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Graphical Abstract

Clinical, haematological, cytokine and acute phase protein changes during experimental *Babesia gibsoni* infection of beagle puppies.

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Abstract

Babesia gibsoni is a haemoprotozoan parasite of emerging global importance. The clinical presentation of babesial infections is diverse and the systemic inflammatory response induced by infection is considered to be a major feature of the pathophysiology of canine babesiosis. An experimental case-controlled longitudinal study was conducted to assess the clinical, haematological, cytokine and acute phase protein changes that occur during experimental *B. gibsoni* infection of beagle puppies. Infected dogs became transiently pyrexic and anaemic, intermittently neutropenic and transiently, but profoundly, thrombocytopenic, although this had no apparent adverse clinical effect. Experimental *B. gibsoni* infection also induced an acute phase response, characterised by a marked increase in the concentration of C-reactive protein, which was delayed in onset following infection but preceded the detection of peripheral parasitaemia. Experimental *B. gibsoni* infection was also associated with marked increases in the concentration of multiple cytokines which were also delayed in onset following infection and occurred subsequent to the detection of peripheral parasitaemia and the acute phase response. This study furthers our understanding of the immune response that occurs during babesial infections and the role that systemic inflammation plays in the pathophysiology of canine babesiosis.

Curriculum Vitae

Peter Irwin graduated from the Royal Veterinary College University of London in 1982 and completed a PhD in the area of canine babesiosis at James Cook University in 1989. He obtained Fellowship of the Australian New Zealand College of Veterinary Scientists in Canine Medicine in 1995 and has worked at Universities throughout Australasia. Peter is currently a Professor in Veterinary Clinical Science at Murdoch University.

Robert Shiel graduated from University College Dublin (UCD) in 1999. After four years in small animal practice in the United Kingdom he returned to UCD and completed a residency in small animal medicine. He obtained Diplomate status of the European College of Veterinary Internal Medicine - Companion Animals (Internal Medicine) in 2007 and completed a PhD in the area of canine thyroid function at UCD in 2010. Robert is currently a Lecturer in Small Animal Medicine at University College Dublin.

Alexa Brown graduated from The University of Sydney in 2002. After six years in small animal practice she returned to The University of Sydney and completed an internship and attained a Graduate Diploma in Veterinary Clinical Studies. She obtained Membership of the Australian New Zealand College of Veterinary Scientists in Small Animal Medicine in 2010. In 2011 she commenced a residency in small animal medicine at Murdoch University and is currently completing a Master of Veterinary Studies.

1. Introduction

Babesia gibsoni is a vector-borne haemoprotozoan parasite of emerging global importance with infections documented throughout Asia, Australia, Africa, North and South America and Europe (Irwin, 2009). Infection occurs following the bite of an infected ixodid tick from the genus *Haemaphysalis* and, possibly, the genus *Rhipicephalus* (Irwin, 2010). Sporozoite transmission into the bloodstream of the canine host results in erythrocyte invasion, intra-erythrocytic multiplication, and subsequent erythrocyte lysis, releasing more parasites into the bloodstream to infect additional erythrocytes or new ixodid vectors (Irwin, 2010). Non-vectorial routes of transmission have also been documented for *B. gibsoni* including transplacental transmission and transmission by blood transfusions or fighting (Stegeman et al., 2003; Fukumoto et al., 2005; Jefferies et al., 2007a).

The clinical presentation of canine babesiosis can vary widely from subclinical to fulminant disease resulting in multiple organ failure and death (Irwin, 2010). The severity of the disease is primarily determined by the species of *Babesia* parasite involved, with *B. gibsoni* considered intermediate in its pathogenicity (Schoeman, 2009). However, host factors, including age and immune response, can also play a role in determining the severity of the disease (Oduye and Dipeolu, 1976; Lewis et al., 1995; Jacobson, 2006). Natural and experimental *B. gibsoni* infections are typically characterised by pyrexia, lethargy, anaemia, thrombocytopenia, icterus and splenomegaly (Jefferies et al., 2007b; Schoeman, 2009).

Infection with babesial parasites induces a systemic inflammatory response in the host and this is considered to be a major feature of the pathophysiology of canine babesiosis which contributes to its diverse clinical manifestations (Welzl et al., 2001; Matijatko et al., 2007; Koster et al., 2009; Schetters et al., 2009). Cytokines play a critical role in the initiation and

development of systemic inflammation and are responsible for mediating and regulating all aspects of the immune response to infection (Borghetti et al., 2009; Lewis et al., 2012). However, while important for host defence, excessive production and release of these immunoregulatory mediators can prove deleterious to the host, initiating widespread tissue injury and organ damage (Borghetti et al., 2009; Lewis et al., 2012). Longitudinal kinetic profiling of a broad range of cytokine alterations that occur during an infection can be useful to expand our understanding of the immunopathogenesis of infectious diseases and host-adaptive humoral and cell-mediated immune responses to infection, as well as potentially allowing the identification of future diagnostic markers and therapeutic interventions.

The immunopathogenesis of canine babesiosis is poorly understood at a cellular level. To date, the only cytokine investigated in canine babesiosis is tumour necrosis factor alpha (TNF-α) which was reported in a single study that assessed TNF-α concentrations in dogs naturally infected with *B. rossi* at the time of presentation to a veterinary hospital with clinical signs of babesiosis (Vaughan-Scott, 2001). In this study higher TNF-α concentrations were found in dogs with higher peripheral parasitaemias and more severe disease, although no association between TNF-α concentration and mortality was identified. The kinetics that occur in other cytokines in canine babesiosis have yet to be characterised.

In response to inflammatory cytokine secretion, increased production of positive acute phase proteins, such as C-reactive protein (CRP), occurs as part of the innate immune response to infection (Murata et al., 2004; Ceron et al., 2005). In dogs, CRP is regarded as a major acute phase protein and can be used as a sensitive biomarker to quantify systemic inflammation (Murata et al., 2004; Ceron et al., 2005). An acute phase response to natural *B. rossi* infections, and both natural and experimental *B. canis* infections, has been documented previously (Ulutas et al., 2005; Matijatko et al., 2007; Koster et al., 2009; Schetters et al.,

2009). Increased concentrations of CRP were identified in dogs naturally infected with *B. rossi* and *B. canis* at the time of presentation to a veterinary hospital with clinical signs of canine babesiosis and persistently increased CRP concentrations were noted in dogs within 2-4 days of experimental infection with *B. canis* (Ulutas et al., 2005; Matijatko et al., 2007; Koster et al., 2009; Schetters et al., 2009). However, no association between CRP concentration and disease outcome was identified. The acute phase response to *B. gibsoni* infection has yet to be characterised.

The aim of this longitudinal study was to investigate the cytokine kinetics together with the clinical, haematological and acute phase protein changes that occur during experimental *B. gibsoni* infection of beagle puppies.

2. Materials and methods

2.1. Experimental animals

Four intact five month old beagle litter-mates were used in an experiment performed as part of the production of microscope slides used commercially in an indirect fluorescent antibody test (IFAT) for the diagnosis of *B. gibsoni* infections. All four dogs were confirmed to be free of *B. gibsoni* infection by polymerase chain reaction (PCR) and IFAT (Vetpath Laboratory Services (VLS), Australia) prior to the start of the experiment. All four dogs were housed together in the Animal House facility at Murdoch University for the duration of the experiment and managed under identical conditions. The experiment was started after an acclimatisation period of two weeks. All four dogs were fed a commercial dry dog food (Supercoat Puppy, Purina) twice daily and provided with *ad libitum* access to water. The

study was approved by the Murdoch University Animal Ethics Committee (Permit Number: R2442/11).

2.2. Experimental B. gibsoni infection

Blood was collected from an American pit bull terrier (red blood cell count 5.2 x 10^{12}/L; reference interval 5.5-8.5 x 10^{12}/L) suspected to be infected with *B. gibsoni*. Parasites were not observed in erythrocytes on manual blood smear evaluation but infection was confirmed by nested PCR amplification of a partial fragment of the 18S rRNA gene of *B. gibsoni* as described previously (Jefferies et al., 2007a). Dogs 1 and 2 were infected with *B. gibsoni* (Day 0) via intravenous injection of 10ml of ethylenediaminetetraacetic acid (EDTA) anti-coagulated whole blood collected from the American pit bull terrier. Dogs 3 and 4 were used as situational controls. The experiment was terminated on Day 23 to allow production of the IFAT slides. Infected dogs were humanely euthanased by barbiturate overdose on Day 24.

2.3. Clinical evaluation

A complete physical examination was performed on Days 0-23 inclusive in all dogs. Heart rate, respiratory rate and body temperature were recorded. Pyrexia was defined as a body temperature greater than 39.3°C.

2.4. Sample collection

Blood (1.3-9.3ml) was collected from each dog every day by jugular venepuncture and placed into potassium EDTA (Sarstedt, Australia) (1.3ml), lithium heparin (Greiner Bio-One, United States of America) (4ml) and serum (Greiner Bio-One, United States of America) (4ml) tubes. Samples were centrifuged within 2 hours of collection at 2000G and serum or plasma supernatant transferred into plain tubes (Sarstedt, Australia) (1.5ml aliquots), frozen and stored at -20°C until analysis.

2.5. Haematological analysis

A complete blood count was performed to assess erythrocyte, leucocyte and platelet numbers using EDTA-anticoagulated whole blood on Days 0-22 inclusive in infected dogs and Days 0, 1, 3, 5, 6 and 8-22 inclusive in control dogs. Samples were analysed on the day of collection at VLS using the CELL-DYN 3700 automated haematology analyser (Abbott Diagnostics, United States of America). Manual blood smear assessment was performed on each sample to manually enumerate platelet numbers, examine erythrocyte and leucocyte morphology and determine differential leucocyte counts. Erythrocytes were specifically evaluated for the presence of intra-erythrocytic parasites and an estimation of the magnitude of peripheral parasitaemia present (expressed as the percentage of the total number of red blood cells that contained parasites) was recorded. Anaemia was defined as an absolute red blood cell count less than 3.7×10^{12}/L, based upon age and breed specific reference intervals previously described for laboratory beagles (Harper et al., 2003). Leucopenia and neutropenia were defined as an absolute white blood cell count less than 6.0×10^{9}/L and an absolute neutrophil count less than 3.0×10^{9}/L respectively. A left shift was defined as a band neutrophil count greater than 0.3×10^{9}/L. Thrombocytopenia was defined as a platelet count

less than 150 x 10^9/L. If platelet numbers were less than 150 x 10^9/L but manual smear assessment identified sufficient platelet clumping that platelet numbers were deemed adequate thrombocytopenia was not considered to be present.

