Reticulocyte count and indices in dogs naturally and experimentally infected with *Babesia rossi*

By

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Submitted in fulfillment of the requirements for the degree Master of Science in the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria

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DECLARATION OF ORIGINALITY

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Ethics

The author, Chandini Seejarim, has obtained applicable research ethics approvals for the work described in this dissertation. These approvals were granted by the Research Ethics Committee of the Faculty of Veterinary Science (REC202-19) and Animal Ethics Committee of the University of Pretoria (V055-11, V091-13 and V003-18). The author declares that she has observed the ethical standards required in terms of the University of Pretoria's code of ethics for researchers and the policy guidelines for responsible research.

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Faculty of Veterinary Science

Research Ethics Committee

Project Title	**Description of the absolute reticulocyte count and reticulocyte indices in dogs naturally and experimentally infected with Babesia rossi**
Project Number	**REC202-19**
Researcher / Principal Investigator	**Dr C Seejarim**

Dlissertation / Thesis submitted for	**Masters**

Supervisor	**Prof A Goddard**

APPROVED	Date: **2019-10-11**
CHAIRMAN: UP Research Ethics Committee	Signature:

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Ref: V055-11

28 September 2011

Prof JP Schoeman
Department of Companion Animal Clinical Studies
Faculty of Veterinary Science
(johan.schoeman@up.ac.za)

Dear Prof Schoeman

V055-11 : Haemostatic changes in canine babesiosis, caused by Babesia rossi, and its association with outcome *(A Goddard)*

The application for ethical approval, dated 25 August 2011 was approved by the Animal Use and Committee at its meeting held on 26 September 2011.

Kind regards

E Mostert

Elmarie Mostert

AUCC Coordinator

Copy Prof A Goddard

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Animal Ethics Committee

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

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

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

KINDLY NOTE:

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

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

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Faculty of Veterinary Science

Animal Ethics Committee

Approval Certificate
New Application

AEC Reference No.: V003-18 (A3)
Title: A dog model of severe haemolytic illness
Principal investigator: Prof. A Leisewitz

Dear Prof. Leisewitz

The **New Application** as supported by documents received on 17 January 2019 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 29 January 2019.

Please note the following about your ethics approval:

1. The use of species 6 canine (Beagles) is approved.
2. Ethics Approval is valid for 1 year and needs to be renewed annually by 1 January 2020.
3. Please remember to use your protocol number (V003-18 (A1, A2 and A3) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

"The important thing is not to stop questioning. Curiosity has its own reason for existing."

-Albert Einstein

Table of contents

Acknowledgements

My greatest acknowledgement extends to my parents, without whom, none of this would be possible. Your support and love has been the ultimate pillar to my success.

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List of abbreviations

ACVIM	American College of Veterinary Internal Medicine
AID	Anaemia of inflammatory disease
ARC	Absolute reticulocyte count
B. canis	*Babesia canis*
B. rossi	*Babesia rossi*
B. vogeli	*Babesia vogeli*
BFU-E	Erythroid burst forming unit
CFU-E	Erythroid colony forming unit
CFU-GEMM	Colony forming unit-granulocyte, erythrocyte monocyte, megakaryocyte
CH	Cell haemoglobin content
CHCM	Cell haemoglobin concentration mean
CHCMr	Reticulocyte cell haemoglobin concentration mean
CHDWr	Distribution width (variability) of CHr
CHr	Reticulocyte haemoglobin content
EDTA	Ethylenediaminetetraacetic acid
HCT	Haematocrit
HGB	Haemoglobin concentration in whole blood
IFN-ɣ	Interferon-gamma
IL	Interleukin
IMHA	Immune-mediated haemolytic anaemia
IQR	Interquartile range
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
MCVr	Mean cell volume of reticulocytes
OVAH	Onderstepoort Veterinary Academic Hospital
OVARU	Onderstepoort Veterinary Academic Research Unit
P. falciparum	*Plasmodium falciparum*
P. vivax	*Plasmodium vivax*
PCR	Polymerase chain reaction

RDWr	Distribution width (variability) of reticulocyte cell size
RET%	Reticulocyte percentage
RLB	Reverse line blot
RNA	Ribonucleic acid
TNF	Tumour necrosis factor

List of tables

List of figures

List of appendices

Summary

Despite haemolytic anaemia being the main consequence of *Babesia rossi* infection in dogs, the bone marrow response has been reported to be mild in the face of severe anaemia. A similar finding has been described in falciparum malaria and has been ascribed to either a decreased production of erythroid precursors or an inability of erythroid precursors to respond to hormonal stimulus.

Recently, more information has become available on the use of various reticulocyte indices in an attempt to describe and explain the underlying pathogenesis of various anaemias as their release describes the recent functional state of the bone marrow.

The objective of this study was to compare the admission absolute reticulocyte count (ARC) and reticulocyte indices in dogs naturally infected with *B. rossi* with dogs suffering from immune-mediated haemolytic anaemia (IMHA), unrelated to babesiosis, as well as healthy control dogs. The ARC and reticulocyte indices were also evaluated in five experimentally *B. rossi* infected dogs throughout the disease course.

This was a retrospective observational study looking at the records generated on a haematology analyser, the ADVIA 2120 (Siemens, Munich, Germany). The haematocrit (HCT), ARC and other reticulocyte indices for 103 dogs, naturally infected with *B. rossi* was compared to 16 dogs with IMHA and 14 control dogs. The experimentally infected dogs consisted of five purpose-bred beagles that were infected with high and low dose *B. rossi* parasite inoculum. Differences between groups were assessed using the Mann-Whitney *U* test, whereas the Friedman's ANOVA was used to assess the change over time during the disease course in the experimentally infected dogs. The Wilcoxon Signed Ranks test was then used to determine the differences within the experimentally infected groups.

The median (IQR) HCT for the *Babesia* (0.16 L/L; 0.12 – 0.27; $P < 0.001$) and IMHA (0.15 L/L; 0.12 – 0.17; $P < 0.001$) groups were significantly lower than the control

group (0.52 L/L; 0.45 – 0.57). For the *Babesia* and IMHA groups the HCT did not differ significantly. Compared to the control group (42.1 x10^9/L; 33.8 – 62.6), the median (IQR) ARC was significantly higher in the *Babesia* (82.1 x10^9/L; 48.6 – 174.9; *P* = 0.006) and IMHA (256.7 x10^9/L; 79.0 – 436.9; *P* = 0.004) groups. The ARC was significantly lower in the *Babesia* group compared to the IMHA group (*P* = 0.011), despite no significant difference for HCT between groups.

On day four of the experimentally infected group, approximately 24 – 48 hours after a peripheral parasitaemia was observed, there was a sudden decrease in the ARC to less than its value on day one, which was inappropriate for the degree of anaemia observed.

