microscopic lesions in 16 dogs that died of canine babesiosis

	Macroscopic haemorrhages		Necrosis	Cell infiltrate	Microscopic haemorrhages	Fibrin micro-thrombi
	Epicardial	Endocardial				
Number	10	9	8	10	11	12
Left atrium	1	1	3	3	2	4
Right atrium	3	3	1	3	1	4
Left ventricle	8	6	2	6	8	8
Right ventricle	4	4	0	4	4	3
IV septum	0	0	2	3	5	5
Apex	N = 0	N = 0	N = 2	N = 3	N = 6	N = 6
Multifocal	60%	56%	80%	96%	81%	94%
Focal	20%	33%	20%	0%	19%	6%
Diffuse	20%	11%	0%	4%	0%	0%
Distinct area	100% (Superficial)	100% (Superficial)	80%	37%	84%	71%
Scattered	0%	0%	20%	53%	16%	29%

In summary, myocardial necrosis, inflammation, haemorrhage or fibrin microthrombi occurred at similar rates in this study. Lesions tended to be multifocal, but were generally limited to one area within the myocardium. The ventricles were the most common site, especially the left ventricle including the apex.

DISCUSSION

Pericardial effusion was seen in four of the 16 dogs that had been necropsied in this study, and it was always clear and watery, consistent with the definition of hydropericardium[179]. In one study it was found to be the most common cardiac lesion in association with myocardial necrosis in dogs and cats[100]. In this study, only one dog with pericardial effusion had myocardial necrosis. Low albumin and increased capillary permeability are two other possible mechanisms of effusion that have been described in canine babesiosis[26,73,91,117,122]. The volume of fluid seen in the pericardium in this study was relatively small, and would not be expected to affect the heart function[179].

Myocardial haemorrhages are seldom seen in dogs, and if seen are more common in the subepicardium[83]. They were, however, not a rare finding in this study. Both macroscopic and microscopic haemorrhages did not show predilection for any specific site. Myocardial haemorrhages have been reported in hypoxia, acute infectious diseases[179] and DIC[175], all of which are part of the described pathogenesis of canine babesiosis[88]. As coagulopathy was diagnosed in this study, based on the presence of haemorrhages, it is not surprising that all dogs that had coagulopathy also had myocardial haemorrhages. Interesting is that 5/10 dogs with subepicardial haemorrhages, 6/9 with subendocardial haemorrhages and 6/11 with microscopic haemorrhages, did not have haemorrhages in any other organ. Haemorrhages limited to the epi- or the endocardial surface of the heart can be an agonal change. This finding is relatively rare in the dog in comparison with horses and cattle[179], however, and thus might instead indicate a specific babesiosis-related lesion.

Myocardial necrosis is a common, non-specific finding in systemic diseases, especially infectious and anaemic diseases. This necrosis is commonly diffuse[179], while in this study the necrosis was commonly multifocal. Myocardial necrosis, seen in eight dogs in this study, has previously been described in canine babesiosis[26]. Necrosis can only be apparent 12

hours after injury[83,179], and could have been overlooked or underscored, because of the acute death seen in all cases in this study (all dogs died within 24 hours of hospitalisation). The nature of the necrotic lesion in our study, namely degeneration with inflammatory infiltrate but no fibroblasts, is consistent with acute necrosis of 12 hours to 4 days duration[153]. Myocardial necrosis can be a consequence of either coronary artery obstruction or inadequate oxygenation of the myocardial tissue[100]. Microthrombi because of a hypercoagulable state due to DIC or IMHA[100], are relatively common obstructive lesions in the dog and cat. Endothelial injury is another cause of thrombosis[179], and is assumed to occur in babesiosis[175]. Hypoxia associated with shock can also cause myocardial necrosis[218].

There is an association between disseminated myocardial necrosis and central neurological diseases[179] possibly through neurologically induced catecholamine release causing coronary spasm[218]. Myocardial necrosis might also be induced by stress associated with a disease process[218]. Association between myocardial necrosis and brain lesions was described retrospectively in a large number of dogs. The brain lesion can be traumatic, infectious or space occupying[101]. Myocardial necrosis was also induced experimentally by creating intracranial haemorrhage in dogs[63]. This necrosis was accompanied by typical ECG changes and echocardiographic myocardial wall motion abnormalities[63]. The necrosis was multifocal and subepicardial, predominantly in the left ventricle[101]. In this study 3/8 dogs with myocardial necrosis had histologic lesions in the brain. Three other dogs, however, had brain lesions and no myocardial necrosis. No cause and effect relationship could thus be established. The acute death can also be a factor in the lack of association, since there is a lag time of up to several days between the brain damage and the myocardial necrosis[101].

All the conditions reported to be associated with myocardial necrosis, namely IMHA, DIC, brain involvement, hypoxia and shock, are reported complications of babesiosis[88], which were seen in this study. However, association between a specific complication and

myocardial necrosis was not found. A cause and effect relationship between microthrombi and necrosis was not detected at any site, but of 10 sites with necrosis in this study, 7 also had fibrin microthrombi, indicating a possible association. Myocardial necrosis and infarctions in dogs are found predominantly in the subepicardium[58]. In necrosis due to hypoxia and arteriosclerosis, the site reported is predominantly the subendocardium of the papillary muscles of both ventricles[65,218]. This was not the case in this study and there was no predilection for any site of necrosis within the myocardium (e.g., endocardium, mid-myocardium or epicardium). The left ventricular papillary muscles were not over presented. Combining the apex and the IV septum with the left papillary muscles as left ventricle sites revealed that 60% of necrosis was within the left ventricle. Thus, the distribution of necrosis is not classical to hypoxia and DIC and might represent a pattern of disseminated necrosis more typical of canine babesiosis.

Inflammatory changes in the myocardium are relatively frequent in the dog[119]. They are commonly secondary to myocardial necrosis or systemic infectious disease[179], but are rarely considered as the primary disease[119]. The inflammatory infiltrate following necrosis is commonly neutrophilic[100]. In this study inflammatory cells were present in eight of the 10 sites with necrosis, but inflammatory infiltrates were seen in 20 other sites without necrosis. The type of inflammatory cells included neutrophils and macrophages/lymphocytes in about equal numbers. Thus, besides the inflammatory reaction secondary to necrosis, there could have been an inflammatory reaction due to another process. Overwhelming inflammatory response was suggested as one of the mechanisms for tissue damage in canine babesiosis[175], and it may be responsible for part of the myocardial inflammation seen in this study.