2.6. C-reactive protein measurement

Plasma CRP concentrations were measured on Days 0, 3, 5, 6, 8, 9 and 11-22 inclusive in all dogs. Samples were shipped on ice and analysed using an immunoturbidimetric CRP assay (Avacta Animal Health, United Kingdom) validated for use in dogs. Increased CRP concentrations were defined as those greater than 15mg/L, based upon age and breed specific reference intervals previously described for laboratory beagles (Kuribayashi et al., 2003).

2.7. Cytokine measurement

The serum concentrations of 13 cytokines (TNF-α, interferon-γ (IFN-γ), monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, interferon-γ-induced protein (IP-10) and keratinocyte-derived chemokine (KC-like)) were measured on Days 0, 1, 4, 7, 10, 13, 15-20 inclusive and 23 in all dogs using a commercially available canine-specific multiplex cytokine immunoassay (MILLIPLEX MAP Canine Cytokine/Chemokine Magnetic Bead Panel CCYTOMAG-90K-PX13, Merck Millipore, United States of America). The selection of cytokines measured was determined by those available in the MILLIPLEX MAP Canine Cytokine/Chemokine Magnetic Bead Panel. All cytokines were assayed in duplicate

according to the manufacturer's instructions. Samples were analysed using a Bio-Plex MAGPIX Multiplex Reader (Bio-Rad, United States of America) equipped with a Luminex XMAP technology detection system. Data were evaluated using the Bio-Plex Manager software (Bio-Rad, United States of America). Seven-point standard curves, created from known concentrations of recombinant canine cytokines, were analysed using a five-parameter logistic regression curve-fitting method to determine the concentration of each cytokine. Intra-assay and inter-assay coefficients of variation stated by the manufacturer for all cytokine analytes were <5% and <17% respectively. The minimum detectable concentrations of the 13 cytokines provided by the manufacturer were regarded as the detection limits in this study (TNF-α 6.1pg/ml; IFN-γ 13.6pg/ml; MCP-1 21pg/ml; GM-CSF 9.2pg/ml; IL-2 3.5pg/ml; IL-6 3.7pg/ml; IL-7 7.5pg/ml; IL-8 21.7pg/ml; IL-10 8.5pg/ml; IL-15: 9.0pg/ml; IL-18 5.8pg/ml; IP-10 3.2pg/ml; KC-like 5.3pg/ml) and values below the detection limit were assigned a value equal to the minimum detectable concentration for the respective cytokine. As reference intervals have not been established in healthy dogs for any of the 13 cytokines measured in this experiment all changes detected in the concentration of cytokines were described in relation to control dogs and baseline values.

3. Results

3.1. Experimental infection

Babesia gibsoni infection was successfully established in Dogs 1 and 2. The peripheral parasitaemia of Dogs 1 and 2 throughout the experiment are shown in Figure 1. Parasites were first detected microscopically in circulating erythrocytes on Day 15 in both

dogs. Peripheral parasitaemia increased in magnitude over the following week and by Day 22 intra-erythrocytic parasites were observed in 5% of circulating erythrocytes in both dogs. Parasites were not detected in the circulating red blood cells of Dog 3 or 4 at any time.

3.2. Clinical observations

No abnormalities in heart or respiratory rate were noted in any dog at any time. Pyrexia developed in Dog 1 on Day 14 and in Dog 2 on Day 13 prior to the detection of peripheral parasitaemia. However, the pyrexia resolved in both dogs by Day 18 and did not reoccur despite further increases in the magnitude of the peripheral parasitaemia over the next week. No abnormalities in body temperature were documented in Dog 3 or 4 at any time.

3.3. Haematological results

The red blood cell counts of all four dogs throughout the experiment are shown in Figure 2. Both Dogs 1 and 2 developed a mild anaemia on Day 15 (Dog 1 red blood cell count 3.1 x 10^{12}/L; Dog 2 red blood cell count 3.7 x 10^{12}/L) coincident with the onset of peripheral parasitaemia. However, this anaemia was transient and resolved in both infected dogs by Day 17 despite further increases in the magnitude of the peripheral parasitaemia over the following week. Mild anisocytosis and polychromasia were noted in the erythron of all dogs throughout the experiment. However, a corrected reticulocyte count greater than 1.5% (maximum 2.9%) and more marked anisocytosis and polychromasia indicative of a regenerative response were detected intermittently between Days 18 and 22 in Dogs 1 and 2.

The neutrophil counts of all four dogs throughout the experiment are shown in Figure 3. Leucopenia and neutropenia developed in Dog 1 on Days 14-16 inclusive and Days 18-19 inclusive. Dog 2 became leucopenic and neutropenic on Days 1, 5, 14-17 inclusive and 21. The nadir in the neutrophil count occurred in both dogs at about the same time that a peripheral parasitaemia was first detected (Dog 1 neutrophil count 1.8 x 10^9/L on Day 15; Dog 2 neutrophil count 2.0 x 10^9/L on Day 16). Toxic changes were noted in the neutrophils of both infected dogs intermittently during the periods of neutropenia but neither infected dog developed a left shift at any point. No abnormalities of lymphocyte, monocyte or eosinophil numbers were detected at any time in any dog.

The platelet counts of all four dogs throughout the experiment are shown in Figure 4. Marked thrombocytopenia, which was absolute on some days, developed in Dog 1 between Days 14-18 inclusive and in Dog 2 between Days 14-16 inclusive. This thrombocytopenia developed prior to the detection of peripheral parasitaemia and resolved despite further increases in the magnitude of the peripheral parasitaemia over the following week.

No abnormalities in the erythron, leucon or thrombon of Dog 3 or 4 were detected.

3.4. C-reactive protein results

The CRP concentrations throughout the experiment are shown in Figure 5. The CRP concentration increased transiently in Dogs 1 and 2 on Day 3. The magnitude of this increase was mild (Dog 1 CRP concentration 20.86mg/L; Dog 2 CRP concentration 19.03mg/L). However, on Day 13, prior to the detection of peripheral parasitaemia, both infected dogs developed a marked increase in CRP concentration. This increase peaked on Day 15 (Dog 1 CRP concentration 161.31mg/L; Dog 2 CRP concentration 161.73mg/L) coincident with the

onset of peripheral parasitaemia. Following this, the concentration of CRP gradually decreased but remained persistently mildly elevated in both infected dogs for the remainder of the experiment. No increase in the CRP concentration occurred at any time in Dog 3 or 4.

3.5. Cytokine results

The concentrations of TNF-α, MCP-1, GM-CSF, IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18 and KC-like in all four dogs throughout the experiment are shown in Figures 6-16.

In Dog 1 the concentrations of TNF-α, GM-CSF, IL-2, IL-6 and IL-18 increased from Day 17 onwards with simultaneous peaks on Day 18 (TNF-α, GM-CSF, IL-2, IL-6 and IL-18 concentrations 59pg/ml, 127pg/ml, 63pg/ml, 134pg/ml and 194pg/ml, respectively). Persistent, albeit less marked, increases in the concentration of all these cytokines were documented for the remainder of the experiment. The concentration of IL-7 increased on Day 18 only (81pg/ml).

In Dog 2 the concentrations of TNF-α, IL-2, IL-7 and IL-18 increased markedly on Day 23 (TNF-α, IL-2, IL-7 and IL-18 concentrations 3527pg/ml, 12662pg/ml, 18253pg/ml and 8533pg/ml, respectively). The concentration of IL-6 was initially increased prior to infection (75pg/ml on Day 0) before gradually decreasing to the minimum detectable concentration by Day 13. Mild increases in the concentration of IL-6 and GM-CSF were then noted on Days 15, 18 and 21 followed by marked increases on Day 23 (8473pg/ml and 8860pg/ml respectively).

The concentration of TNF-α, GM-CSF, IL-2, IL-6, IL-7 and IL-18 did not increase substantially above the minimum detectable concentration for the respective cytokine at any time in Dog 3 or 4.

The concentration of IL-8 in Dogs 1, 3 and 4 ranged from 22-3133pg/ml throughout the experiment. In Dog 2 the IL-8 concentration increased markedly above this range between Days 16-20 inclusive with a peak on Day 20 (7056pg/ml).

Increased IL-10 concentrations were noted on Days 13-16 inclusive and 23 in Dog 1 with a peak on Day 15 (679pg/ml) and on Days 15 (140pg/ml) and 18 (133pg/ml) in Dog 2. The concentration of IL-10 did not increase above the minimum detectable concentration at any time in Dog 3 or 4.

Increased IL-15 concentrations were noted from Day 17 onwards in Dog 1 with a peak on Day 18 (266pg/ml) and from Day 15 onwards in Dog 2 with a peak on Day 23 (16463pg/ml). In Dog 3 the concentration of IL-15 increased on Day 1 (169pg/ml) and remained persistently elevated throughout the experiment while in Dog 4 no increases in the concentration of IL-15 above the minimum detectable concentration occurred at any time.

The concentration of KC-like in infected dogs increased markedly above the range of concentrations seen in control dogs throughout the experiment (125-884pg/ml) on Day 16 only (Dog 1 KC-like concentration 2336pg/ml; Dog 2 KC-like concentration 1134pg/ml).

No apparent difference in the MCP-1 concentration between infected and control dogs was noted throughout the experiment. The concentration of IFN-γ and IP-10 did not increase above the minimum detectable concentration at any time in any dog.

4. Discussion

Experimental *B. gibsoni* infections were successfully established in Dogs 1 and 2 in this experiment. The interval between experimental infection and the onset of peripheral

parasitaemia in the dogs in this study was consistent with previously reported prepatent periods for experimental *B. gibsoni* infection (Meinkoth et al., 2002; Jefferies et al., 2007b).

