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

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ABSTRACT

C-reactive protein (CRP) is a positive major acute-phase protein in dogs and can be used as a predictive marker for risk of disease and to monitor the response to treatment. Increased concentrations in certain diseases are associated with poor outcome. This cross-sectional, observational study of 75 dogs naturally infected with *Babesia rossi* was designed to examine the relationship between outcome and CRP concentration at admission and the magnitude of CRP change 24 hours after admission. Diagnosis was confirmed by polymerase chain reaction (PCR) and reverse line blot. CRP concentrations were determined by an automated human CRP Turbidometric Immunoassay, previously validated for use in dogs. There was no significant difference in mean CRP concentration between survivors ($n = 57$), 107.5 ± 49.5 mg/ℓ and non-survivors ($n = 11$), 122.1 ± 64.6 mg/ℓ at admission and using the exact logistic regression, adjusting for age and sex, there was no association with outcome ($P = 0.53$). Multiple regression analysis failed to show a significant relationship between admission CRP concentration and number of days of hospitalisation in the survivors, adjusting for age and sex ($P = 0.65$). Similarly, no significance was found in the relationship between the magnitude of change in CRP concentration 24 hours after admission, and the number of days of hospitalisation in survivors, ($P = 0.34$). It is concluded that CRP concentration, as a measure of the acute phase response, is not associated with outcome in canine babesiosis, and inflammation is unlikely to be the only cause of severity of disease.

Keywords: *Babesia rossi,* C-reactive protein prognosis.

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INTRODUCTION

Canine babesiosis in South Africa is caused by the intra-erythrocytic protozoan parasites *Babesia rossi* and *Babesia vogeli*[33]. The disease is characterised by haemolytic anaemia and a course ranging from mild to peracutely fatal, with the subspecies differing in their pathogenecity. *Babesia rossi* infections may be fatal despite intensive treatment, whereas *B. vogeli* usually causes a mild, clinically unapparent infection[17,46]. The virulent form of canine babesiosis, caused by *B. rossi*, is associated with various complications including acute renal failure (ARF), cerebral babesiosis, disseminated intravascular coagulation (DIC), icterus, hepatopathy, immune-mediated haemolytic anaemia (IMHA), acute respiratory distress syndrome (ARDS), haemoconcentration, shock and pancreatitis[24,35,49]. These complications are proposed to be the result of a Systemic Inflammatory Response Syndrome (SIRS) present in most cases of canine babesiosis, mediated by cytokines, nitric oxide and free oxygen radicals, which eventually progress to a Multiple Organ Dysfunction Syndrome (MODS)[17,49]. In a study by Welzl[49], using the criteria for SIRS, 87 % of complicated canine babesiosis cases in South Africa were positive for SIRS. Of the dogs diagnosed with organ dysfunction/damage, 52 % had single organ involvement and the remaining 48 % had MODS.

Several studies have found a number of indices to be predictors of mortality or associated with fatal outcome in canine babesiosis. An association has been demonstrated between tumour necrosis factor (TNF) concentration, clinical severity and parasitaemia[48]. A higher capillary and venous parasitaemia and a collapsed state were associated with mortality[4]. Hypoglycaemia at admission, hyperlactataemia that fails to decline by more than 50 % after 24 hours and specific organ involvement have been associated with a poor prognosis[37,49]. In a more recent study, mortality was shown to be associated with endocrine predictors, with significantly higher cortisol and adrenocortical trophic hormone concentrations and lower thyroxin and free thyroxin concentrations occurring in the group of dogs that died as compared with the survivors[43].

Interestingly, the severity of the anaemia or the presence of SIRS, MODS, and IMHA had no impact on outcome[49]. Furthermore, although nitric oxide is proposed to be a mediator of MODS, which is seen in complicated canine babesiosis, reactive nitrogen intermediates failed to correlate to the severity of disease and were not predictive of outcome[18]. Although collapse is associated with poor outcome and hypotension correlates with disease severity, blood pressure has not been shown to be a predictor of mortality[19].