Fibrin microthrombi throughout the body are the hallmark of DIC[184]. In this study 12/16 dogs had myocardial fibrin microthrombi, but only four had coagulopathy and were suspected to have DIC. However, the morphological diagnosis has serious limitations due to the

fibrinolytic process that eliminates fibrin thrombi, and the similarities between fibrin thrombi and post-mortal clots[184]. Immune peroxidase stains are assumed to increase the sensitivity of the morphological diagnosis because they can react with fibrinogen and fibrin degradation products[188].

Immune-mediated haemolytic anaemia is another condition associated with myocardial thrombosis[58,100] that has been described in canine babesiosis. Fibrin microthrombi containing aggregates of parasitised erythrocytes within blood vessels is another possible cause for fibrin microthrombi[73]. Parasites were seen in capillaries within the myocardium in this study, but parasitic plaques similar to the description above were not identified.

In summary, myocardial lesions were seen in high prevalence in this study, but they were all non-specific lesions. It can, however, justifiably be said that the heart suffers from the same pathological processes, which have been described for other organs involved in canine babesiosis, namely inflammatory reaction and ischaemia[88].

The study showed no correlation between ECG changes and histopathological changes. This is consistent with the literature and other similar studies[153]. It reflects the non-specific properties of the ECG, which cannot differentiate cardiac and extracardiac causes of altered heart conduction, as well as the fact that the numbers of necropsies in clinical studies is always limited. Heart pathology is also often non-specific, since there are similar changes involved in many disease and agonal processes[179,218]. The changes seen in these studies are multifactorial, and canine babesiosis has a variety of metabolic and pathologic abnormalities that can contribute to both ECG and pathological changes[88,218].

The long-term implications of the cardiac pathology detected in this study were not investigated. Chronic cardiac complications are exceptionally rare in malaria[187]. The prevalence of chronic myocardial diseases at the OVAH is low (0.03%) compared to the

literature (0.5-1.1%)[190], which may also indicate a lack of chronic changes from babesiosis, despite the high prevalence of the disease.

CHAPTER 6

SERUM PROTEIN RESPONSE

INTRODUCTION

Alterations in total serum proteins, serum protein quantification, serum protein electrophoretic changes, and the acute phase response in naturally occurring canine babesiosis have been poorly described. They have, however, been well described in pyrexia, haemolytic disease, some infectious diseases, acute inflammation and shock[207], all of which are known to occur, in various combinations, in canine babesiosis. Alterations and patterns of changes in serum protein electrophoresis have been described in other haemoprotozoan diseases of dogs, cats, other domestic animals[72,146,207], and in malaria[39,56], a disease that has many similarities to babesiosis[88]. There is thus reason to believe that serum protein alterations are likely to occur in canine babesiosis.

The interpretation of electrophoretograms in dogs suffering from babesiosis and with suspected concurrent disease is currently hampered by unfamiliarity with the electrophoretic changes in both uncomplicated and complicated babesiosis cases. In the literature only 3 studies have been documented that report on serum protein electrophoresis changes in canine babesiosis[122,128,208]. However, these findings are not consistent, nor are the severity or complications of the disease reported. The one study was in experimental dogs[208], whereas the other two were in naturally occurring disease[122,128].

The purpose of this investigation was to retrospectively evaluate total serum protein, albumin, globulin, globulin fractions (alpha, beta and gamma globulins), and α1-acid glycoprotein (αGP), an acute-phase protein, and to identify any patterns, if present, in dogs with naturally

occurring *B. canis* infection. *Ehrlichia canis* titres were also determined to evaluate the possible effect of concurrent ehrlichiosis on the serum protein values.

MATERIALS AND METHODS

The study was retrospectively done on 29 stored serum samples of dogs with babesiosis, diagnosed on a stained, thin capillary blood smear (stained with Cams Quick stain (CA Milsch, Krugersdorp, South Africa)). Samples from 10 healthy dogs were used as a comparison group. All the dogs were from the Onderstepoort area presented at the Onderstepoort Veterinary Academic Hospital (OVAH) and all samples were collected prior to any treatment. Three groups of babesiosis cases were identified: mild uncomplicated, severe uncomplicated, and complicated. Mild cases had mild-to-moderate anaemia (haematocrit 20-30%) with no clinical or biochemical signs of concurrent disease. Severe cases had severe anaemia (haematocrit < 15%) with no clinical or biochemical signs of concurrent disease. Complicated cases had one or more of the following complications: cerebral signs, acute renal failure, acute respiratory distress syndrome, hypotensive shock, haemoconcentration, or immune-mediated haemolytic anaemia. This classification system is a clinically based system as proposed by Jacobson and Clarke[88]. Samples from dogs that were presented to the OVAH for routine ovariohysterectomy and thus from the same area as the babesiosis cases, were used as a comparison group.

The Section of Clinical Pathology of the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria, used standard methods for total serum protein, albumin, and globulin determination. Total serum protein and albumin were determined using a Technicon RA-1000 system (Technicon Instruments Corporation, Tarrytown, New York, USA). Total serum proteins were determined with the automated Biuret reagent method. The albumin was determined with the Bromcresol method, whereas the serum globulins were calculated by subtracting the albumin value from the total serum protein.

The albumin-globulin (A/G) ratio was calculated. Serum protein electrophoresis to identify alpha (α), beta (β) and gamma (γ) peaks was performed on a standard Beckman Microzone electrophoresis system, utilising a cellulose acetate membrane and Barbital buffer (0.5 ionic strength and pH 8.6). This system identifies α1 and 2, β1 and 2, and γ peaks. The αGP was quantitatively determined using a commercially available radial immuno-diffusion test kit (Canine α1 AG plate, Cardiotech Services, Louisville, Kentucky, USA).

Ehrlichia canis titres were determined utilising a standard canine ehrlichiosis fluorescent antibody substrate slide using the Oklahoma strain as antigen and fluorescein conjugated anti-dog IgG (VMRD, PO Box 502, Pullman, Washington, USA). A positive reaction was taken as $\geq$ 1:40, with sera not reactive at this dilution being regarded as negative[133].

Statistical analyses of the data were performed on a commercial statistical software package (SigmaStat software, Jandel Scientific Software, USA). The data were compared using the Kruskal-Wallis one-way analysis of variance on ranks comparing the serum protein parameters between the groups. The Tukey test was used for pairwise comparisons between individual parameters within the groups. Significance was set at $p < 0.05$.