A mild anaemia developed in both infected dogs in this study coincident with the onset of peripheral parasitaemia. Anaemia is a well-documented finding in canine babesiosis with direct parasite-induced damage to erythrocytes resulting in haemolysis of infected red blood cells (Irwin, 2010). However, the anaemia in both dogs in this experiment was transient and resolved despite further increases in the magnitude of the peripheral parasitaemia. This is not an unexpected finding as the severity of the anaemia in canine babesiosis is seldom proportional to the magnitude of the peripheral parasitaemia and additional mechanisms, such as oxidative and immune-mediated injury to erythrocytes, are suspected to contribute to erythrocyte loss (Otsuka et al., 2002; Irwin, 2010; Scheepers et al., 2011).

Intermittent leucopenia and neutropenia were noted in both infected dogs throughout the experiment. Although neutropenia has previously been documented in *B. gibsoni* infections, the aetiology remains unknown. Sequestration, increased utilisation and reduced production are postulated as potential causes (Meinkoth et al., 2002; Matijatko et al., 2007).

Both infected dogs in this experiment became profoundly, albeit transiently, thrombocytopenic. In contrast to the anaemia, thrombocytopenia developed prior to the onset of peripheral parasitaemia but, like the anaemia, also resolved despite further increases in the magnitude of the peripheral parasitaemia. Thrombocytopenia is one of the most consistent haematological features of canine babesiosis (Kettner et al., 2003; Matijatko et al., 2007). It is generally considered to be an immune-mediated phenomenon, although consumption and sequestration of platelets may also play a role (Jefferies et al., 2007b). Interestingly, neither infected dog developed any clinical evidence of haemorrhagic diatheses despite the presence of severe thrombocytopenia. This phenomenon is reflected throughout the veterinary literature

pertaining to canine babesiosis where severe thrombocytopenia without apparent clinical effect is frequently reported (Kettner et al., 2003; Jefferies et al., 2007b).

Both infected dogs demonstrated a marked acute phase response to experimental *B. gibsoni* infection. A mild, transient increase in the concentration of CRP was noted in both infected dogs on Day 3. C-reactive protein concentrations then returned to within reference intervals until Day 13 when a more marked increase occurred, which peaked on Day 15 coincident with the onset of peripheral parasitaemia. Following this the concentration of CRP gradually decreased despite further increases in the magnitude of the peripheral parasitaemia, but remained mildly elevated in both infected dogs for the remainder of the experiment. As a major acute phase protein in dogs the concentration of CRP typically increases rapidly after infection. However, the timing of the acute phase response can be affected by the nature and dose of inoculum received (Ceron et al., 2005). Six month old beagle puppies experimentally infected with 10^2, 10^4 or 10^6 *B. canis* infected erythrocytes all developed marked increases in the concentration of CRP within 2-4 days of infection regardless of the dose of inoculum received and these increases were sustained in all dogs throughout the duration of the two week long experiment (Schetters et al., 2009). This demonstrates the marked difference in the timing of the acute phase response induced by different species of babesial parasite and indicates that the peak of the acute phase response induced by experimental *B. gibsoni* infection may be delayed by as much as two weeks following infection.

Increased concentrations of CRP have also previously been documented in dogs naturally infected with *B. rossi* and *B. canis* at the time of presentation to a veterinary hospital with clinical signs of canine babesiosis (Ulutas et al., 2005; Matijatko et al., 2007; Koster et al., 2009). However, these dogs were all parasitaemic at the time that increased concentrations of CRP were detected. Yet, in this study, the acute phase response induced by experimental *B.*

gibsoni infection preceded the detection of peripheral parasitaemia. Increased concentrations of CRP were also noted in beagle puppies experimentally infected with *B. canis* prior to the detection of peripheral parasitaemia (Schetters et al., 2009). This is of clinical significance, as dogs infected with babesial organisms could potentially present with clinical signs of canine babesiosis without a detectable parasitaemia and the absence of a peripheral parasitaemia in a sick dog should not exclude a diagnosis of babesiosis.

Increased concentrations of multiple cytokines were detected in both infected dogs in this study. However, these increases were delayed by more than two weeks following infection and did not develop until after a peripheral parasitaemia was detected. Cytokines are fundamentally involved in the pathophysiology of the systemic inflammatory response to infection (Borghetti et al., 2009; Lewis et al., 2012). The interval between experimental *B. gibsoni* infection and the cytokine alterations detected in this study indicates that the systemic inflammatory response induced by experimental *B. gibsoni* infection, like the acute phase response, may be delayed by more than two weeks following infection.

In this study the acute phase response preceded the cytokine alterations. This is surprising given that inflammatory cytokines, particularly TNF-α and IL-6, are reported to be responsible for stimulating the acute phase response. This may reflect transient increases in the concentration of cytokines which were not detected by the intermittent measurement of cytokine concentrations in this experiment; biologically significant increases in the concentration of cytokines that were below the limit of detection of the assay used in this experiment; local production and release of cytokines within organ systems which were not reflected in the systemic concentration of cytokines; or that other mechanisms were involved in triggering the acute phase response.

Marked increases in the concentration of TNF-α and IL-6 were noted from Day 17

onwards in Dog 1 and on Day 23 in Dog 2. Concentrations of TNF-α have previously been evaluated in a cross-sectional study of dogs at the time of presentation to a veterinary hospital with clinical signs of canine babesiosis following natural *B. rossi* infection (Vaughan-Scott, 2001). Higher TNF-α concentrations were documented in dogs with higher peripheral parasitaemia and more severe disease but no association between TNF-α concentration and mortality was identified. Tumour necrosis factor alpha is a potent pleiotropic inflammatory cytokine responsible for inducing the production of multiple other cytokines and mediating many of the physiological disturbances characteristic of sepsis (Blackwell and Christman, 1996). Interleukin-6 is a multifunctional immunoregulatory cytokine which plays a crucial role in B and T cell activation and the induction of the acute phase response (Scheller et al., 2011). Together, TNF-α and IL-6 drive the initial inflammatory response to infection and have been implicated as playing a pivotal role in the pathophysiology of sepsis. Increased concentrations of both TNF-α and IL-6 have been correlated with increasing disease severity and mortality rates in septic dogs (Otto et al., 1997; Rau et al., 2007; Lewis et al., 2012). Given the importance of these cytokines in systemic inflammatory states it is highly likely that they also play an important role in the immunopathogenesis of canine babesiosis.

Increased concentrations of multiple other cytokines including GM-CSF, IL-2, IL-7, IL-10, IL-15, IL-18 and KC-like were noted in both infected dogs in this study while the concentration of IL-8 increased only in Dog 2. All cytokines exhibit a degree of pleiotropy and redundancy with multiple overlapping actions (Akdis et al., 2011). Interleukins 2, 7 and 15 are all members of the IL-2 cytokine family and all play a role in B and T cell growth, differentiation and activation (Akdis et al., 2011). Likewise, IL-18, a member of the IL-1 cytokine family, also plays a role in T cell development (Akdis et al., 2011). In contrast, IL-8 is a chemokine and potent chemoattractant for neutrophils and eosinophils while IL-10 is an

important anti-inflammatory cytokine that regulates the immune response by inhibiting T cell activation and the production of pro-inflammatory cytokines (Akdis et al., 2011). The role of these cytokines in systemic inflammatory states in dogs is currently poorly understood.

While the concentrations of multiple cytokines were documented to increase simultaneously in an infected dog, the timing of these alterations was often different between dogs. We suggest that this reflects differences in individual host's immune response to infection. Even though the beagles used in this experiment were litter-mates and managed under identical environmental conditions, differences in genes and their regulatory elements likely existed between dogs, which may explain the different cytokine profiles seen. Further characterisation of the different cytokine profiles seen in infectious diseases may help delineate different patterns of immunological response which are associated with distinct clinical presentations and outcomes and allow the identification of specific diagnostic markers and future therapeutic interventions.

Other cytokines, such as IFN-γ and IP-10, that did not increase above the minimum detectable concentration at any time in either infected or control dogs are considered unlikely to play an important role in the immunopathogenesis of canine babesiosis. This is not a surprising finding given that the predominant role of IFN-γ is in the inhibition of viral infections and the production of IP-10 is induced by IFN-γ.

This study has a number of limitations. Due to ethical considerations only a small number of dogs were able to be used in the experiment. This precluded any statistical analysis of the results from being performed. The young age of the beagles used in the experiment makes it difficult to directly compare the immune responses of the dogs in the study to those of adult dogs, as dogs are generally not considered fully immunocompetent until 12 months of age (Day, 2007). However, by four months of age the proportion and functional competency

of circulating B and T lymphocyte subpopulations in puppies has been documented to be the same as those in adult dogs (Felsburg, 2002). Additionally, following the decline in maternal immunoglobulins in puppies, normal adult concentrations of serum IgM and IgG can be reached by as early as two and six months of age respectively (Felsburg, 2002). Thus it is possible that the dogs used in this study were immunocompetent, but interpretation of the immune response of the dogs in this study as equivalent to those of adult dogs must be done with caution.

The experimental model used in this study may not accurately reflect what occurs during a naturally-acquired *B. gibsoni* infection. An intravenous route of inoculation is different to that of a naturally acquired tick-based infection, but is not dissimilar to an infection acquired via blood transmission during a fight. However, the number of parasites passaged into the dogs in this study was likely much higher than would occur in a naturally acquired infection from either a tick bite or blood exchange during a fight. These factors could impact the resultant immune response, cytokine alterations and acute phase response seen in the dogs in this experiment although to date no studies have been conducted evaluating the effect of the dose or route of inoculum on the cytokine kinetics in canine babesial infections. The relatively limited duration of the study also restricted our understanding of the clinical, haematological, cytokine and acute phase protein changes that occur during experimental *B. gibsoni* infection of beagle puppies to the initial phase of infection only. Finally, as the range of cytokines measured in this experiment was predetermined by assay availability, some biologically important cytokines may not have been evaluated by this study.