The reticulocytes of *B. rossi* naturally infected dogs were larger and more hypochromic with a greater difference in cellular haemoglobin (CH) between reticulocytes and mature erythrocytes compared to the control group, whereas the reticulocytes of the experimentally infected dogs were smaller and more hypochromic than day one of infection. All reticulocyte indices demonstrated significant differences on day four compared to day one of the experimental study. The changes noted in the reticulocyte indices are postulated to be a contribution of anaemia of inflammation (AID), shift reticulocytosis and iron-restricted erythropoiesis.

This study concludes that the regenerative response in dogs naturally infected with *B. rossi* is inappropriate, despite the severity of anaemia observed, compared to dogs with IMHA. Furthermore, the findings of the experimentally group may indicate a possible direct suppressive action of the *Babesia* parasite on the bone marrow during the time of parasitaemia, resulting in insufficient erythropoiesis. Similar findings have been reported in falciparum malaria and other factors will require further investigation.

Chapter 1: Introduction

Babesiosis is a common intra-erythrocytic tick-borne disease that affects canines in South Africa, resulting in both intra- and extravascular haemolysis. *Babesia rossi* is considered to be the most common as well as the most virulent of the species (Schoeman, 2009).

Haemolytic anaemias are usually highly regenerative. In regenerative anaemias, the bone marrow responds appropriately to blood loss or destruction by producing red cell precursors, which can later be recognised in circulation as reticulocytes in dogs. The absolute reticulocyte count (ARC) is a variable commonly used to determine the adequacy of the regenerative response in anaemic patients (Cowgill et al., 2003). The ADVIA 2120 is an automated haematology analyser that is able to measure reticulocytes and their indices using a nucleic acid-binding dye (oxazine 750) that differentiates them from mature erythrocytes (Fry and Kirk, 2006; Schaefer and Stokol, 2015). Recent studies have reported that certain reticulocyte indices may be earlier indicators of iron deficiency anaemia in dogs than conventional haematological or biochemical indices (Foy et al., 2015; Fry and Kirk, 2006).

Human malaria and canine babesiosis have been described as related intra-erythrocytic protozoa that share a similar pathogenesis and clinical course (Jacobson, 2006). The anaemia seen in *Plasmodium falciparum* and *P. vivax* malaria is associated with a poor reticulocyte response in the face of ongoing haemolysis, coupled with high serum erythropoietin levels (Joice et al., 2014; Wickramasinghe and Abdalla, 2000). There is a dearth of information available on the reticulocyte response in dogs with babesiosis, specifically with *B. rossi* infection. To date, only two previous studies recorded an inadequate regenerative response in *Babesia* infected dogs with moderate to severe anaemia (Reyers et al., 1998; Scheepers et al., 2011). To our knowledge, there is no available literature that specifically describes the reticulocyte response in dogs with *B. rossi* infection.

This study describes the regenerative response and reticulocyte indices in dogs naturally and experimentally infected with *B. rossi*, and drew conclusions based on similarities or differences seen in human falciparum malaria. The results may provide more understanding on the pathophysiology of the disease and allow for better clinical management.

Chapter 2: Literature review

2.1 Canine erythropoiesis

In dogs, erythropoiesis occurs in the bone marrow parenchyma. As erythrocytes develop, they become smaller, their chromatin becomes aggregated and ribonucleic acid (RNA) is lost which changes the cytoplasmic colour from blue to orange (Harvey, 2012). Figure 1 summarises the sequence of erythropoiesis of the erythroid cell line. Specific colony-stimulating factors, cytokines, inhibitory factors and growth factors, such as erythropoietin, mediate the differentiation of pluripotential stem cells (Weiss et al., 2010). Furthermore, erythropoietin prevents apoptosis of progenitor cells, and stimulates the release of shift reticulocytes during anaemia (Cowgill et al., 2003). Shift reticulocytes are basophilic macroreticulocytes that are prematurely released into circulation during severe anaemia (Harvey, 2012). The myeloid stem cell, colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), has the potential to develop into more than one cell line. In the erythroid cell line, CFU-GEMM differentiates into a primitive progenitor cell, the erythroid burst forming unit (BFU-E), which further differentiates into an erythroid colony forming unit cell (CFU-E). Following this is the development of erythroid precursors, which are the first cells that can be recognised as part of a specific cell line. Reticulocytes are immature erythrocytes and usually remain in the bone marrow for two to three days before being released into circulation (Harvey, 2012). Mature erythrocytes appear in circulation approximately five days after the start of maturation of prorubricytes (Weiss et al., 2010).

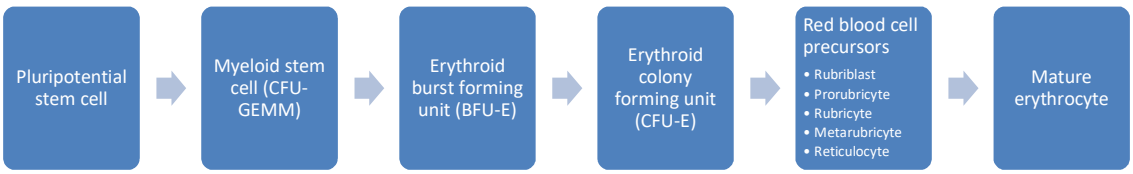

Figure 1: A summary of the sequence of erythropoiesis of the erythroid cell line in canines.

Once canine erythrocytes reach their lifespan of approximately 110 days, they are removed from the circulation by a normal process of phagocytosis by macrophages of mainly the spleen and liver. Intravascular lysis also forms a small part of erythrocyte removal (Latimer and Duncan, 2011). This process of phagocytosis is exacerbated in erythrocytes with increased membrane injury. Anaemia would result if the rate of erythrocyte destruction exceeds the response of the bone marrow to replace lost cells (Harvey, 2012).

2.2 Anaemia

Anaemia is defined as a decrease in erythrocyte mass in the body, which can be caused by decreased production and/or an increased loss (including destruction) of erythrocytes (Ettinger et al., 2017; Harvey, 2012). Anaemias are commonly classified as regenerative or non-regenerative, based on the response of the bone marrow. In regenerative anaemia, the bone marrow responds appropriately to blood loss (haemorrhage) or destruction (haemolysis) by increased production of erythrocyte precursors. Haemolytic anaemias are usually more regenerative than haemorrhagic anaemias, as the liberated iron from lysed erythrocytes is recycled into the production of new cells (Ettinger et al., 2017). Non-regenerative anaemias result from decreased erythrocyte production or defective erythropoiesis, which would indicate a poorly or non-responsive bone marrow. In these cases, the reticulocyte response would be normal or only minimally increased for the degree of anaemia (Harvey, 2012). Non-regenerative anaemias commonly present as normocytic and normochromic, and may be a result of extramedullary disease, such as anaemia of inflammatory disease (AID) (Ettinger et al., 2017).