RESULTS

Twenty-nine babesiosis cases were evaluated: Group 1 comprised of 10 mild uncomplicated cases; Group 2 of 10 severe uncomplicated cases; and Group 3 of nine complicated cases. Ten healthy dogs were used a comparison (Group 4). Results are tabulated in Table 6.1. The complications recorded in this study were immune-mediated haemolytic anaemia (2), haemoconcentration (3), acute respiratory distress syndrome (2), and hypotensive shock (2).

Table 6.1: Age, breed, *Ehrlichia canis* titres, serum protein values, means and standard deviations in mild, severe, and complicated canine babesiosis cases

		Breed	Age (Months)	Serum protein (g/l)	Albumin (g/l)	Globulin (g/l)	A/G ratio	Alpha (g/l)	Beta (g/l)	Gamma (g/l)	αGP (µg/ml)	*E. canis* Titre
GROUP 1 Mild cases	1	Bull terrier	18	56.2	34.9	21.3	1.64	10.8	15.8	5.0	1400	-
	2	Toy pom	36	49.2	32.9	16.3	2.02	11.2	11.9	3.5	1720	-
	3	Staffie[a] X	12	59.1	33.4	25.7	1.3	13.1	17.5	4.3	2580	-
	4	Doberman	36	57.9	33.8	24.1	1.4	12.4	13.5	6.9	1300	+ (w)
	5	Great Dane	36	56.6	28.5	28.1	1.01	12.6	13.6	9.3	2840	-
	6	Bull terrier	6	49.7	32.6	17.1	1.91	9.8	11.8	4.0	2200	-
	7	Boer-boel	12	52.4	28.3	24.1	1.17	10.8	13.4	7.1	2480	+ (s)
	8	Staffie	20	50.0	30.1	19.9	1.51	11.0	13.0	5.0	1370	+ (s)
	9	Chow X	36	50.4	32.8	17.6	1.86	9.4	13.8	5.0	1280	-
	10	GSD[b] X	132	66.9	36.1	30.8	1.17	13.7	17.1	8.0	340	+ (w)
	Mean	**N/A****	**34.40**	**54.84**	**32.34**	**22.5**	**1.49**	**11.48**	**14.14**	**5.81**	**1751**	**N/A**
	SD*	**N/A**	**36.24**	**5.63**	**2.95**	**4.89**	**0.34**	**1.41**	**1.99**	**1.91**	**766.79**	**N/A**
GROUP 2 Severe cases	1	Labrador	24	49.0	21.5	27.5	0.78	9.8	9.7	13.8	2450	+ (w)
	2	Dalmatian	156	54.7	30.8	23.9	1.29	9.9	14.2	5.3	2120	-
	3	Spaniel	10	55.1	24.9	30.2	0.82	11.3	9.7	16.0	2180	+ (s)
	4	Staffie X	36	77.3	25.2	52.1	0.48	8.8	16.3	32.5	2180	+ (s)
	5	Ridgeback X	9	77.0	20.7	52.3	0.4	11.6	13.8	32.5	1300	-
	6	Rottweiler	108	61.5	25.4	36.1	0.7	13.0	15.8	13.7	2840	+ (w)
	7	Pug	5	47.6	28.3	19.3	1.47	12.2	14.6	3.3	2450	+ (w)
	8	Boer-boel	36	51.0	19.4	31.6	0.61	10.3	13.9	12.1	2240	+ (w)
	9	Fox terrier	5	39.4	18.8	20.6	0.91	9.8	8.3	6.6	2840	+ (s)
	10	Labrador	5	45.3	26.5	18.8	1.41	11.7	8.7	5.7	1820	-
	Mean	**N/A**	**39.4**	**55.39**	**24.15**	**31.24**	**0.88**	**10.84**	**12.5**	**14.15**	**2242**	**N/A**
	SD	**N/A**	**51.53**	**12.04**	**3.94**	**12.39**	**0.38**	**1.31**	**3.05**	**10.57**	**458.23**	**N/A**

Table 6.1 (continued): Age, breed, *Ehrlichia canis* titres, serum protein values, means and standard deviations in mild, severe, and complicated canine babesiosis cases