5. Conclusions

Experimental *B. gibsoni* infection of beagle puppies is characterised by the development of a transient pyrexia and anaemia, intermittent neutropenia and a profound, but likewise transient thrombocytopenia without apparent clinical effect. Experimental *B. gibsoni* infection of beagle puppies induces an acute phase response, characterised by a marked increase in the concentration of CRP, which is delayed in onset by some weeks following infection but precedes the detection of peripheral parasitaemia. Experimental *B. gibsoni* infection of beagle puppies is also associated with marked increases in the concentration of multiple cytokines. However, these cytokine alterations were delayed in onset by more than two weeks following infection and occurred subsequent to the detection of peripheral parasitaemia and the acute phase response.

Conflict of interest statement

Vetpath Laboratory Services contributed financial support towards this study. However, VLS played no role in determining study design, or in the analysis or interpretation of data or the decision to submit the manuscript for publication. None of the authors of this paper has any financial or personal relationships with any other people or organisations that could inappropriately influence or bias the content of the paper.

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Figure legends

Fig.1. Peripheral parasitaemia (expressed as the percentage of the total number of red blood cells that contained parasites) of infected dogs (Dogs 1 and 2) during experimental *B. gibsoni* infection.

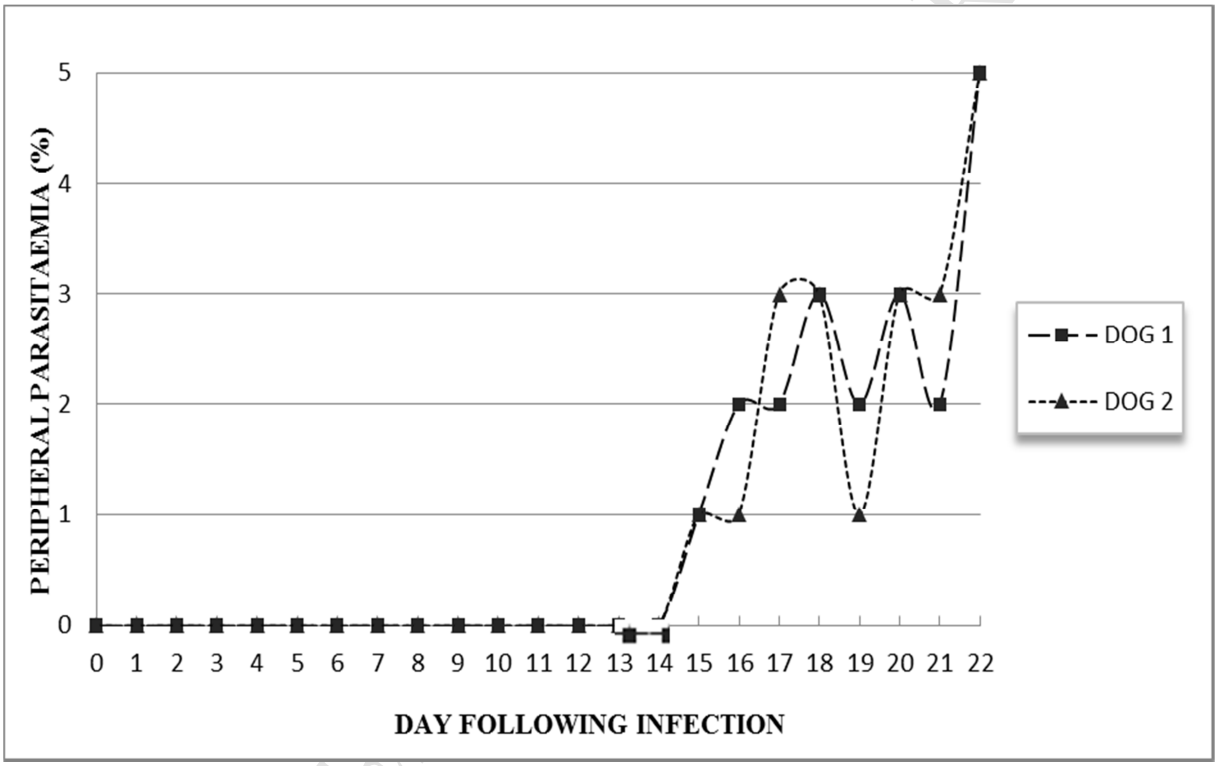


Fig.2. Red blood cell count of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection. Based on age and breed specific reference intervals previously described for laboratory beagles dogs were considered anaemic when the absolute red blood cell count was less than 3.7 x 10^{12}/L (Harper et al., 2003). The arrow indicates the day that peripheral parasitaemia was first detected.

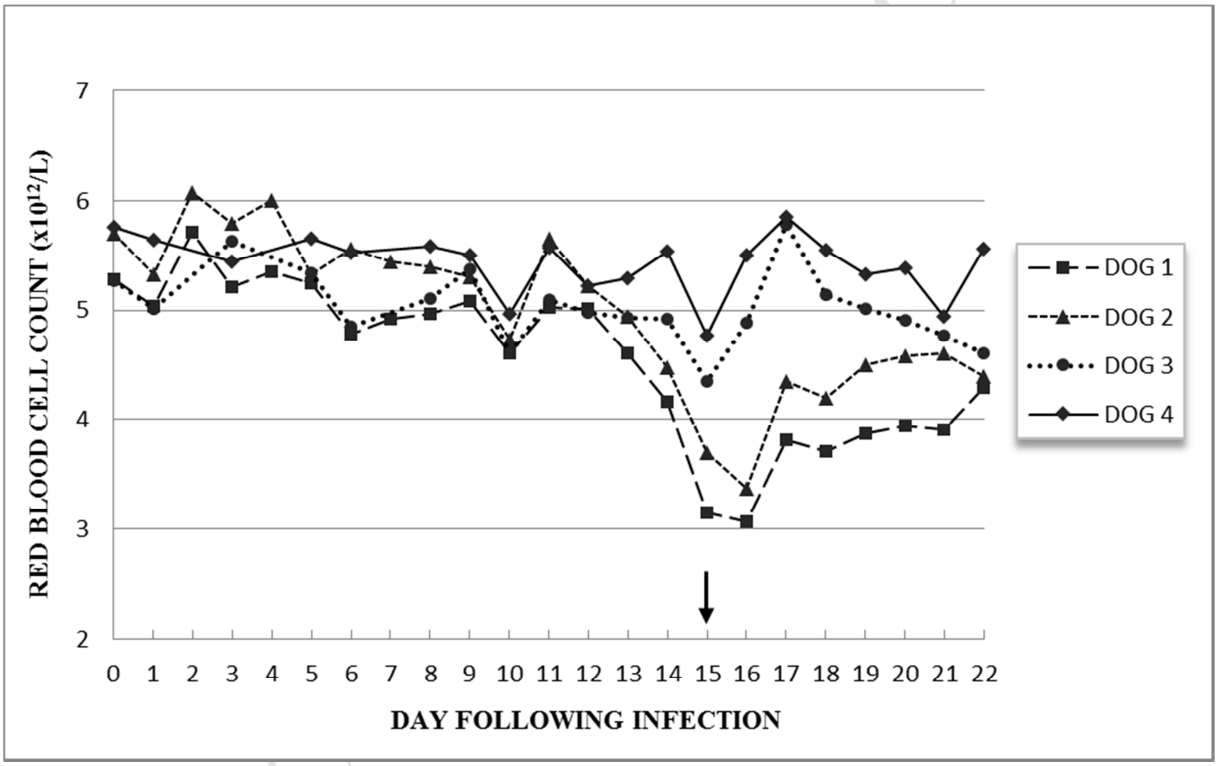


Fig.3. Neutrophil count of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection. Dogs were considered neutropenic when the absolute neutrophil count was less than 3.0 x 10^9/L. The arrow indicates the day that peripheral parasitaemia was first detected.

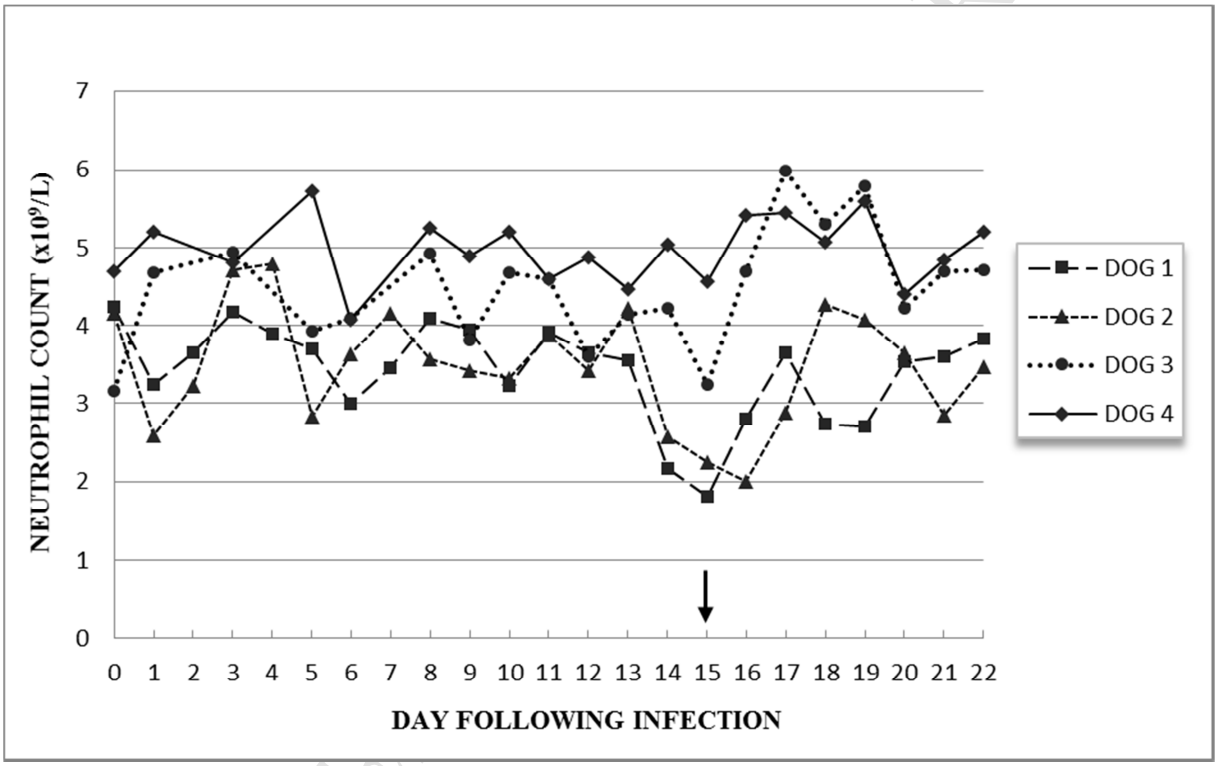


Fig.4. Absolute platelet count of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection. Dogs were considered thrombocytopenic when the platelet count was less than 150 x 10^9/L unless manual smear assessment revealed sufficient platelet clumping that platelet numbers were deemed adequate. The arrow indicates the day that peripheral parasitaemia was first detected.