Determining the reticulocyte percentage (RET%) alone can overestimate the bone marrow response, as the result is not corrected to the degree of anaemia, but is rather expressed as a percentage of the total erythrocytes in circulation (Harvey, 2012). The ARC is a variable commonly used to determine the adequacy of regeneration in anaemic patients, and is considered the best indicator of the bone marrow response (Barger, 2003; Cowgill et al., 2003; Ettinger and Feldman, 2017). Sufficient and appropriate increases in the ARC points towards a functioning bone marrow (Latimer

and Duncan, 2011). The ARC should be higher in cases of severe anaemia versus mild anaemia. A reticulocyte response will not usually be seen in early acute cases of anaemia, given that the reticulocyte response takes three to four days to occur. This may create the impression of the anaemia being initially non-regenerative in nature. Reticulocytes peak within four to seven days after an anaemic insult and disappear in 10 to 14 days if the anaemia resolves (Cowgill et al., 2003; Harvey, 2012).

More information regarding the nature and possible cause of anaemia can be provided by other variables, which include erythrocyte indices, evaluating erythrocyte morphology on blood smears, the appearance of the plasma, plasma protein concentration, serum iron measurements, serum bilirubin determination, direct antiglobulin test, and bone marrow evaluation (Harvey, 2012). The mean cell volume (MCV) and mean cell haemoglobin concentration (MCHC) are erythrocyte indices that respectively describe the average size and haemoglobin concentration of the erythrocyte population. These variables can help determine the nature of the anaemia, but should not be solely relied upon as they are usually normal in regenerative anaemias (Ettinger et al., 2017).

2.3 Immune-mediated haemolytic anaemia

Immune-mediated haemolytic anaemia (IMHA) is a type of regenerative anaemia, characterised by premature destruction of circulating erythrocytes by intravascular and extravascular haemolysis. The process is mediated by the attachment of immunoglobulins to the erythrocyte membrane, which may subsequently activate the complement cascade. Complement activation results in the production of the membrane attack complex which directly damages the cell membrane, causing an influx of extra-cellular fluid into the cell and ultimately, erythrocyte lysis within circulation (Balch and Mackin, 2007; Manev and Marincheva, 2018). The binding of immunoglobulins and complement to erythrocyte membranes can also enhance extravascular lysis and produce spherocytes, which are one of the diagnostic criterions of the condition (Garden et al., 2019; McCullough, 2003). Extravascular haemolysis is mediated by the mononuclear-phagocyte system, during which macrophages phagocytose erythrocytes in the liver and spleen (Balch and Mackin, 2007). Dogs are

commonly affected by IMHA and present with mortalities between 21 and 83%, which usually occur within the first two weeks after onset, and are mainly attributed to thromboembolism or organ failure (Ettinger et al., 2017). The condition may be primary or secondary, based on its aetiology. The former is idiopathic, while the latter is a result of an underlying condition, categorised as neoplasia, infectious disease, inflammatory disease, toxins, drugs and vaccines (McCullough, 2003). However, a recent consensus statement by the American College of Veterinary Internal Medicine (ACVIM) suggested using the terms “non-associative” and “associative” instead of “primary” and “secondary,” respectively. The consensus is to rather use the term “associative”, instead of secondary, when a comorbidity is identified because in some instances the comorbidity might have caused the IMHA, whereas in others it might be coincidental. The term “non-associative” should be used for IMHA cases in which comorbidities are not identified during an extensive workup, and include primary (“idiopathic”) and cryptogenic cases, meaning that perhaps an underlying cause was not identified because the underlying pathomechanisms are not yet understood, or available testing is insufficient to detect the comorbidity (Garden et al., 2019). Similar to babesiosis, the clinical signs may be related directly to the degree of the anaemia. In a study done on 42 dogs with IMHA, severe anaemia (HCT < 0.20 L/L) was observed in 37 dogs (Klag et al., 1993). The anaemia is described as normocytic and normochromic, with a marked regenerative response that occurs after two to five days (Balch and Mackin, 2007; McCullough, 2003). According to the recent ACVIM consensus statement, the diagnosis of IMHA should involve the presence of anaemia, signs of immune-mediated destruction of erythrocytes and evidence of haemolysis. (Garden et al., 2019).

2.4 Canine babesiosis

Babesiosis is a common intra-erythrocytic tick-borne disease that affects canines worldwide. The three large *Babesia* species that affect dogs are *B. vogeli, B. canis* and *B. rossi.* Of the three, *B. vogeli* and *B. rossi* occur in South Africa, with *B. rossi* having a higher prevalence. In a study determining the prevalence of babesiosis around the Onderstepoort region (the study area), 32.1% of 56 canine blood samples tested positive for *B. rossi*, while only 1.8% tested positive for *B. vogeli* (Matjila et al.,

2004). *B. rossi* is also considered to be the most virulent of the species, associated with mortalities of 12 to 15% (Nel et al., 2004; Schoeman, 2009). The parasite is transmitted by the *Haemaphysalis elliptica* tick through trans-stadial and transovarial mechanisms (Schoeman, 2009).

Canine babesiosis can present as uncomplicated or complicated syndromes. The uncomplicated form produces clinical signs that are attributed directly to anaemia (Jacobson, 2006). These include pale mucous membranes, depression, tachycardia, tachypnea, water-hammer pulse, anorexia, weakness, splenomegaly and pyrexia (Jacobson and Clark, 1994b; Schoeman, 2009). The uncomplicated form may be sub-classified according to the degree of anaemia, with mild disease producing a mild to moderate anaemia and life-threatening disease producing an anaemia that is severe, usually with a HCT less than 0.15 L/L (Jacobson, 2006; Jacobson and Clark, 1994a). The South African form of babesiosis caused by *B. rossi* can produce severe haemolytic disease (Jacobson, 2006; Schoeman, 2009). One study that included 662 hospitalised dogs with *B. rossi* infection reported a severe anaemia (HCT < 0.15 L/L) in 50% of cases while 32% of cases were moderately anaemic and 18% non-anaemic on presentation (Reyers et al., 1998).