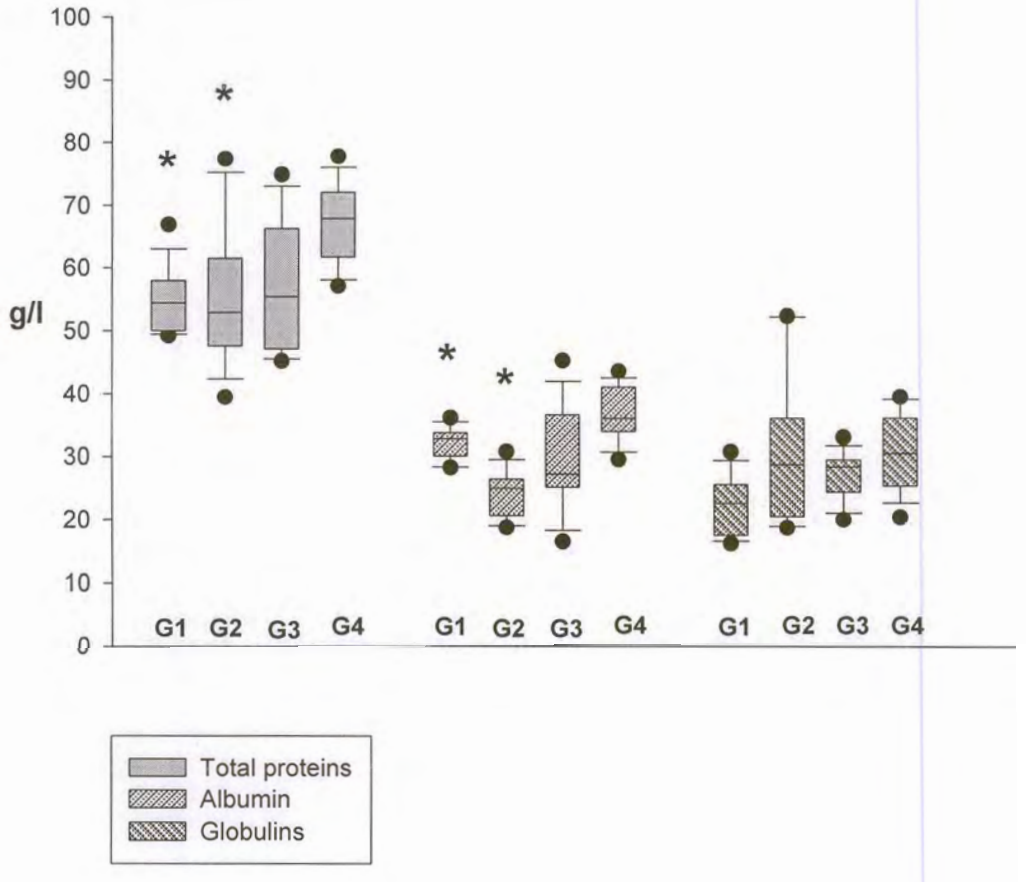
GROUP 3 Complicated cases	1	Maltese	18	55.3	28.1	27.2	1.03	13.6	11.4	9.3	1680	-
	2	GSD	5	46.2	21.1	25.1	0.84	10.2	6.8	11.1	1860	-
	3	Bull terrier	36	65.0	36.5	28.5	1.28	12.7	20.2	9.7	1220	-
	4	Bull terrier	54	74.8	45.3	29.5	1.54	17.7	25.5	7.1	2800	+ (w)
	5	Staffie	108	49.4	26.6	22.8	1.17	10.6	12.6	6.1	1750	+ (s)
	6	Boer-boel	18	70.2	37.1	33.1	1.12	16.2	20.0	7.2	1640	-
	7	Bull dog	36	56.5	26.6	29.9	0.89	12.5	21.4	8.8	620	-
	8	GSD X	8	47.4	27.3	20.1	1.36	13.2	7.8	6.6	2660	-
	9	Staffie	20	45.2	16.6	28.6	0.58	11.5	10.3	11.3	1040	+ (w)
	Mean	**N/A**	**33.67**	**56.66**	**29.46**	**27.2**	**1.09**	**13.13**	**15.11**	**8.57**	**1696.67**	**N/A**
	SD	**N/A**	**31.83**	**10.97**	**8.78**	**3.95**	**0.29**	**2.46**	**6.73**	**1.92**	**706.67**	**N/A**
GROUP 4 Healthy	1	Staffie X	60	67.4	34	33.4	1.02	15.7	19.1	6.8	480	-
	2	Terrier X	36	77.7	41.5	36.2	1.15	17	17.6	3.9	440	+ (w)
	3	Boxer	48	68.4	29.6	38.8	0.76	12.6	26.9	3.8	590	-
	4	Boxer	18	57	31.9	25.1	1.27	10.8	14.2	6.1	400	-
	5	Boxer	36	72	37.2	34.8	1.07	15.2	20.6	5.6	400	-
	6	Border collie	84	74.3	34.8	39.5	0.88	15.2	26.2	5.5	460	+ (w)
	7	Staffie	20	66.6	41.1	25.5	1.61	15.1	14.3	6.6	660	-
	8	Terrier X	6	59.1	38.6	20.5	1.88	13.4	10.3	4.1	360	-
	9	Chow	26	71.5	43.6	27.9	1.56	16	13.8	7.8	680	-
	10	GSD	16	61.7	34.9	26.8	1.3	11.4	15.9	7.4	620	+ (w)
	Mean	**N/A**	**35**	**67.57**	**36.72**	**30.85**	**1.25**	**14.24**	**17.89**	**5.76**	**509**	**N/A**
	SD	**N/A**	**23.56**	**6.68**	**4.49**	**6.51**	**0.35**	**2.07**	**5.42**	**1.45**	**117.80**	**N/A**

*Standard deviation **Not applicable ***Weakly positive ***Strongly positive

[a] Staffordshire bull terrier [b] German shepherd dog

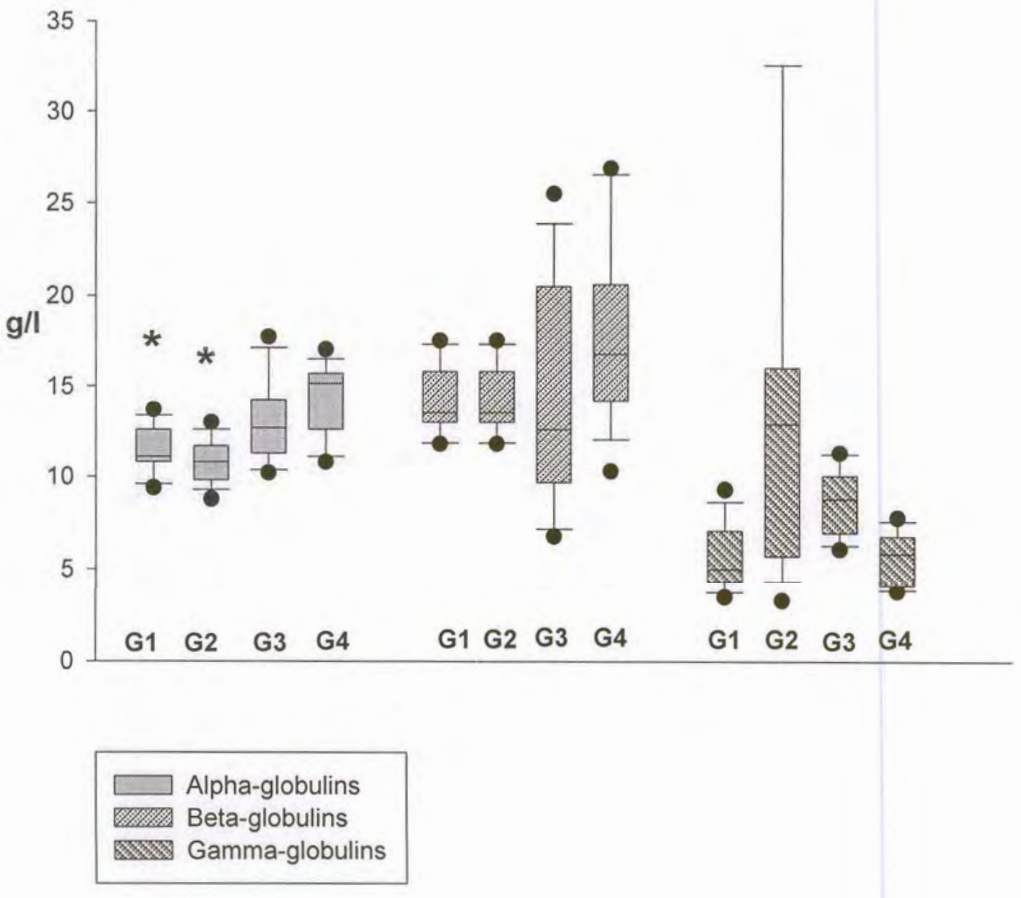
Total serum protein was statistically different between Groups 1 and 2 and Group 4, in those that Groups 1 and 2 showed a lower total serum protein. A similar finding was evident with albumin and the A/G ratio. No statistical difference was present in the total globulin values (Figure 6.1).