The acute-phase response is thought to be an innate host defence mechanism, occurring during the early stages of infection, tissue injury or immunological disorders. It is responsible for accumulation and activation of granulocytes and mononuclear cells, which in turn release acute-phase cytokines, including interleukin-1 (IL-1), IL-6 and tumour necrosis factor alpha (TNF-α)[6,9]. During an acute-phase response the serum concentration of acute-phase proteins (APP) changes in response to these cytokines. Some APP will decrease (referred to as negative APP), while others will increase in concentration (positive APP)[1,6]. In dogs, CRP has been isolated from the acute-phase serum, and was found to be analogous in its properties to the human serum CRP fraction[8]. C-reactive protein is classified as a major positive APP due to the magnitude of its response[5,51]. A positive CRP reaction has been shown as early as 4 hours and is dramatically increased within 24 hours after surgical stimulation or injection of an irritating substance, followed by a rapid return to baseline concentrations at the end of the inflammatory process[5,7,52].

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The rapid changes in CRP concentration are valuable when used as a biomarker for inflammation as is the lack of modulation by anti-inflammatories. Even though corticosteroids participate as a co-factor in the regulation of APP, no statistically significant changes occurred in the concentrations of CRP in dogs dosed with exogenous glucocorticoids, nor did they modulate the CRP response in dogs experimentally infected with *Bordetella bronchiseptica*[28,50]. Very young dogs appear to show a less marked acute-phase response to inflammation. Quantifiably lower CRP concentrations, in response to injected turpentine oil, were measured in 1-month-old dogs as compared with 3- and 18-month-old dogs[14]. Samples that can be used to measure CRP concentration include serum, saliva and effusions[21,41,42]. Haemolysis, lipaemia and hyperbilirubinaemia have been shown to cause a statistically significant change in the CRP concentrations measured, but differences observed are unlikely to be clinically relevant[27].

It has been shown that CRP concentrations will increase in pathological conditions, such as infection with *Babesia rossi, Ehrlichia canis, Leishmania infantum,* in induced acute gastric mucosal injury, and neoplasia[29,40,44,45,47]. Serum CRP concentration has been a useful index in differentiating pyometra from cystic endometrial hyperplasia/mucometra, and has shown a good correlation with the canine Inflammatory Bowel Disease (IBD) activity index[10,20]. It has been shown to be a suitable laboratory test to assess the effect of therapy in patients with IBD[20]. Serum CRP concentrations are also elevated in dogs with naturally occurring acute pancreatitis, with concentrations decreasing by day 3 in dogs that recover[15]. In diseases such as *Leishmania infantum* and *Trypanosoma brucei* infection, which show resistance to treatment or are prone to relapse, CRP can be used to monitor response to treatment and predict relapses[26,36]. CRP was useful in predicting complete remission status in dogs with multicentric lymphoma treated with cytotoxic drugs, but concentrations were not able to detect or predict relapse[38]. In dogs naturally infected with *B. canis* and treated with an antibabesial drug (imidocarb diproprionate), a serial decline in CRP concentrations has been demonstrated, approaching baseline by about the 8th day post-treatment, and it was correlated with traditional markers of inflammation including erythrocyte sedimentation rate and white cell count[31]. CRP concentrations have been shown to be significantly higher in dogs with complicated babesiosis than in dogs with the uncomplicated form of the disease and was proposed to be able to predict severity of canine babesiosis[30].

The aim of the current study was 2-fold: to assess the association between admission CRP concentration and outcome in dogs with canine babesiosis caused by a natural infection with *B. rossi,* and secondly to determine if the failure of a significant decline in CRP concentration is associated with a worse outcome.

MATERIALS AND METHODS

This was a prospective, cross-sectional, observational study that included 75 client-owned dogs that were diagnosed with canine babesiosis and admitted to the Intensive Care Unit (ICU) of the Onderstepoort Veterinary Academic Hospital (OVAH), Faculty of Veterinary Science, University of Pretoria, South Africa. Diagnosis of canine babesiosis was made on the basis of morphological demonstration of the intra-erythrocytic *Babesia* trophozoite on stained thin capillary blood films. Dogs that exhibited concurrent infections on clinical examination or had a history of being treated for an infection in the last 7 days were excluded from the study. Confirmation of infection with *B. rossi* was by PCR reverse line blot (RLB) hybridisation assay. Dogs infected with *B. vogeli,* or concurrent *Ehrlichia canis* or *Theileria* infection, and dogs euthanased for reasons other than poor prognosis were excluded. Poor prognosis was based on previously established negative prognosticators which included cerebral babesiosis, ARDS, hyperlactataemia and hypoglycaemia.