The complicated form of canine babesiosis, caused by *B. rossi*, presents with severe and sometimes life-threatening syndromes and are usually characterised by systemic inflammatory response syndrome and multiple organ dysfunction syndrome (Jacobson and Clark, 1994b). These complications include haemoconcentration ('red-biliary'), acute kidney injury, hepatopathy, IMHA, pulmonary oedema, rhabdomyolysis, pancreatitis, acid-base abnormalities, consumptive coagulopathy and cerebral signs (Goddard et al., 2013; Jacobson and Clark, 1994b; Schoeman, 2009; Welzl et al., 2001). These complications may or may not be associated with severe haemolytic anaemia, and are frequently associated with a poorer prognosis than the uncomplicated form (Jacobson and Clark, 1994b). One study recorded a mortality rate of 45% in 85 dogs with complicated babesiosis (Welzl et al., 2001) and it is reported that mortality may even reach 100% with some complications, despite treatment interventions (Schoeman, 2009). Cases of uncomplicated babesiosis that are appropriately treated and managed are associated with a survival rate of 85 to 88%

(Nel et al., 2004). Treatment of uncomplicated babesiosis aims to eliminate the parasite, which is done by anti-babesial drugs; and reverse the life-threatening anaemia which is done by supportive treatments such as intravenous fluid therapy and blood transfusions (Schoeman, 2009).

2.5 Haematology of babesiosis

The main consequence of babesiosis is an acute haemolytic anaemia, which may be intravascular as well as extravascular. The *Babesia* parasite causes direct damage to erythrocytes during replication, resulting in intravascular haemolysis while extravascular haemolysis results from increased phagocytosis of parasitised and non-parasitised erythrocytes in the spleen and liver (Ettinger and Feldman, 2017; Maegraith et al., 1957). The anaemia caused by *Babesia* is multifactorial with a number of other mechanisms resulting in erythrocyte destruction, namely binding of antibodies to the erythrocyte surface and complement activation, production of serum haemolytic factors, erythrocyte oxidative damage, creation of spherocytes and an increase in the osmotic fragility of erythrocytes (Maxie, 2016). One study reported that dogs that died of *B. rossi* infections had significantly higher capillary and venous parasitaemias than those that survived (Böhm et al., 2006). This may suggest a parasite-induced anaemia but should not be used to prognosticate clinical course of infection due to the development of complications which may result in clinical collapse and shock (Maegraith et al., 1957). Additionally, sequestration of parasitised erythrocytes in capillaries have been a common finding which may contribute to the reduction in circulating erythrocyte numbers, further contributing to the anaemia of babesiosis (Böhm et al., 2006; Jacobson and Clark, 1994a; Maegraith et al., 1957).

The haematological description of dogs naturally infected with the large *Babesia* species is limited to three main studies. The first and earliest study aimed to describe the clinical pathology of a small group of experimentally *Babesia* infected dogs and over 100 *Babesia* infected dogs that presented to the then outpatients clinic at the Faculty of Veterinary Science in Onderstepoort (Malherbe, 1968). The second is a retrospective analysis of 662 hospitalised cases at the Onderstepoort Veterinary Academic Hospital (OVAH), University of Pretoria, South Africa of which the aim was

to establish whether there were significant differences in haematological variables of *B. rossi*-infected dogs grouped according to their degree of anaemia and whether mortality was associated with changes in variables within each group (Reyers et al., 1998). The third prospective longitudinal study investigated the progression of haematological changes in 32 transfused and 54 non-transfused dogs naturally infected with *B. rossi* over the first six days following diagnosis and treatment (Scheepers et al., 2011).

The first and second study both recorded a normocytic, normochromic anaemia in the babesiosis cases. However, the first study only described the reticulocyte response of a single acute and chronic case out of a few experimentally *Babesia* infected dogs. In the acute case, the reticulocytes showed a moderate rise a few days after clinical signs started, followed by a drop during the few days before the patient died. This was postulated to be from possible bone-marrow depression. The chronic babesiosis case demonstrated a marked reticulocyte response during the period of severe anaemia. The reticulocyte response and HCT then normalised after a few days of treatment (Malherbe, 1968). Unfortunately, the study did not provide clear definitions for acute or chronic babesiosis. In the second and third study, inadequate regenerative responses were recorded. Reyers et al noted that the mean RET% of single admission blood samples of patients were non-regenerative (RET% = 0.74%) for moderately anaemic dogs (HCT 0.15 – 0.30 L/L) and mildly regenerative (RET% = 3.43%) for severely anaemic dogs (HCT < 0.15 L/L) (Reyers et al., 1998). The ARC was not determined in these cases, nor was the timeline of infection known as blood was collected only once, upon presentation to the OVAH. Scheepers et al described the regenerative response to be mild (ARC = 60 – 150 x 10^9/L) to moderate (ARC = 150 – 300 x 10^9/L) for dogs that received a blood transfusion. The transfused group had a median HCT of 0.09 L/L at presentation. Similarly, a mild to moderate response was seen in dogs that did not receive a blood transfusion. These responses were recorded over six days of study. The response was never marked, despite the severity of anaemia observed (Scheepers et al., 2011).

Other literature have suggested an inadequate regenerative response, possibly from a poor bone marrow response (Schoeman, 2009) or a suppressive action of the

Babesia parasite on the bone marrow (Scheepers et al., 2011). Meanwhile, multiple other reports have described the anaemia of canine babesiosis to be adequately or strongly regenerative (Irwin, 2010; Jacobson, 2006; Jacobson and Clark, 1994a). In an early study, an appropriate regenerative response to the severity of anaemia was recorded in all 60 dogs experimentally infected with *B. canis*, except in those with peracute deaths (Maegraith et al., 1957). These conclusions were however, based only on RET% and not ARC.

2.6 Malaria

Human malaria and canine babesiosis have been described as related intra-erythrocytic protozoa that share a similar pathogenesis and clinical course (Lau, 2009). Similar to *Babesia*, the *Plasmodium* parasite is transmitted by an arthropod vector. *Plasmodium falciparum* causes the most severe form of malaria, with almost one million deaths every year (Joice et al., 2014). The anaemia is classified as normocytic and normochromic with a reticulocytosis that only occurs after several days (White, 1992). The pathophysiology of the anaemia is described as multifactorial and is not only due to haemolysis of infected as well as uninfected erythrocytes, but also due to inability to replenish erythrocytes lost by haemolysis through an inadequate erythroid response (Pathak and Ghosh, 2016). Acute malaria can also present with complications, such as cerebral malaria amongst others (Wickramasinghe and Abdalla, 2000).