Figure 6.1: Total serum proteins, albumin, and globulins for dogs with mild uncomplicated (G1), severe uncomplicated (G2) and complicated (G3) canine babesiosis. G4 = control. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes), and 10th and 90th percentiles (T-bars). An asterisk indicates significant group differences. Black dots represent outliers.



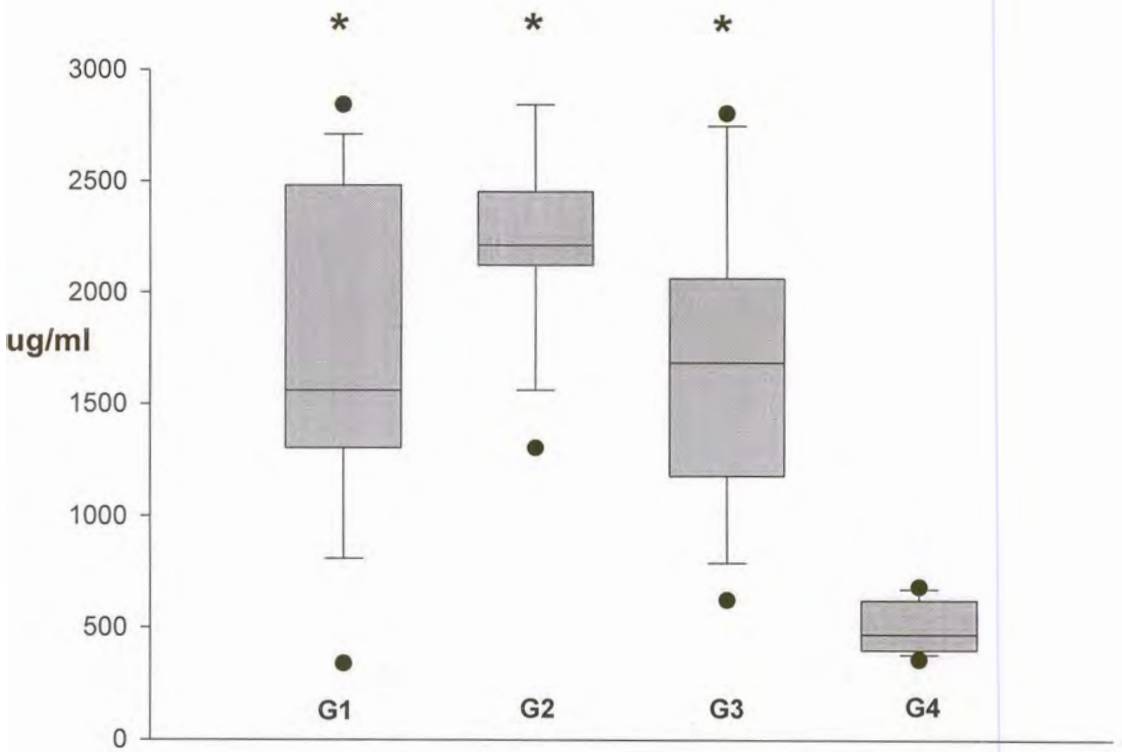
The α globulins were statistically different between Groups 1 and 2 and Group 4 in that the α globulins were lower in Groups 1 and 2. There was no statistical difference between Groups 1, 2 and 3 and there were no specific patterns with the various complications. Both the serum β and γ globulins were not significantly different between the three babesiosis groups (Figure 6.2).

Figure 6.2: Serum protein fractions for dogs with mild uncomplicated (G1), severe uncomplicated (G2) and complicated (G3) canine babesiosis. G4 = control. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes), and 10th and 90th percentiles (T-bars). An asterisk indicates significant group differences. Black dots represent outliers.



Twenty-seven out of the 29 babesiosis cases showed a massive αGP response when compared to Group 4, which was statistically significant. There was, however, no statistical difference in the αGP response between Groups 1, 2, and 3 (Figure 6.3).

Figure 6.3: α1- acid glycoprotein for dogs with mild uncomplicated (G1), severe uncomplicated (G2) and complicated (G3) canine babesiosis. G4 = control. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes), and 10th and 90th percentiles (T-bars). An asterisk indicates significant group differences. Black dots represent outliers.



All groups showed positive *E. canis* titres: four of the dogs in Group 1, seven of dogs in Group 2, and three dogs in both Groups 3 and 4. A positive titre was taken as ≥ 1:40. At this titre, only six dogs were strongly positive, at a titre of ≥ 1:40. Of the *E. canis* positive dogs, only one dog in Group 2 showed a corresponding increase in the total and γ globulins.

There was no age difference between the 4 groups. The mean age for all groups was approximately 35 months. Group 3 appeared to have more fighting breeds (bull terriers and Staffordshire terriers) than the other groups.

DISCUSSION

The results of this study suggest that dogs with mild and severe babesiosis had low total serum proteins, albumin, A/G ratio, and α globulins, whereas dogs with complicated babesiosis showed no typical serum protein changes or patterns. It was also shown that dogs with babesiosis had no evidence of an acute phase response detectable on serum protein electrophoresis. A massive acute phase response, as measured by the αGP was present in all the babesiosis groups, however.

In a study of pathological processes in *B. canis* infection, Maegraith *et al.*[122] identified a decrease in serum albumin concentration with a concomitant increase in serum globulin concentration and postulated that the latter might be due to increased immunoglobulin production. Quantification of the various globulin fractions was, however, not performed to test this hypothesis. The decreased albumin concentration corresponds to the findings in this study. It is difficult to correlate the globulin findings in that study[122], however, as the serum protein fractions were not quantified and the babesiosis cases were not classified into mild, severe, or complicated forms. In another study[208], the electrophoretic changes in 6 puppies with experimentally induced babesiosis showed a decrease in the albumin fraction, increased α1, α2 and γ globulins, while β globulins remained unchanged. A corresponding finding in this study was the decrease in the albumin fraction, whereas there was no increase in the α globulins. In the work done by Malherbe[128], dogs with unclassified babesiosis showed a decrease in serum albumin, no significant change in α1 globulin, a significant decrease in α2 globulin, and a significant rise in γ globulin fractions. The β globulin levels were not recorded,

as free haemoglobin was judged to have falsely elevated this fraction. The decrease in albumin corresponds to the findings in this study.