Study design

History, including duration of illness, and signalment were recorded. Blood samples for measuring CRP concentration and PCR/RLB were collected from the jugular vein in serum and EDTA vacutainer brand tubes (Beckton Dickinson Vacutainer Systems, UK) respectively, by needle venipuncture prior to any therapy being instituted. Thereafter serum was collected daily until discharge or death. Serum samples were allowed to clot at room temperature and then centrifuged at 3000 *g* for 10 min. Serum was stored at –80 °C until analysis, which took place within 6 months.

All dogs in this study were treated with an antibabesial drug (Diminazene aceturate, Intervet, South Africa) and blood transfusions as needed. In addition, any complications were treated at the discretion of the attending clinician. The primary investigator was blinded to the results of the CRP concentrations and parasite PCR/RLB results due to the delay in the processing of these samples. Outcome was recorded as the patient survived and was discharged or died.

PCR and RLB

DNA was extracted from 200 μl of each whole blood specimen. The QIAmp blood and tissue extraction kit (Qiagen, Hilden, Germany) was used for DNA extractions, following the manufacturer's protocols. The *Babesia/Theileria/Hepatozoon* PCR and *Ehrlichia/Anaplasma* PCR were performed as described previously[2,13,32,39]. Reverse line blot hybridisation was subsequently conducted on amplified products (*Babesia, Theileria, Hepatozoon, Anaplasma* and *Ehrlichia*) as described previously[32].

CRP assay

CRP measurements were performed in 2 batches. CRP concentrations in serum samples were measured by an automated human CRP Turbidometric immunoassay (TIA) previously validated for use in dogs (Bayer CRP TIA, Newbury, UK)[22]. The analysis was performed using an automated analyser (Nexct, Alfa Wasserman, Bayer, South Africa) according to the manufacturer's instructions[22]. Haemoglobin concentrations were measured and bilirubin concentrations were visually estimated at the clinical pathology laboratory. None of the samples exceeded the limit of 10 mg/mℓ (10 g/ℓ) of haemoglobin or 25 g/ℓ (425 mmol/ℓ) of bilirubin set by the manufacturer.

Statistical analysis

Data were analysed using Stata 10.0 statistical software (StataCorp, College Station, TX, USA). Significance was set at $\alpha = 0.05$. The association between admission CRP concentration and outcome was analysed using exact logistic regression analysis. The independent variable of interest was CRP concentration. The effect of CRP on survival was adjusted for potential confounding by including sex and age as covariates. Exact logistic regression was used because of the relatively small number of mortalities in the dataset. A Fisher's exact test was then used to compare the survival rates between 2 groups defined by a cut-off CRP concentration below which 100 % survival occurred, determined retrospectively. Among survivors, multiple regression analysis was used to examine the relationship between CRP concentration at admission and length of stay in ICU, adjusting for age and sex. A Wilcoxon rank-sum test was used to compare the median number of days of hospitalisation required in survivors between those dogs with an admission CRP concentration below the selected cut-off that predicted 100 % survival and those dogs that

exceeded this cut-off. Multiple linear regression was again used in the same group of dogs, to examine the relationship between the magnitude of CRP concentration change over the 1st 24 hours and length of stay in the ICU, once again adjusting for age and sex.

RESULTS

The results of 75 dogs were available, 69 of which met the inclusion criteria; 1 dog was euthanased due to other reasons and excluded, 11 dogs died and 57 dogs survived. None of the serum samples had haemoglobin or bilirubin concentrations that exceeded the reagent manufacturers' recommendations. The mean ± SD CRP concentration on admission for survivors (n = 57) was 107.5 ± 49.5 mg/ℓ and for non-survivors (n = 11), 122.1 ± 64.6 mg/ℓ. The median admission CRP concentrations were compared between survivors and non-survivors in a box plot (Fig. 1). Using exact logistic regression, adjusting for age and sex, there was no statistically significant association between admission CRP concentration and survival (P = 0.53). None of the non-survivors had an admission CRP concentration less than 63.2 mg/ℓ. When an admission CRP concentration cut-off of 60 mg/ℓ was selected, the survival rate for dogs with an admission CRP concentration <60 mg/ℓ was 100 % (9/9) and those dogs with an admission CRP concentration ≥ 60 mg/ℓ was 81.4 % (48/59). However, these 2 proportions did not differ significantly using a Fisher's exact test (P = 0.34).