Several reports have attributed the anaemia seen in *falciparum* malaria to direct invasion of erythrocytes, iron shunting for utilisation by parasites, hypersplenism from intense stimulation of the monocyte-macrophage system, haemophagocytosis of parasitised and non-parasitised erythrocytes, cytokine dysfunction, endothelial injury, splenic sequestration, bone marrow suppression, dyserythropoiesis and impaired erythropoiesis response (Ghosh Kanjaksha and Ghosh; Joice et al., 2014; Wickramasinghe and Abdalla, 2000). Despite these numerous mechanisms involved, the main pathogenesis of the anaemia of *falciparum* malaria can be summarised as the haemolysis of infected and uninfected erythrocytes, as well as a suppressed erythropoiesis (Wickramasinghe and Abdalla, 2000). The general findings of bone

marrow cellularity in acute, uncomplicated *P. falciparum* malaria are described as hypocellular, normocellular or mildly hypercellular with a normal or reduced percentage of erythroblasts (Abdalla et al., 1980; Phillips et al., 1986). Dyserythropoiesis is the production of morphologically defective erythrocytes and is mainly seen in chronic and cerebral malaria versus acute malaria (Wickramasinghe and Abdalla, 2000).

There are contrasting findings regarding the erythropoietin concentration in malaria infected cases, with some studies indicating normal to increased concentrations (Burchard et al., 1995; Kurtzhals et al., 1997) and others indicating suppressed erythropoietin (Burgmann et al., 1996; Hassan et al., 1997). Impaired erythropoiesis in the presence of sufficient erythropoietin may be due to 1) an inability of the erythroid progenitor cells to respond to the hormone, and 2) a decreased production of erythroid progenitors and/or a diminished production of multipotential stem cells (Maggio-Price et al., 1985; Silverman et al., 1987). It has also been reported that the release of reticulocytes in malaria is suppressed by the presence of parasites (Jacobson and Clark, 1994a).

A study on malaria-infected mice showed that bone marrow cellularity was markedly decreased. This was postulated to be as a result of the migration of BFU-E from the bone marrow to the spleen and differentiation to CFU-E in the bone marrow, since histologic evaluation of the spleen revealed erythropoiesis within the splenic red pulp at the same time that bone marrow erythropoiesis returned. Regardless of this response, bone marrow CFU-E was insufficient, and proliferation and differentiation of erythroid progenitors in all locations failed to produce enough reticulocytes to provide a viable red cell count (Maggio-Price et al., 1985). The erythroid progenitor cells were also evaluated in 21 humans with complicated (n=15) and uncomplicated (n=6) *P. falciparum* infections. The bone marrow CFU-E and BFU-E were cultured with sera obtained during parasitaemia as well as post-parasitaemia. In the acute cases, the CFU-E in bone marrows post-parasitaemia was suppressed by the sera obtained during parasitaemia. This implies that the sera during parasitaemia contains reduced erythropoietin or suppressive factors such as increased tumour necrosis factor (TNF) (Jootar et al., 2008). Tumour necrosis factor as well as interferon-gamma (INF-ɣ) has

been shown to be increased in malaria, which subsequently inhibits progenitor cells, thus inhibiting erythropoiesis (Harpaz et al., 1992; Wickramasinghe and Abdalla, 2000). It has been suggested that the malarial pigment, haemozoin, is responsible for the increase of TNF, along with other inflammatory cytokines (Sherry et al., 1995). This would imply a direct suppressive action by the malaria parasite on erythropoiesis.

2.7 Reticulocyte indices

Recently, more information has become available on the use of various reticulocyte indices in an attempt to describe and explain the underlying pathogenesis of various anaemias. These indices include the mean cell volume of reticulocytes (MCVr), haemoglobin content (CHr), haemoglobin concentration (CHCMr); percentage values for subpopulations of hypochromic reticulocytes, microcytic reticulocytes, macrocytic reticulocytes and reticulocytes with low or high haemoglobin content (Fry and Kirk, 2006). The ADVIA 2120 (Siemens, Munich, Germany), an automated haematology analyser, is able to provide information regarding the above mentioned reticulocyte indices and their respective variabilities by two mechanisms. A nucleic acid-binding dye (oxazine 750) identifies reticulocytes while light scatter is used to determine reticulocyte cell size and haemoglobin concentration (Fry and Kirk, 2006; Schaefer and Stokol, 2015). Reticulocytes contain more residual RNA and have a greater cell volume, which allows for a greater scatter of light compared to mature erythrocytes (Cowgill et al., 2003).

Studies have provided evidence that suggest that several reticulocyte indices are better indicators of iron deficiency anaemia in dogs than conventional haematological or biochemical indices, because they demonstrate real-time function of the bone marrow (Foy et al., 2015; Fry and Kirk, 2006; Fuchs et al., 2017; Schaefer and Stokol, 2015). Furthermore, it has been described that a decrease in CHr is a more sensitive indicator of iron deficiency than other reticulocyte or erythrocyte markers (Fry and Kirk, 2006; Steinberg and Olver, 2005). One particular study describes the association of iron deficiency to other conditions such as anaemia of chronic disease, porto-systemic shunts and breed associated microcytosis, suggesting that reticulocyte indices can be of value in the further workup of other causes of anemia and microcytosis in dogs

(Schaefer and Stokol, 2015). The above-mentioned literature describes reticulocyte responses to microcytic, hypochromic, and non-regenerative anaemias. To our knowledge, there is only one study that describes the reticulocyte response of a haemolytic anaemia. By evaluating the CHr in dogs with IMHA, the authors found that affected dogs had iron-restricted erythropoiesis as a result of the concurrent inflammatory response of the condition, which could have resulted in iron sequestration or a functional iron deficiency (Schaefer and Stokol, 2016).

Chapter 3: Materials and methods

3.1 Objectives

- To describe the admission ARC and reticulocyte indices in dogs naturally infected with *B. rossi* and compare the results to healthy control dogs, as well as dogs with acute erythrocyte destruction (unrelated to babesiosis).
- To describe the ARC and reticulocyte indices in dogs experimentally infected with *B. rossi* throughout the disease course.

3.2 Hypotheses

- H_0: The admission ARC and reticulocyte indices in dogs naturally infected with *B. rossi* will be similar to those seen in healthy control dogs and dogs with acute erythrocyte destruction (unrelated to babesiosis).
  H_a: The admission ARC and reticulocyte indices in dogs naturally infected with *B. rossi* will be significantly different from those seen in healthy control dogs and dogs with acute erythrocyte destruction (unrelated to babesiosis).
- H_0: There will be no changes in the ARC and reticulocyte indices in dogs experimentally infected with *B. rossi,* over the course of the disease.
  H_a: The ARC and reticulocyte indices in dogs experimentally infected with *B. rossi* will change significantly over the course of the disease.

3.3 Benefits

- This study provides information regarding the pathophysiology of a common and highly virulent canine disease in South Africa.
- Further understanding of the disease process of babesiosis, as well as the host response, will assist in better clinical management of the condition.
- The findings may serve as a model for human falciparum malaria, which has a similar disease process to canine babesiosis.