In other haemoprotozoal infections, similar serum protein changes have been described. Feline babesiosis has been associated with decreases in α and β globulins, and an increase in γ globulins[72], whereas feline haemoplasmosis has been associated with increases in α3, β2, and occasionally γ globulins[207]. The serum protein electrophoretic pattern in guinea pigs and horses infected with *Trypanosoma evansi* showed a highly significant decrease in albumin levels and an increase in γ globulins, leading to a very low A/G ratio. No significant differences in total protein levels between healthy and infected animals were registered. Likewise, α globulins were not significantly affected. A decrease in β globulins was observed in one horse and in guinea pigs with experimental infection, while in horses with natural infections this decrease was not constant[146]. In one malaria study it was shown that there was an increase in the γ globulins, a decrease in the serum albumin, and no consistent pattern of change in either the α or β globulins[56]. The decrease in the serum albumin corresponds to the present study. In humans with *Plasmodium falciparum* infection there is a decrease in the A/G ratio, an increase in the α and γ globulins and a decrease in the β globulins[39]. The A/G ratio changes correspond to this study.

The acute phase response occurs during the early stage of infection, tissue injury, or immunological disorders[227]. The systemic reaction of the acute phase response includes dramatic changes in acute phase protein (APP) concentrations, primarily due to increased hepatic APP production[227]. Acute phase proteins are serum proteins identifiable by electrophoresis in the α globulin zone, which increase in concentration during the acute phase response to inflammation or infection[61]. As the incubation period of canine babesiosis is 10-21 days[26], it would have been expected that an increase in the α globulins (representing APP) would have been evident on serum protein electrophoresis. The lack of α

globulin elevations in all three groups of canine babesiosis cases in this study may be indicative of no APP; however, the significant elevation of αGP indicated a massive increase in at least this one APP. Therefore, serum protein electrophoresis may not be sensitive enough to detect elevated APP, as was demonstrated in this study, presumably because the concentration was insufficient to raise the total α globulin value above the normal range. In this study, complicated cases showed the highest mean α globulin value of the 3 groups, although none of the dogs showed an increase in the α globulin values.

Inflammatory changes following infection are central to the clinical manifestation of disease, and APP alterations may reflect the severity of inflammatory damage[176]. Complicated babesiosis has been suggested to be the consequence of dogs developing the systemic inflammatory response syndrome (SIRS), and the multiple organ dysfunction syndrome (MODS), both of which are cytokine-mediated phenomena[88]. Pyrexia also develops, which is attributed to the release of endogenous pyrogens from erythrolysis, parasite destruction, and activation of inflammatory mediators[26,88].

A study correlating serum cytokine activity with APP levels in dogs with acute inflammation demonstrated significantly raised serum interleukin-6 (IL-6) and tumour necrosis factor (TNF) levels, which preceded the APP increase[227]. Dogs developing complicated babesiosis (SIRS or MODS) may be expected to show similar APP alterations, which was shown in this study.

An increased blood fibrinogen concentration in the early phase of experimental canine babesiosis infection has also been linked to the acute phase response[182]. In human falciparum malaria, cytokine-mediated activation of hepatocytes induce acute phase responses, which may contribute to the stabilisation of parasitaemia[217].

Higher serum globulin concentrations have been associated with death in dogs with

babesiosis having an admission haematocrit of $\geq$ 30%[175]. It was postulated that if these globulins were APP, they could reflect an overwhelming inflammatory process and/or be a reflection of tissue destruction. Although the haematocrits in all but one of the dogs in our study were below 30%, and therefore reflect a different disease sub-group under discussion in the afore-mentioned study, the postulated reasons for increases in APP may still hold true. This same study postulated that if the increased globulins in this disease sub-group represented immunoglobulins, the theory of an "over-enthusiastic immune response" would be strengthened[175]. The lack of a statistically significant elevation in γ globulins in this study does not support this latter postulation.

Marked increases in two APP (C-reactive protein (CRP) and haptoglobin) have been demonstrated in dogs following infection with another haemoprotozoan parasite, *Trypanosoma brucei*. The highest levels of CRP were observed immediately after the peak of parasitaemia[155]. Measurement of APP in acute canine ehrlichiosis demonstrated significant elevations in CRP and αGP, 4-6 days post exposure[176].

Albumin has been termed a negative APP, and its serum concentration may decrease during the acute phase response[208]. Significant decreases in serum albumin concentrations have been shown to follow significant increases in serum IL-6 and TNF activities[227]. In correlation with previous studies[122,128,208], the mean albumin values for all 3 babesiosis groups in this study were lower than the comparison group with a statistically lower albumin in Groups 1 and 2 as compared to the control group . It has been reported that albumin values decrease with advancing age[98]. As the mean age of dogs in this study was approximately 35 months, the decreased albumin values were attributed to the babesiosis alone.

Ehrlichia canis infection has no pathognomonic clinical signs, and typical *E. canis* morulae in monocytes can only be found during the acute phase following infection, rendering it a difficult disease to diagnose[133,169]. None of the dogs in this study showed *E. canis* morulae on

peripheral blood smear, nor had any haematological changes or clinical signs suggestive of ehrlichiosis. Although the majority of the dogs showed positive *E. canis* titres, only 1 dog in the severe uncomplicated group showed a corresponding increase in the serum globulins and γ globulins that could indicate concurrent ehrlichiosis. A study performed in the Bloemfontein area of South Africa identified 42% of apparently healthy dogs with significant antibody titres (≥ 1:64) against *E. canis*[169], and a similar study conducted in Zimbabwe reported the same figure of 42% seropositivity (≥ 1:40) against *E. canis* in apparently healthy dogs[133]. It has been reported that hyperglobulinaemia is commonly detected in all phases of this disease[133]. The Zimbabwean study identified hyperglobulinaemia in only 5% (2/39) of these dogs, however, while none of the dogs showed hypoalbuminaemia[132]. A study conducted on dogs infected with *B. canis* and *E. canis* found the prevalence of hyperglobulinaemia to be significantly higher in dogs with babesiosis that had a significant *E. canis* antibody titre (≥ 1:20), compared to dogs with significant antibodies to *E. canis* without concurrent babesiosis[132]. This suggests that the presence or absence of a significant *E. canis* titre does not significantly affect the prevalence of hyperglobulinaemia in dogs with babesiosis. The latter study also failed to demonstrate any relationship between *E. canis* antibody titre, and abnormal laboratory values and/or the presence or absence of clinical signs[132]. A study performed in Virginia in the USA found a significant *E. canis* antibody titre (≥ 1:64) in 38% (28/74) of dogs studied[51]. However, PCR analysis on blood samples from these dogs obtained negative results for *E. canis* in all samples. The results do, however, indicate that *E. canis* serology is much less specific than previously believed. It is therefore not believed that the positive *E. canis* titres in this study necessarily reflect concurrent ehrlichiosis, or that such co-infection, if indeed present, would appreciably interfere with or skew the results of this study. The effects of other concurrent diseases could not be evaluated, but it is unlikely that all of the dogs would have been suffering from concurrent diseases with similar effects.