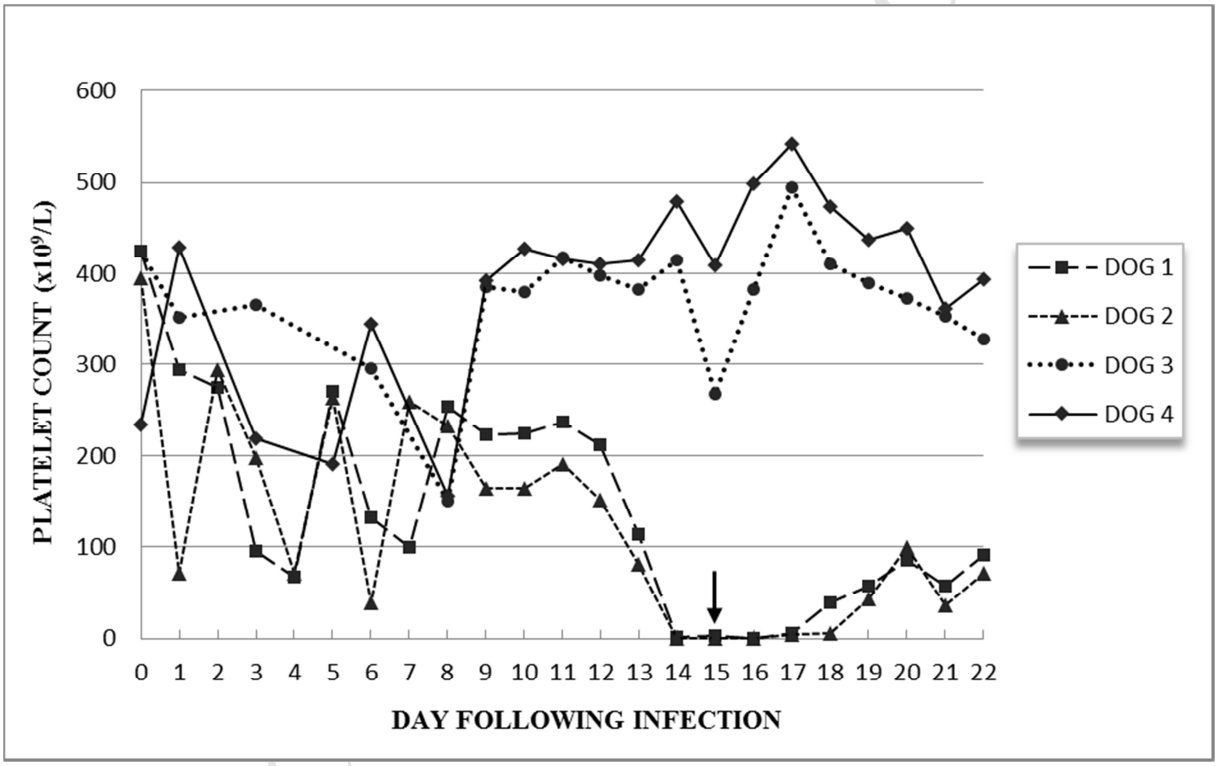


Fig.5. CRP concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection. Based on age and breed specific reference intervals previously described for laboratory beagles the concentration of CRP was considered increased when it was greater than 15mg/L (Kuribayashi et al., 2003). The arrow indicates the day that peripheral parasitaemia was first detected.

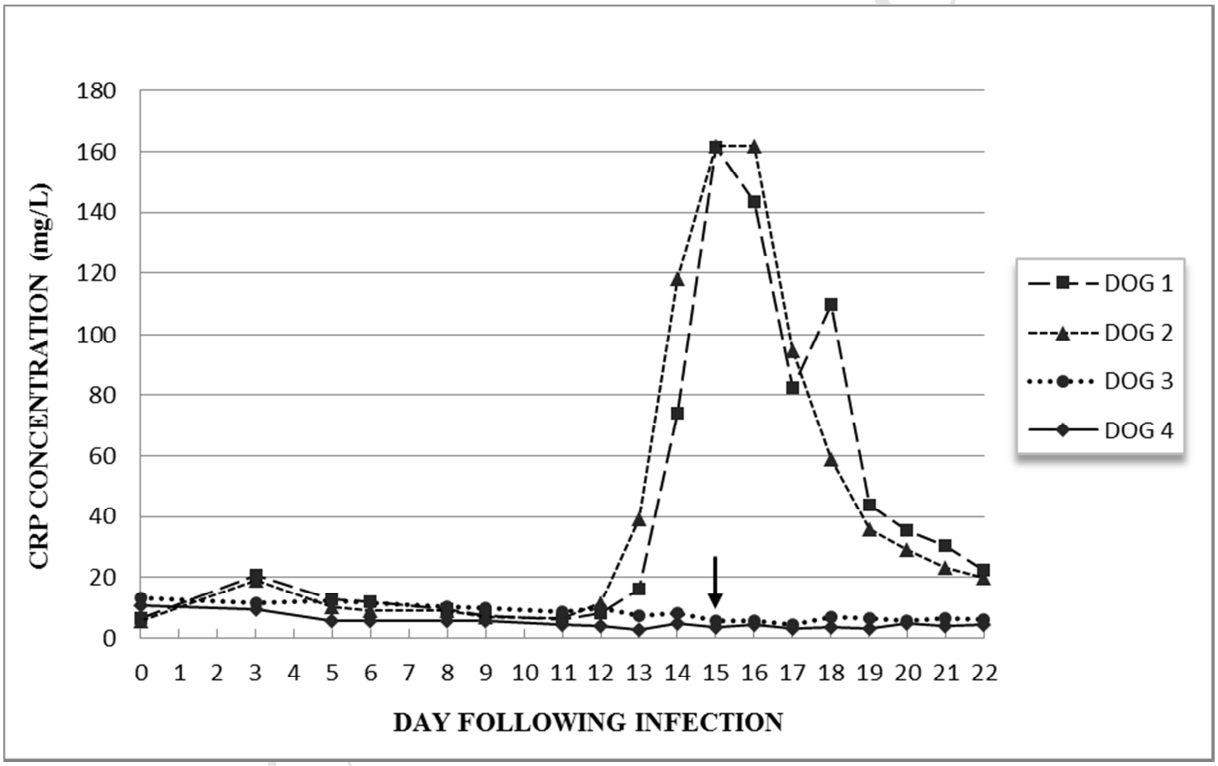


Fig.6. TNF-α concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection.

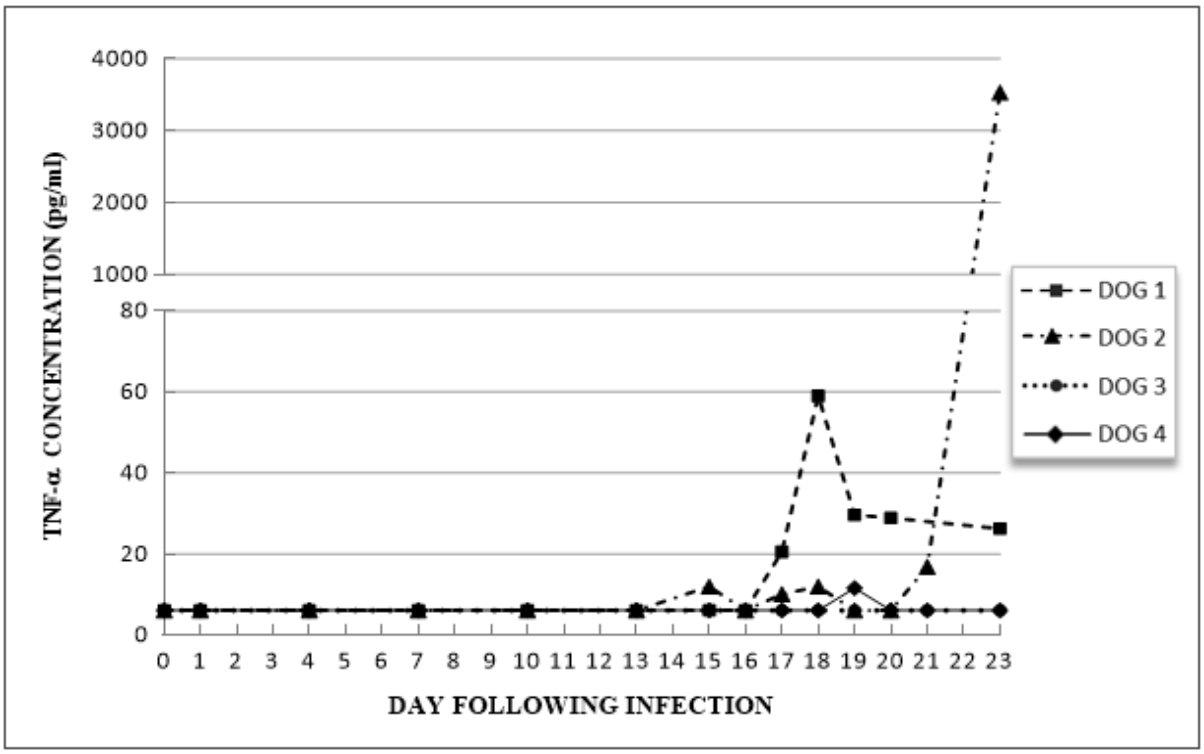


Fig.7. MCP-1 concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection.