3.4 Study design

The records generated on the ADVIA 2120 (Siemens, Munich, Germany), an automated haematology analyser, at the OVAH, Clinical Pathology laboratory were retrospectively analysed, as part of this retrospective, observational study. The ARC and various erythrocyte and reticulocyte indices were evaluated in naturally and experimentally infected dogs with babesiosis:

- Naturally infected dogs: The first study cohort included 85 *Babesia*-infected dogs and 14 healthy control dogs, collected from October 2013 to July 2015, whereas the second study cohort included 18 *Babesia*-infected dogs collected from January to December 2014. Both studies were approved by the University of Pretoria's Animal Ethics committee (V055-11 and V091-13, respectively). In addition, these dogs were compared to 16 dogs with IMHA, unrelated to babesiosis that presented as clinical cases to the OVAH between 2014 and 2020.
- Experimentally infected dogs: Five purpose-bred sterilised male beagle dogs experimentally infected with *B. rossi*, collected from 28 February to 8 March 2019. This study was approved by the University of Pretoria's Animal Ethics committee (V003-18).

3.4.1 Study design for naturally infected dogs

For both study cohorts, *Babesia*-infected dogs were included if they were >12 weeks of age, weighed >3 kg and had demonstrable parasitaemia. Infection with *B.rossi* was confirmed by polymerase chain reaction (PCR) assay. There was no gender or breed predilection. Dogs were excluded if they were infected with *B. vogeli* or *Ehrlichia canis*, based on the results of PCR assay and reverse line blot (RLB). Dogs were also excluded if they had any comorbidities such as systemic inflammatory, cardiac, neoplastic, infectious or traumatic conditions or diseases. In addition, any treatment with anti-inflammatory drugs at presentation or within 4 weeks prior to presentation was reason for exclusion. The standard treatment for the dogs with babesiosis included diminazene aceturate, an antibabesial drug (Berenil RTU 0.07 g/mL, Intervet, Kempton Park, South Africa) at 3.5 mg/kg. Additional supportive treatments of packed red cell transfusions and intravenous fluids were administered as needed. Any

complications were treated accordingly at the discretion of the attending clinician. Fourteen healthy dogs formed part of the control group. They were client-owned and presented to the OVAH for routine procedures such as ovariohysterectomy, castration or blood donation. The control dogs were deemed healthy based on history, a full clinical examination, peripheral blood smear evaluation, complete blood count, full biochemistry profile, as well as PCR assay and RLB to rule out infection with *B. rossi, B. vogeli* and *E. canis*. Owner consent was obtained for enrolment of infected cases and healthy control dogs.

At presentation and before any treatments were administered, blood was collected in ethylenediaminetetraacetic acid (EDTA) and serum vacutainer tubes from *Babesia*-infected and control dogs. The EDTA blood was used to perform a complete blood count on the ADVIA 2120 within one hour, which included ARC and various reticulocyte indices.

The database for the Clinical Pathology laboratory was retrospectively searched for dogs with IMHA, unrelated to babesiosis. This was confirmed by evaluation of the blood smears for *Babesia* parasites during the complete blood count, absence of haematological changes typically seen in babesiosis, as well as the medical history. This cohort of cases was included to compare their reticulocyte response with that seen in canine babesiosis. The inclusion criteria for this cohort of cases were in line with those set out by the ACVIM consensus statement on the diagnosis of IMHA (Garden et al., 2019) which include:

- Anaemia (below the laboratory generated reference interval).
- Signs of immune mediated erythrocyte destruction: Spherocytosis (≥5 spherocytes per ×100 oil immersion field on blood smear evaluation), positive saline agglutination test and demonstration of anti-erythrocyte antibodies.
- Evidence of haemolysis: Spherocytosis, hyperbilirubinaemia (in the absence of functional hepatic disease, cholestasis or sepsis), haemoglobinaemia, haemoglobinuria or erythrocyte ghosts.

A diagnostic algorithm for IMHA in dogs is illustrated in Figure 2 below.

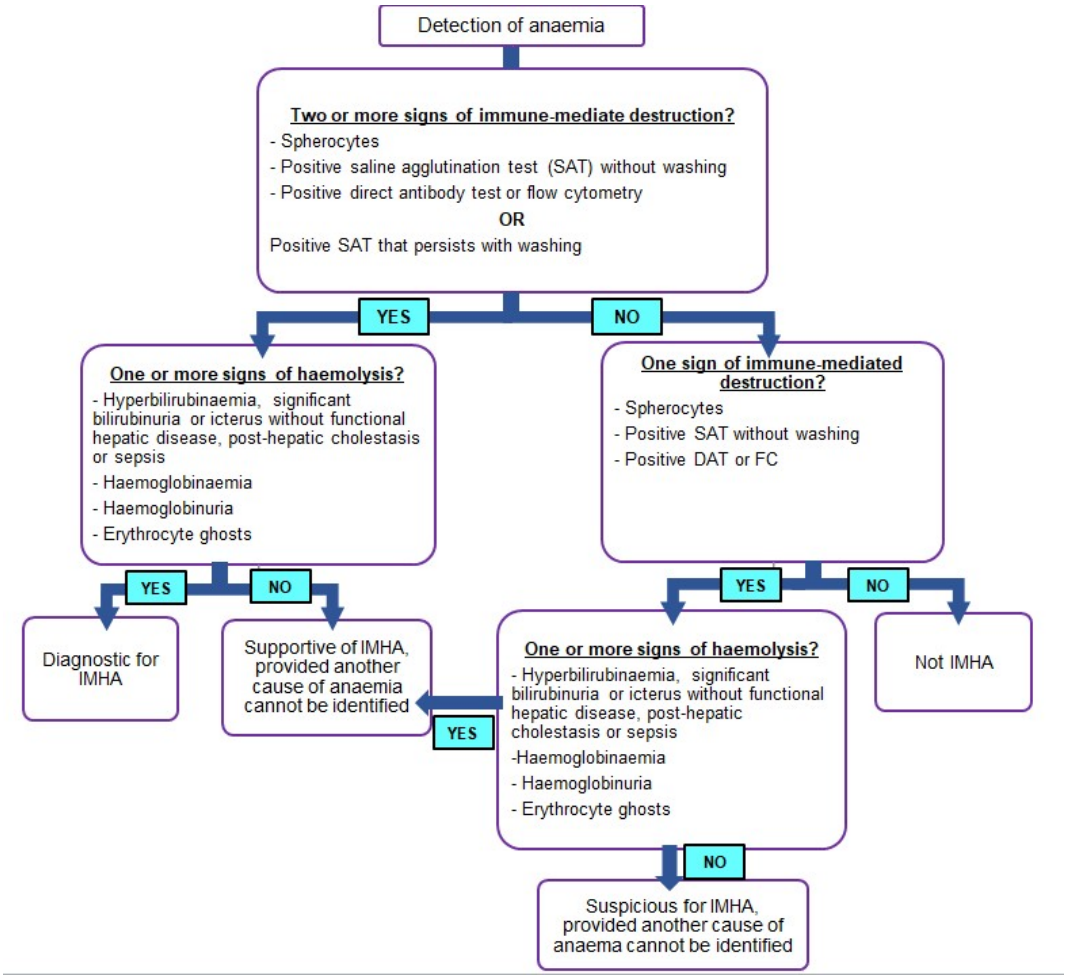

Figure 2: A diagnostic algorithm for IMHA in dogs (Garden et al., 2019)

Owner consent for use of the data forms part of the standard disclaimer form that owners have to sign when their pets are admitted to the OVAH.