The findings from this study suggest that dogs with mild and severe babesiosis have low total serum proteins, albumin, A/G ratio, and α globulins; dogs with complicated babesiosis show no typical serum protein changes or patterns; and all dogs with babesiosis show no evidence of an acute phase response detectable on serum protein electrophoresis. There is, however, evidence of a significant APP response in the mild, severe, and complicated canine babesiosis, which did not correlate with the α globulin concentrations.

CHAPTER 7

CONCLUSIONS

Canine babesiosis is an extremely common tick-borne disease throughout South Africa. In a survey done approximately 10 years ago, the results showed that on average, a veterinary practice treats between 100 and 500 babesiosis cases each year, at a cost to the dog-owning public of more than R20 million[38]. The average number of canine babesiosis cases at the Onderstepoort Veterinary Academic Hospital (OVAH) from 1988 to 1993 was 1253 per year, which represented 11.69% of all dogs presented to the OVAH[186]. Of the 1253 dogs diagnosed with babesiosis, 393 or 31.4% had to be hospitalised for more intensive treatment.

It is highly unlikely that the situation has changed and thus babesiosis remains an extremely important canine disease. Possible explanations for this is that there is currently no effective vaccine for the disease in southern Africa and that tick control in the canine population is not effective.

The nature of the disease is intra-erythrocytic parasitaemia that results in haemolysis, which manifests as regenerative anaemia, haemoglobinaemia, haemoglobinuria, and bilirubinuria. However, there are various clinical manifestations that are difficult to relate to an organism that is solely restricted to the erythrocyte. Over the years many complications of canine babesiosis have been documented and although the clinical manifestations are diverse, the mechanisms promoting them are likely to be more uniform. Chapter 1 of this thesis gives an overview of canine babesiosis together with a brief report of the complications, excluding the complications that are described in this thesis.

This thesis showed that dogs with natural infection with *B. canis* had both renal and cardiac dysfunction, both of which can be classified as complications of babesiosis and would thus necessitate supportive therapy.

Renal function

In the past renal changes in babesiosis were attributed to the haemoglobinuria and were referred to as haemoglobinuric nephropathy as babesiosis can result in a swollen kidney, dark in colour, with red-brown urine in the bladder. Microscopically the renal tubular epithelial (RTE) cells can be swollen and contain haemoglobin droplets and small vacuoles[122].

In a study where the relative roles of haemoglobinaemia and hypoxia on renal function and pathology in the dog were investigated, it was shown that severe haemoglobinuria, of the magnitude seen in canine babesiosis, did not induce significant nephropathy, regardless of whether or not concomitant anaemia was present[115]. That study also showed that the glomerular filtration rate (GFR) was reduced in the groups of dogs that were rendered anaemic to the same degree that would occur in severe cases of babesiosis. It is thus possible that the renal effects of canine babesiosis can be attributed to a reduction in the GFR, which occurred secondary to the anaemic state, reduced cardiac output resulting from myocardial dysfunction, and hypotension.

This thesis demonstrated that RTE celluria, proteinuria, and variable enzymuria and azotaemia occurs in dogs with babesiosis. These were all minimal changes, however, and all could be consistent with hypoxia, reduced GFR, or reduced cardiac output.

This thesis showed that dogs with naturally occurring babesiosis had significant urine met-haemoglobin with no evidence of blood met-haemoglobin. The possibility would be that the urinary met-haemoglobin was either produced in the kidney or possibly by oxidation of haemoglobin to met-haemoglobin in the bladder. It has been shown experimentally that met-haemoglobin can be toxic[228].

The combination of reduced GFR, anaemic hypoxia, and met-haemoglobin can all act synergistically to cause renal damage. Renal haemodynamics are also much more likely to

be abnormal when cardiac dysfunction is present. Reduced renal blood flow and glomerular filtration rate are evidence of redistribution of blood flow that commonly occurs in early heart failure[27]. This thesis showed that the renal changes in canine babesiosis are most likely secondary to cardiac dysfunction and that the cardiac complications are thus more important, which has not heretofore been demonstrated in canine babesiosis.

An important finding in this thesis was that dogs with babesiosis had lower serum sodium than control dogs but there was no difference between mild, severe, or complicated cases of babesiosis. Also dogs with babesiosis had a lower Fc_{Na} than clinically healthy control dogs, which can be interpreted as sodium retention by the kidneys. Children with malaria may show hyponatraemia, which is speculated to be associated with dehydration and having an inappropriate secretion of vasopressin (antidiuretic hormone)[64]. This sodium retention would also result in water retention, which will result in an expansion of the plasma volume. This finding has a bearing on the cardiac changes, as an increase in plasma volume will result in an added strain on myocardial function.

Cardiac function

In the past heart lesions in canine babesiosis were regarded as rare complications[88,116], with the majority of lesions being reported as incidental findings at post-mortem examination of complicated babesiosis cases[148,204]. This thesis demonstrated that cardiac lesions are common in canine babesiosis.

This thesis showed that that ECG changes in babesiosis were similar to the pattern described for myocarditis and myocardial ischaemia, and together with the histopathological findings indicated that the heart suffers from the same pathological processes described in other organs in canine babesiosis, namely inflammation and hypoxia. As the clinical

application of the ECG changes found in this study was limited, cardiovascular assessment should be based on functional and biochemical monitoring rather than an ECG.

Using cardiac troponin as a marker of myocardial injury, this thesis showed that myocardial cell injury occurs with canine babesiosis. Cardiac troponins, especially troponin I, are sensitive markers of myocardial injury in canine babesiosis, and the magnitude of elevation of plasma troponin I concentrations appears to be proportional to the severity of the disease.