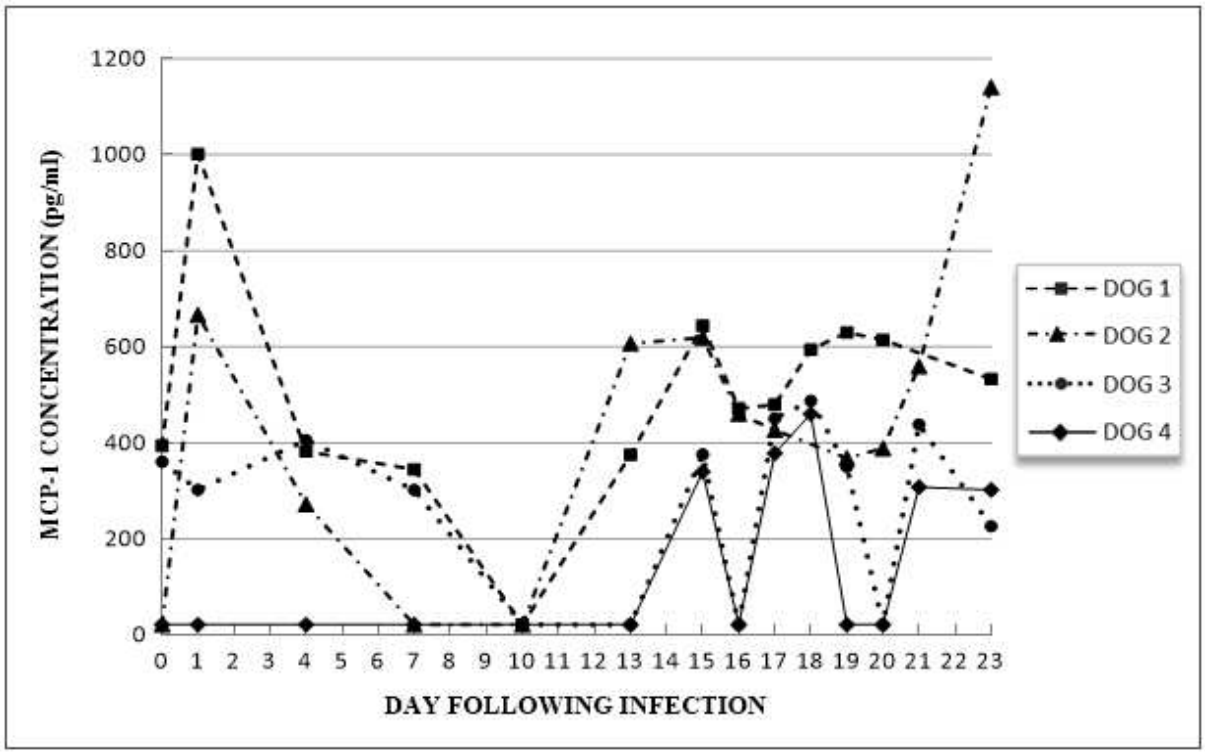


Fig.8. GM-CSF concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection.

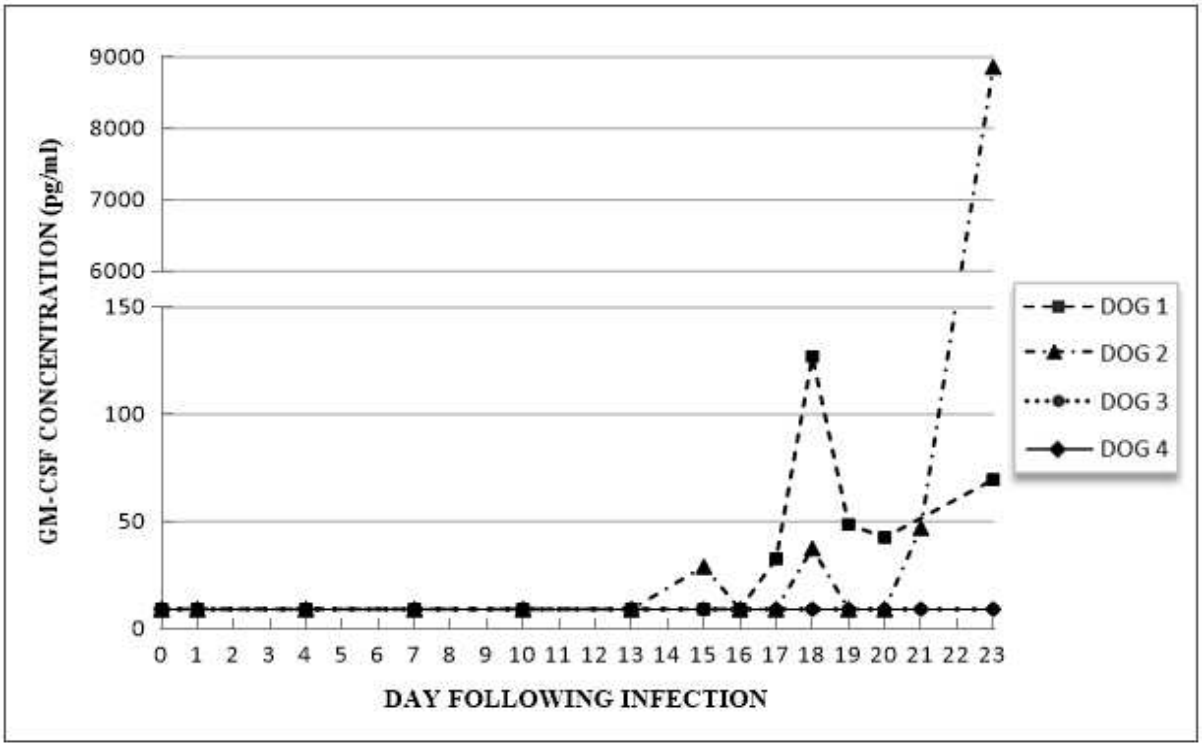


Fig.9. IL-2 concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection.

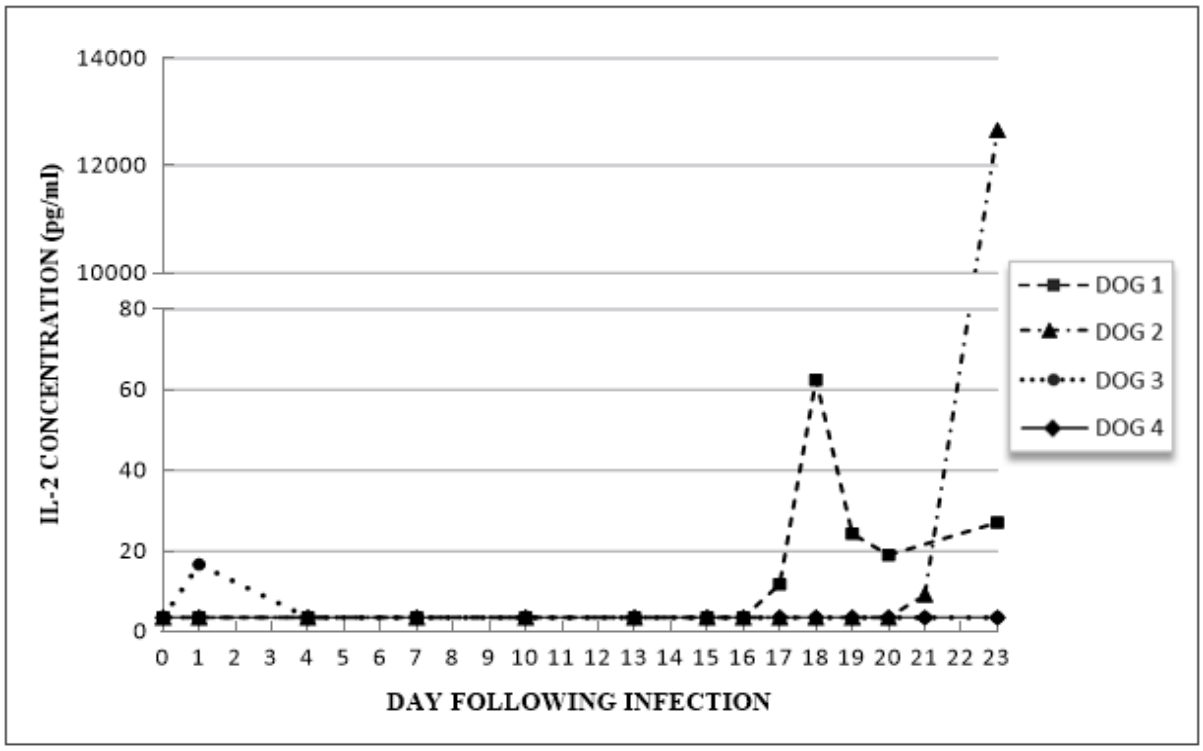


Fig.10. IL-6 concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection.