Only the admission ARC, erythrocyte and reticulocyte indices were evaluated for all naturally infected babesiosis, control and IMHA dogs.

3.4.2 Study design for the experimentally infected dogs

Data collected during a prospective longitudinal observational study that included 6 purpose-bred sterilised male beagle dogs were retrospectively included in our study. The beagles were housed at the Onderstepoort Veterinary Academic Research Unit (OVARU) from eight weeks of age until the end of the experimental period, thereafter

the dogs were rehomed. One dog was randomly selected, splenectomised and used to raise a viable parasite inoculum from cryopreserved wild type *B. rossi*. The parasitaemia of the splenectomised dog was determined manually twice daily on central venous blood collected 12 hourly from one day post infection. The remaining five dogs were experimentally infected with intravenous injection of fresh whole blood, diluted to achieve the two parasite doses required. Two of the five dogs received a low dose *B. rossi* parasite inoculum (10^4 parasitised RBC/mL) and three dogs received a high dose (10^8 parasitised RBC/mL). The five dogs finally infected with the *B. rossi* inoculum acted as their own baseline controls. The splenectomised dog was drug cured with diminazine aceturate. Blood was collected on a daily basis in EDTA and serum vacutainer tubes from all 5 dogs for the duration of the experiment until the predetermined end points of the infection were reached. Once these end points were reached, the dogs were drug cured with diminazene aceturate. The EDTA blood was used to perform a complete blood count on the ADVIA 2120 within one hour of collection.

3.5 Methodologies

The ADVIA 2120 (Siemens, Munich, Germany) is a flow cytometer that evaluates haematological parameters individually. Erythrocytes are analysed by light scatter, which allows for the measurement of the volume and haemoglobin concentration (HGB) of individual erythrocytes, thus providing information on a cell-by-cell basis. The erythrocyte data is graphically represented in a cytogram (Harris et al., 2005).

Reticulocytes are selectively stained with oxazine 750, a nucleic acid-binding dye that allows immature erythrocytes to be distinguished from mature erythrocytes. As for the erythrocytes, measurement of the volume and haemoglobin content of reticulocytes is done by light scatter of individual cells. Analysing reticulocytes and mature erythrocytes by the same method at the same time provides information on the interrelationship of the two cell populations. The results of other reticulocyte indices are derived from the reticulocyte volume and CHr.

Specific variables produced by the ADVIA 2120, from both study data sets that were evaluated included:

1. Haematocrit (HCT)
2. Mean cell volume (MCV)
3. Haemoglobin concentration in whole blood (HGB)
4. Mean cell haemoglobin concentration (MCHC)
5. Cell haemoglobin concentration mean (CHCM)
6. Reticulocyte percentage (RET%)
7. Absolute reticulocyte count (ARC)
8. Reticulocyte mean cell volume (MCVr)
9. Reticulocyte cell haemoglobin concentration mean (CHCMr)
10. Reticulocyte haemoglobin content (CHr)
11. Distribution width (variability) of CHr (CHDWr)
12. Distribution width (variability) of reticulocyte cell size (RDWr)
13. Difference in the cellular haemoglobin (CH) between reticulocytes and mature erythrocytes (CH delta)

3.6 Statistical analysis

Statistical analysis was conducted using a commercial software package, (SPSS Statistics version 26 ®IBM, New York, USA). The normality assumption was evaluated using the Shapiro Wilk test and data distribution was determined to be non-parametric. The Kruskal-Wallis test was used to determine significance across groups for each variable. If significance was present, the Mann-Whitney *U* test was used as posthoc analysis. For determination of the change over time during the disease course in the experimentally infected dogs, Friedman's ANOVA was used. The Wilcoxon Signed Ranks test was then used to determine the differences within the experimentally infected groups. Data is presented as median and interquartile range (IQR). The significance level was set at 5%.

Chapter 4: Results

4.1 Study population characteristics

Data from 103 client-owned dogs naturally infected with *B. rossi*, 16 dogs with IMHA and 14 healthy control dogs were included. There were significant differences in age and weight between the groups. The age of the *Babesia* group (18 months; 9 – 36) was significantly lower than the IMHA group (72 months; 60 – 93; $P < 0.001$) and control group (56 months; 18 – 84; $P = 0.034$). There was no significant age difference between the IMHA and control groups. The weight of the *Babesia* group (18 kg; 9 – 26.8) was significantly lower than the control group (29 kg; 12 – 34.5; $P = 0.042$). There was no significant weight difference between the *Babesia* and IMHA (15 kg; 11.2 – 29.1) groups, as well as between the IMHA and control groups. The ratio of male: female for each group was as follows: control group (5:9), *Babesia* (69:34) and IMHA (7:9), with a significant difference found between groups ($P = 0.022$). The experimentally infected dogs were all from the same litter of beagle dogs, were all males, and there were no differences regarding age or weight.

4.2 Haematological variables, including ARC, for naturally infected babesiosis dogs, compared to dogs with IMHA and healthy control dogs

Table 1 contains a summary of all the variables for the various groups at presentation. For the *Babesia* and IMHA groups the HGB ($P < 0.001$ for both), HCT ($P < 0.001$ for both) and CHCM ($P < 0.001$ for both) were significantly lower than the control group. The MCHC was significantly lower in the *Babesia* group ($P < 0.001$), compared to the control group, but there was no difference for the IMHA group. For the *Babesia* group the HGB ($P = 0.018$) and CHCM ($P < 0.001$) was significantly higher compared to the IMHA group; however, the HCT and MCHC did not differ significantly between the two groups. The MCV was significantly higher in the IMHA group compared to the *Babesia* and control groups ($P < 0.001$ for both); however, there was no significant difference between the *Babesia* and control groups. Compared to the control group, the RET% and ARC were significantly higher in the *Babesia* ($P < 0.001$ and $P = 0.006$,

respectively) and IMHA ($P < 0.001$ and $P = 0.004$, respectively) groups (Fig. 3). The RET% and ARC was significantly lower in the *Babesia* group compared to the IMHA group ($P = 0.001$ and $P = 0.011$, respectively), despite no significant difference for the HCT between groups.