Although there appeared to be an association between admission CRP concentration among the survivors and eventual number of days the dogs were hospitalised (Fig. 2), the multiple regression analysis failed to show a significant relationship between these 2 variables, adjusting for age and sex (P = 0.65). Similarly, there was no significant association between the magnitude of change in CRP concentration over the 1st 24 hours from admission and the number of days of hospitalisation (P = 0.34) (Fig. 3). There was also no significant difference in the median number of days hospitalised in those dogs that survived with an admission CRP concentration <60 mg/ℓ (3 days, range 2–4 days; n = 9) compared with those survivors with an admission CRP concentration ≥ 60 mg/ℓ (2 days, range 1–6 days; n = 48), using the Wilcoxon rank-sum test (P = 0.25).

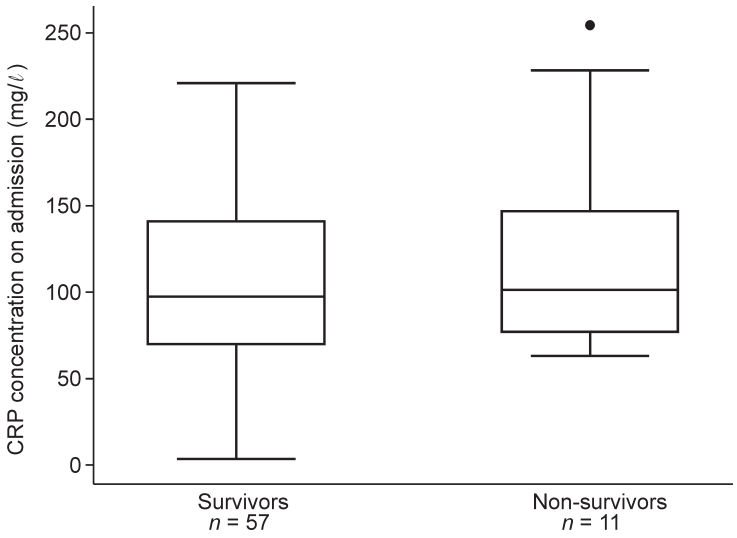

Fig. 1: **Box plot showing admission CRP concentrations in dogs with severe canine babesiosis, grouped according to outcome, namely survivors or non-survivors. The box incorporates the middle 50 % of the observations with the line inside the box as the median. The whiskers extend to the smallest** (25th percentile) **and largest** (75th percentile) **observations, indicating the range of the data. Outliers, values that are 1.5 times removed from the interquartile range, are plotted as dots.**

DISCUSSION

Previous categorisation of canine babesiosis, based on the World Health Organisation classification for malaria, is considered artificial and probably unnecessary. It should be replaced by simple clinical measures such as state of collapse, or biomarkers like lactate and endocrine markers, as these parameters are predictive of outcome[4,16,17,37,43]. In human medicine there are various examples of conditions/diseases where serum CRP has fulfilled the function as a predictor of mortality or morbidity: CRP was correlated with an increased risk of organ failure and death in a heterogeneous human ICU population; CRP accurately predicted severity of malaria; CRP accurately predicts survival in humans suffering from non-Hodgkin's lymphoma; and CRP can predict heart-failure-associated mortality in the year following patients suffering acute myocardial infarctions[3,12,23,25]. In dogs, serum CRP concentration has previously been correlated to disease severity and outcome in several inflammatory conditions, as in the case of canine IBD, where CRP was shown to be positively correlated to disease severity, as well as in dogs naturally infected with *B. canis* in Croatia[20,30]. This does not seem to be the case in *B. rossi* infection in dogs in South Africa, as in this study no statistically significant difference in admission CRP concentration between survivors