ECG changes and serum cardiac troponin were correlated with histopathology. On cardiac histopathology from dogs that succumbed to babesiosis, haemorrhage, necrosis, inflammation and fibrin microthrombi in the myocardium were documented, all of which would have resulted in ECG changes and elevations in cardiac troponin. A similar phenomenon occurs in people with myocardial infarction. Myocardial infarction causes left ventricular failure, which will result in hypotension and a compensatory expansion of the plasma volume due to homeostatic mechanisms[78,163], which would include activation of the renin-angiotension-aldosterone system.

Hypotension in clinically severe babesiosis has been documented[91], with possible causes speculated to be increased capillary permeability with movement of fluid to the interstitium, reduced vascular tone with venous pooling, and myocardial depression. Myocardial infarction will result in both vascular pooling and myocardial depression. A feasible explanation for the hypotension in canine babesiosis would be myocardial pathology, which this study has shown to be a common finding.

Increased plasma volume has been reported in both canine babesiosis and malaria[7,112,183], possibly due to movement of interstitial fluid into the vasculature secondary to hypotension[182], and with evidence of increased blood volume in the presence of hypotension in some patients with malaria[112,192]. The pathogenesis of this phenomenon is thought to be

reduction of effective blood volume - through peripheral vasodilation (mediated by nitric oxide), followed by the release of vasopressors, noradrenalin, renin activation, and reduced renal haemodynamics[192].

This thesis showed that dogs with babesiosis had hypoalbuminaemia, which may either be because of intravascular volume dilution due to fluid retention or being a negative acute phase protein. As babesiosis is an acute disease, decreased albumin production from the liver would be a very unlikely cause for the hypoalbuminaemia. In the light of the cardiac changes, hyponatraemia, and hypotension, a probable cause would be fluid retention due to myocardial disease. It has been shown that hypoalbuminaemia can cause interstitial oedema in people, which then causes a reduction in the serum sodium levels[48]. Patients with malaria, where the pulmonary capillary wedge pressure has been assessed using a Swan-Ganz catheter, have an increased pressure that would be suggestive of either a cardiogenic or hypervolaemic mechanism[24,50]. Hypoalbuminaemia with low colloid oncotic pressure and elevated pulmonary artery wedge pressure have been shown in people with acute systolic heart failure, which may facilitate the onset of pulmonary oedema[8]. This thesis showed that dogs with babesiosis had left ventricular lesions, which can result in systolic heart failure.

Summary

As mentioned above, babesiosis can result in various clinical manifestations that are difficult to relate to an organism that is solely restricted to the erythrocyte. The results of this thesis showed that homeostatic mechanisms of the body to the anaemic state could contribute to the cardiac and renal complications. The probable pathophysiological explanation for the cardio-renal changes would be:

- By the parasites invading and replicating in the erythrocyte, babesiosis results in destruction of the erythrocyte with the development of anaemia and consequently hypoxia. The destruction of the erythrocyte is multi-factorial, including direct parasite

damage to the erythrocyte membrane, splenic removal of damaged and parasitized erythrocytes, complement activation, and the presence of anti-erythrocyte antibodies, which result in a secondary immune-mediated haemolytic anaemia.

- The kidneys are very susceptible to the effects of hypoxia as 20% of cardiac output goes through the kidney. The resultant effect of hypoxia on the kidney is reduced GFR and tubular hypoxia. In addition dogs with babesiosis tend to be hypotensive, which will further reduce renal blood flow. The clinical manifestation of this would be pre-renal azotaemia (elevated urea/creatinine with high urine specific gravity).

- Tachycardia is a common clinical manifestation of babesiosis, which can be attributed to the anaemia, pyrexia, and acidotic state. During the cardiac cycle, tachycardia will result in a longer period of systole and a shorter period of diastole. As myocardial perfusion, via the coronary circulation, occurs during the diastolic period, a shorter diastolic period will result in reduced coronary artery blood flow with compromised myocardial perfusion. The net result is myocardial hypoxia.

- Another effect of the tachycardia is that the heart is forced to work at a faster rate with a reduced oxygen supply, the latter by a combination of reduced coronary circulation and less oxygen in the blood because of the anaemia. Dogs with babesiosis not only have less haemoglobin but the remaining haemoglobin has been shown to be sub-functional, which will further contribute to the hypoxic state and thus myocardial hypoxia.

- Prolonged myocardial hypoxia will result in myocardial depression, which in turn can result in hypotension. Hypotension will further reduce coronary circulation, thus aggravating the myocardial hypoxia.

- Dogs with babesiosis have pathological changes within the myocardium (myocardial necrosis, inflammation, haemorrhage, fibrin microthrombi) that are usually in the left ventricle, which has the highest metabolic requirement. These changes may be due to an inflammatory reaction to the pavementing of parasitized cells against the endothelium via adhesion molecules, coagulopathy, or from myocardial hypoxia. Hypotension can aid in parasite sequestration.

Therapeutic recommendations

From this thesis a number of therapeutic recommendations can be advocated in order to improve the management of canine babesiosis.

- *Cardiac pathology*

  Taking cognisance that myocardial lesions may be more common than previously thought which can result in hypotension, intravascular fluid overload and pulmonary oedema. This will necessitate the careful use of intravenous fluids as well assessing the patient for the presence of myocardial pathology by means of serum troponins. Additional therapy that may be required would be arrhythmia control, positive ionotropic support, strict cage rest, oxygen supplementation, and diuretics.

- *Serum sodium*

  Monitoring of serum sodium and utilizing fluids to maintain and/or correct hyponatraemia.

- *Serum albumin*

  Monitoring serum albumin and replacing with fresh plasma and/or synthetic colloids, if necessary.

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APPENDIX

SCIENTIFIC PUBLICATIONS CONNECTED WITH THIS THESIS

1. Lobetti, R.G. & Reyers, F. 1996. Met-haemoglobinaemia in naturally occurring *Babesia canis* infection. *Journal of the South African Veterinary Association*. 67:88-90

2. Lobetti, R.G. 1998. Canine Babesiosis. *Compendium on Continuing Education for the Practicing Veterinarian*. 20:418-431

3. Lobetti, R.G., Möhr, B., Dippenaar, T. & Myburgh, E. 2000. A preliminary study on the serum protein response in canine babesiosis. *Journal of the South African Veterinary Association*. 71: 38-42

4. Lobetti, R.G. & Jacobson, L.S. 2001. Renal involvement in dogs with babesiosis. *Journal of the South African Veterinary Association*. 72:23-28

5. Lobetti, R.G., Dvir, E. & Pearson, J. 2002. Cardiac troponins in canine babesiosis. *Journal of Veterinary Internal Medicine*. 16: 63-68