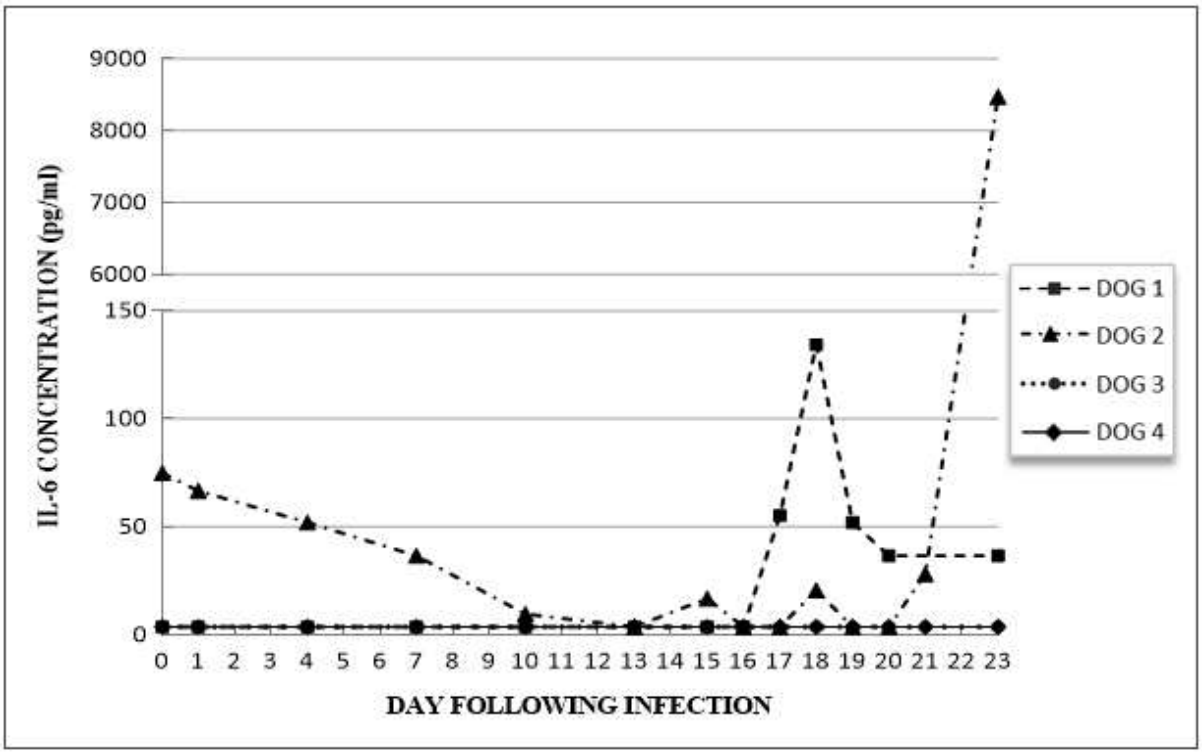


Fig.11. IL-7 concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection.

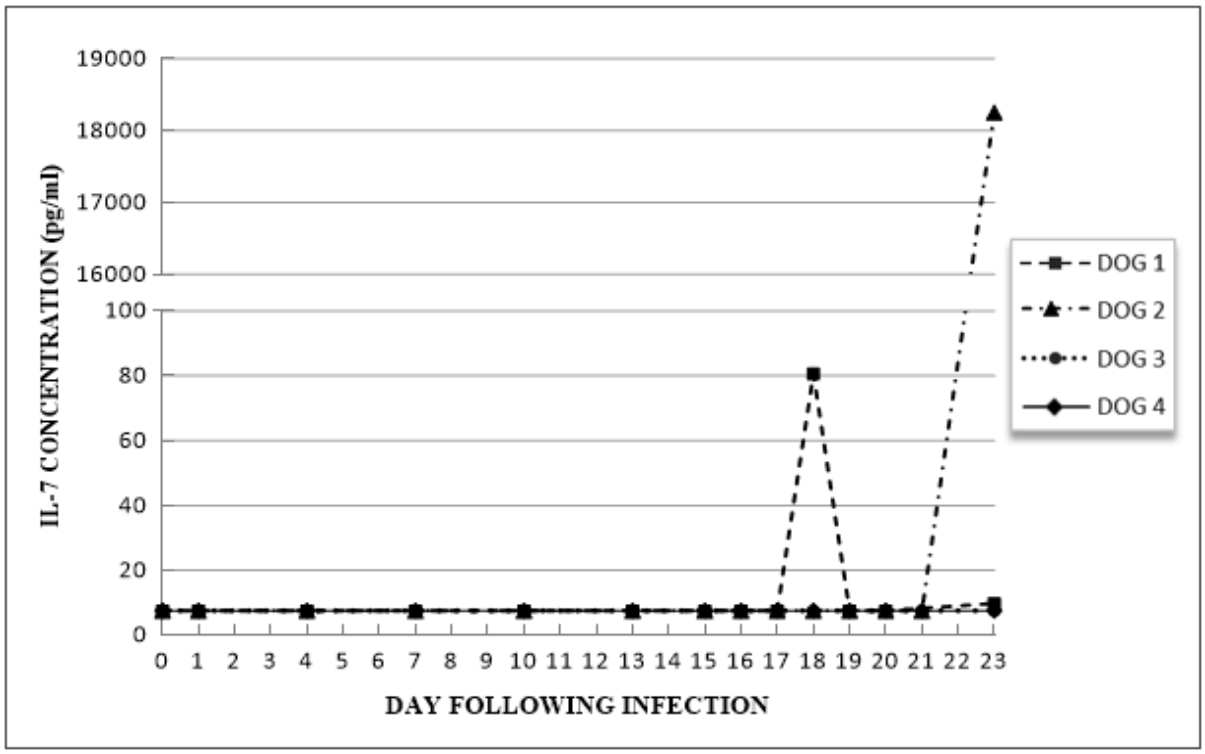


Fig.12. IL-8 concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection.

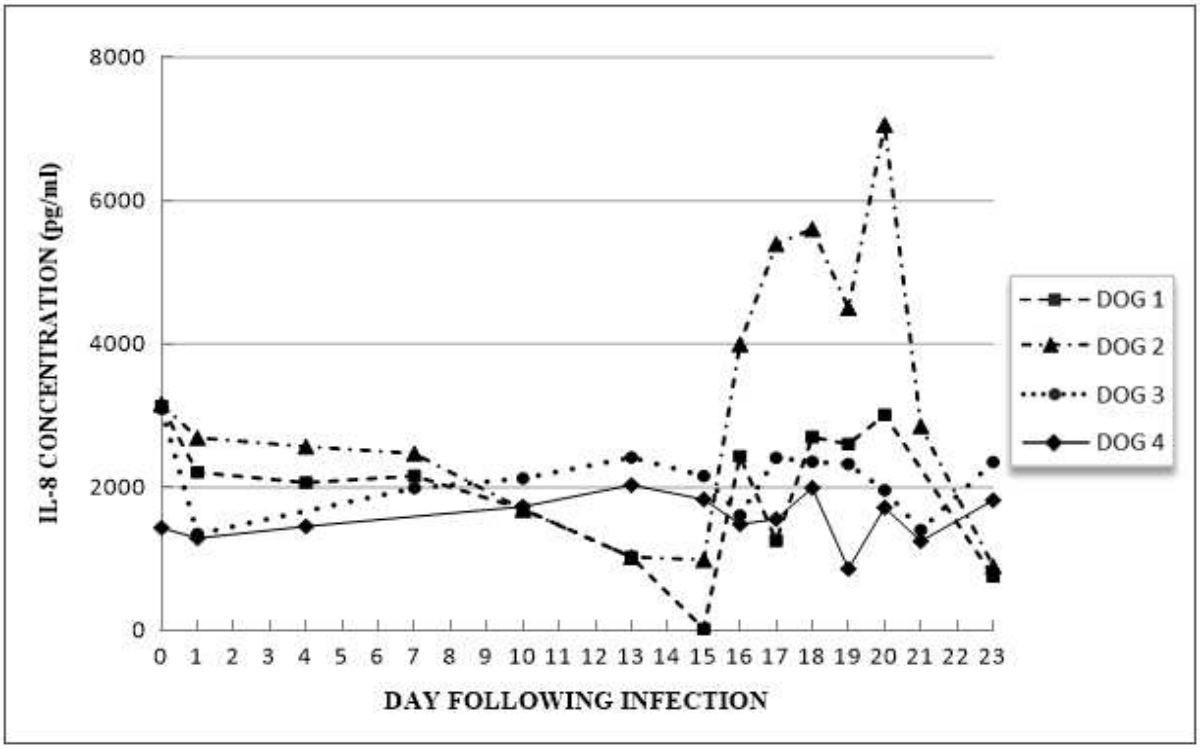


Fig.13. IL-10 concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection.

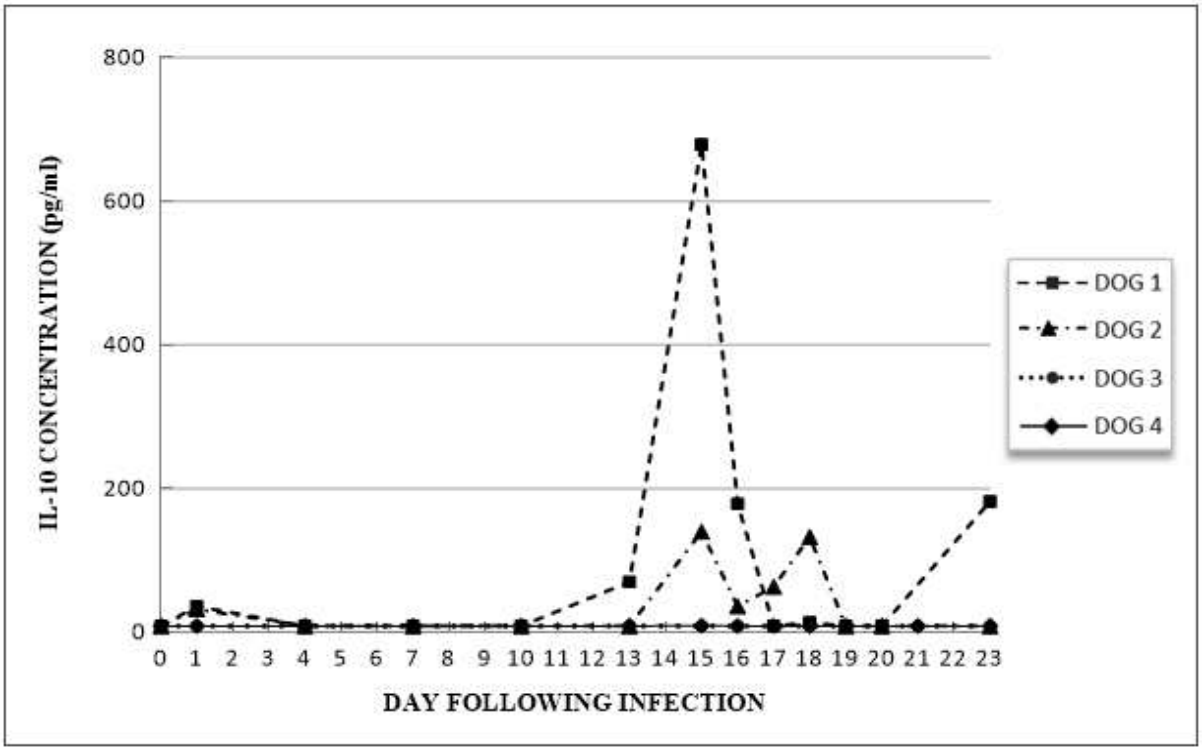


Fig.14. IL-15 concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection.