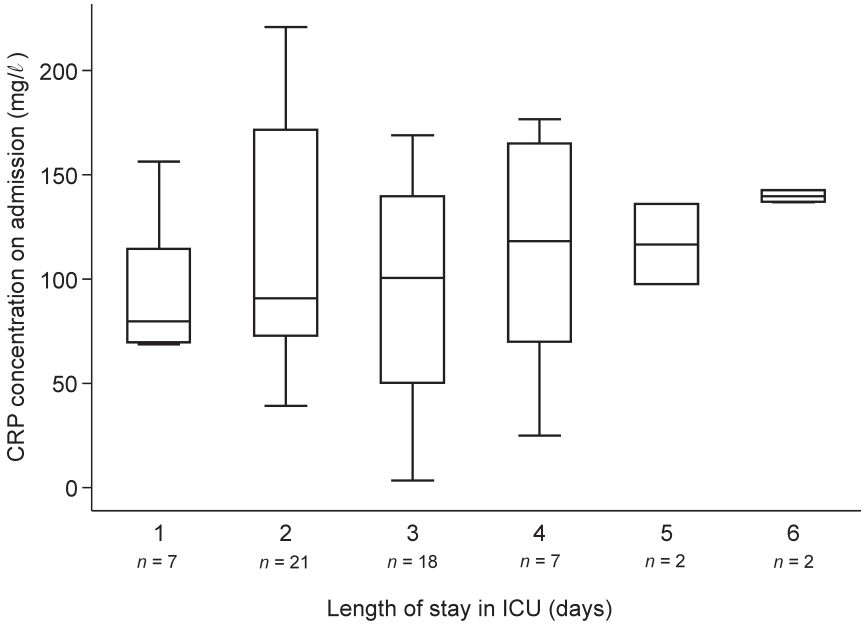

Fig. 2: **Box plot showing admission CRP concentrations** (mg/ℓ) **in dogs with different durations of hospitalisation. See Fig. 1 caption for an explanation. Although there is an apparent linear correlation between the 2 variables, there is no significant relationship when examined with a multiple regression analysis** (P = 0.64), **when adjusted for sex and age.**

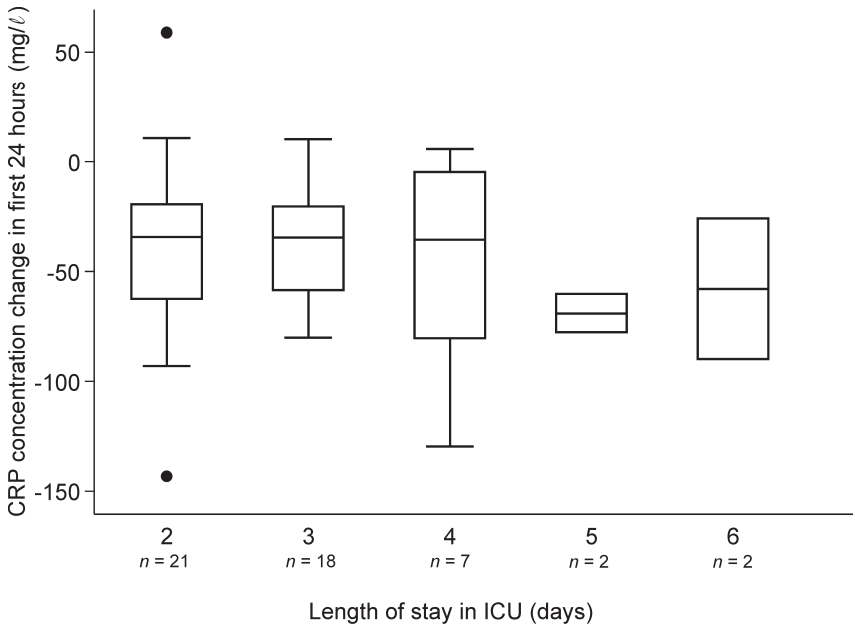

Fig. 3: **Box plot representing the magnitude of decline in CRP concentration, in the 1st 24 hours, for each number of days until discharge. See Fig. 1 caption for an explanation. A multiple regression analysis failed to show a significant relationship between the magnitude of CRP decline and days hospitalised** ($P = 0.34$)**.**

and non-survivors could be demonstrated. The reason for variable outcome in dogs from the same geographic area, naturally infected with the same subspecies of parasite as demonstrated by PCR RLB hybridisation technique, treated identically with interventional therapy as dictated by the complications that developed, was not due to a difference in the acute-phase response. This supports the hypothesis that although inflammatory mechanisms are important in the pathogenesis of babesiosis, tissue hypoxia plays a major role in the disease process[16]. In diseases with low mortality rates, biomarkers can be used to predict morbidity-based outcome including length of hospital stay. In dogs diagnosed with primary IMHA, CRP concentration returning to baseline within 30 days of discharge was associated with a favourable outcome[34]. In dogs with neoplastic diseases, a favourable response to therapy was associated with a decrease in CRP concentration[45]. CRP was associated with SIRS status in dogs diagnosed with pyometra and positively associated with prolonged hospitalisation[11]. In this study CRP concentration at admission or a measure of the decline in concentration in the 1st 24 hours after admission was not helpful in predicting length of hospitalisation.

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

This study showed that there is insufficient evidence to prove that CRP is a prognostic indicator for mortality or morbidity in dogs suffering from canine babesiosis caused by *B. rossi* in South Africa.

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