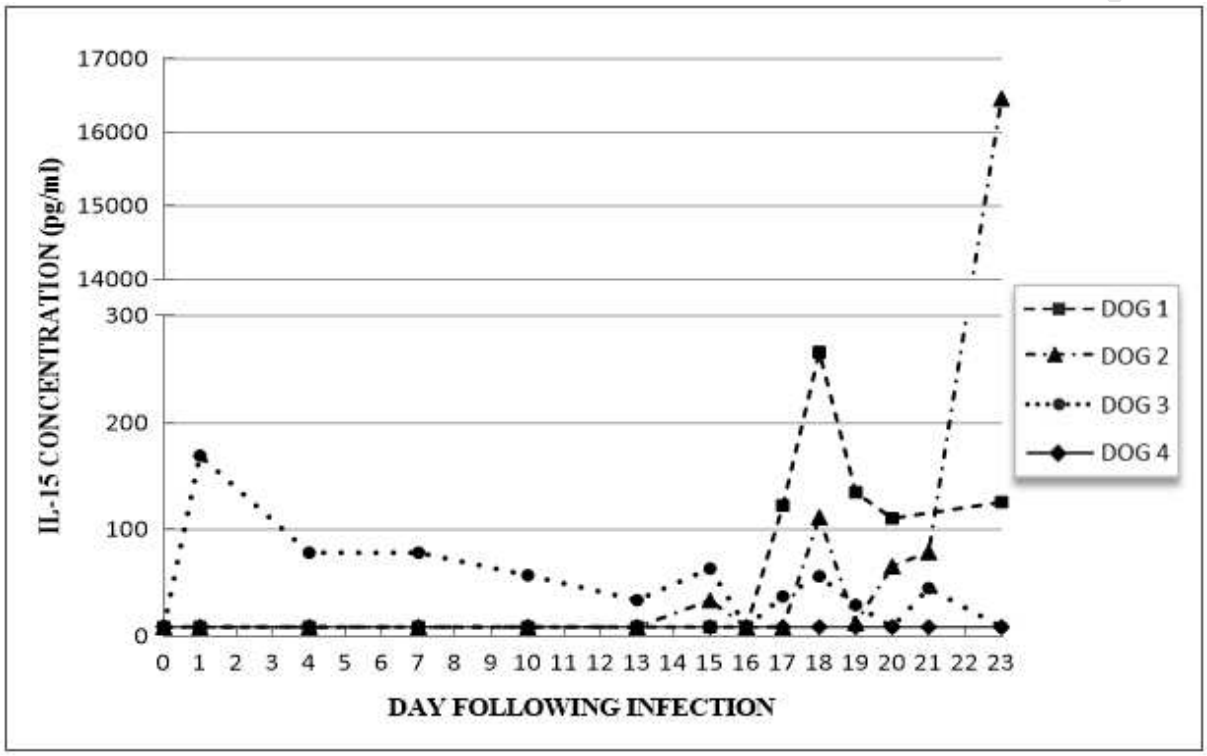


Fig.15. IL-18 concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection.

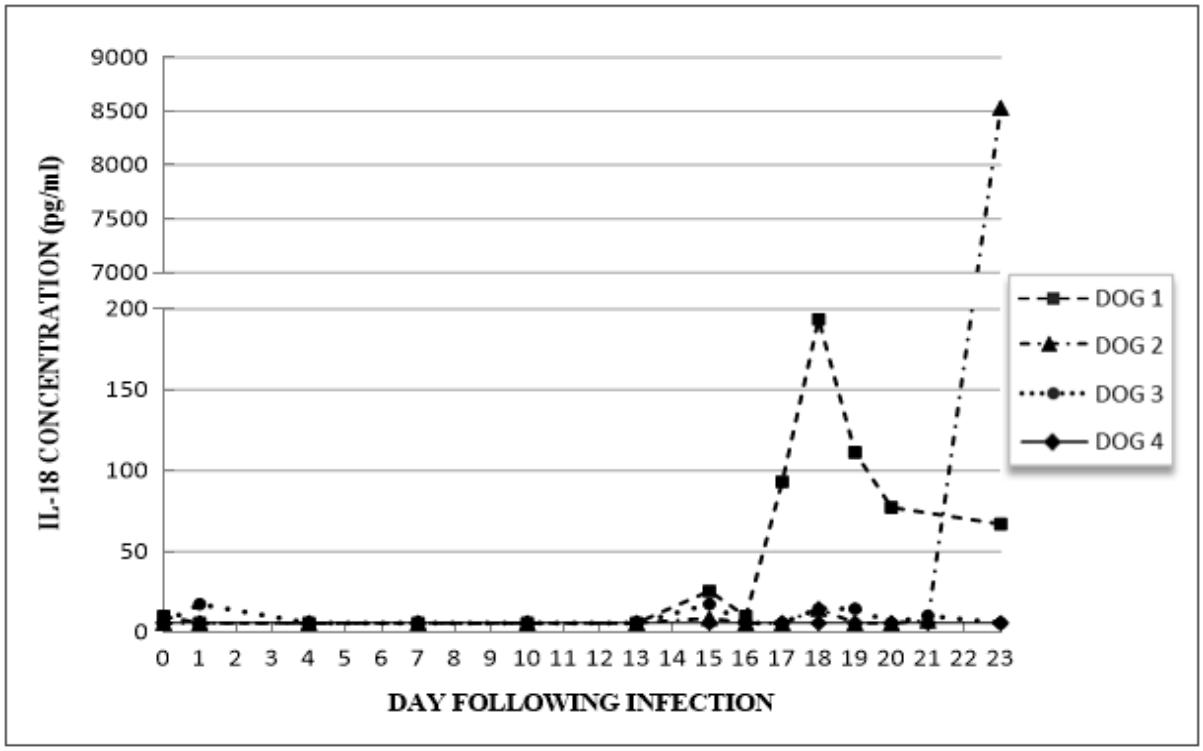
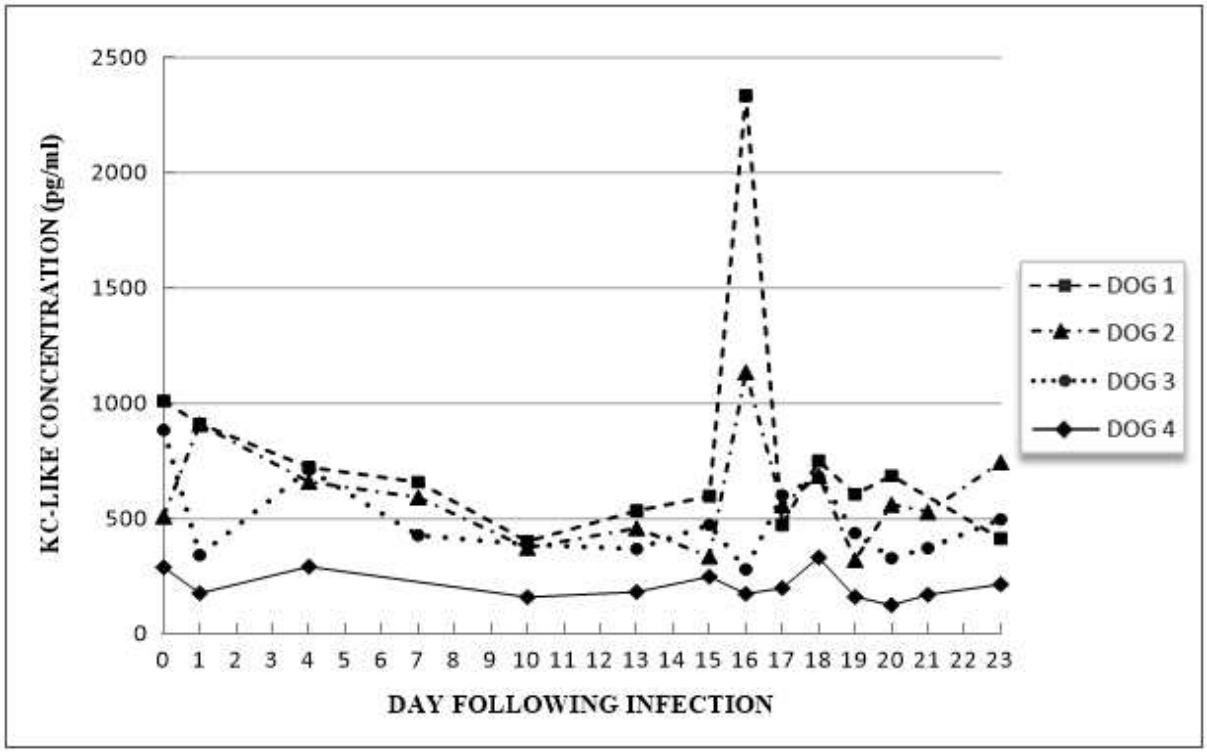


Fig.16. KC-like concentration of infected (Dogs 1 and 2) and control (Dogs 3 and 4) dogs during experimental *B. gibsoni* infection.



Highlights

- Experimental *B. gibsoni* infection caused transient pyrexia and anaemia.
- Infection induced a transient thrombocytopenia without apparent clinical effect.
- The acute phase response was marked but delayed and preceded parasitaemia.
- *B. gibsoni* infection induced marked but delayed increases in multiple cytokines.
- Cytokine alterations occurred following the acute phase response and parasitaemia.