4.3 Reticulocyte indices for naturally infected babesiosis dogs, compared to dogs with IMHA and healthy control dogs

Table 1 contains a summary of all the variables for the various groups at presentation. The MCVr ($P = 0.005$; Fig. 4) and RDWr ($P = 0.003$) were significantly higher in the *Babesia* group compared to the control group, but there were no significant differences between the IMHA and control groups, as well as the *Babesia* and IMHA groups. Although there were no significant differences between groups for CHr, the CHCMr was significantly lower in the *Babesia* ($P = 0.007$) and IMHA ($P < 0.001$) groups compared to the control group, and significantly higher in the *Babesia* group compared to the IMHA group ($P = 0.003$). Compared to the control group, the CHDWr was significantly higher in the *Babesia* ($P = 0.002$) and IMHA ($P = 0.001$) groups, with no significant difference between the *Babesia* and IMHA groups. The CH delta was significantly higher in the *Babesia* group ($P = 0.038$) compared to the control group, with no significant differences between the other groups.

Table 1: Haematological and reticulocyte variables at presentation for naturally infected *B. rossi* dogs, dogs with IMHA and healthy control dogs

Variable	**Unit**	**Control group Median (IQR)**	***Babesia* group Median (IQR)**	**IMHA group Median (IQR)**
Haemoglobin concentration (HGB)	g/L	179.5 (154.0 – 190.8)	50.0 (39.0 – 86.0)	41.0 (29.8 – 48.8)
Haematocrit (HCT)	L/L	0.52 (0.45 – 0.57)	0.16 (0.12 – 0.27)	0.15 (0.12 – 0.17)
Mean cell volume (MCV)	fL	68.0 (66.6 – 69.5)	70.5 (66.7 – 76.3)	89.5 (78.9 – 102.8)
Mean cell haemoglobin concentration (MCHC)	g/L	340 (336 – 341)	322 (300 – 333)	310 (279 – 380)
Cell haemoglobin concentration mean (CHCM)	g/L	346 (339 – 353)	325 (299 – 340)	282 (266 – 293)
Reticulocyte percentage (RET%)	%	0.61 (0.44 – 0.75)	3.55 (1.46 – 7.64)	18.18 (4.41 – 33.46)
Absolute reticulocyte count (ARC)	x 10^9/L	42.1 (33.8 – 62.6)	82.1 (48.6 – 174.9)	256.7 (79.0 – 436.9)

Reticulocyte mean cell volume (MCVr)	fL	88.6 (86.3 – 89.3)	95.9 (88.0 – 103.5)	98.8 (83.5 – 119.3)
Reticulocyte cell haemoglobin concentration mean (CHCMr)	g/L	286 (278 – 303)	273 (262 – 289)	254 (234 – 266)
Reticulocyte haemoglobin content (CHr)	pg/cell	25.6 (23.6 – 26.8)	25.9 (24.5 – 27.4)	24.7 (22.6 – 28.8)
Distribution width (variability) of CHr (CHDWr)	pg	3.0 (2.9 – 3.2)	3.4 (3.1 – 3.8)	3.8 (3.3 – 4.0)
Distribution width (variability) of reticulocyte cell size (RDWr)	%	13.1 (12.1 – 14.3)	14.6 (13.4 – 15.8)	13.8 (12.4 – 20.9)
CH delta	pg	2.0 (1.0 – 2.9)	3.1 (1.3 – 4.7)	2.6 (0.5 – 4.5)

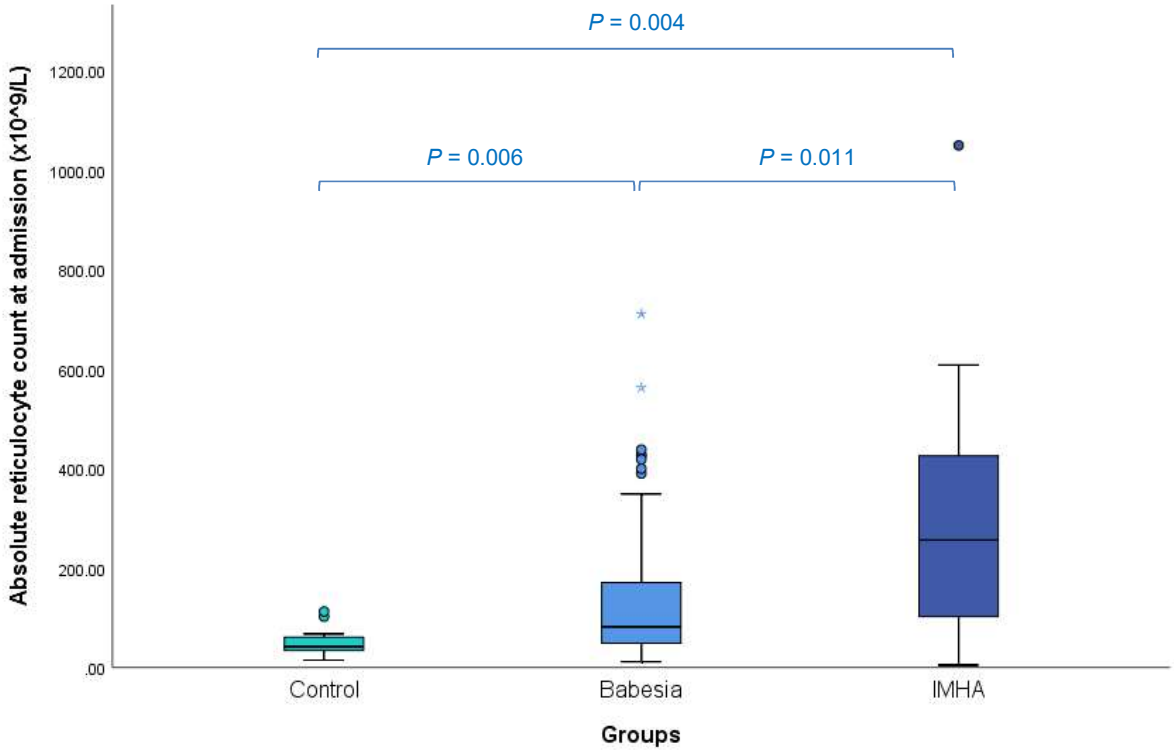

Figure 3: Boxplot demonstrating the admission ARC results for control, *Babesia* and IMHA groups. The median is represented by the horizontal line that runs through the box. The lower and upper edges of the box represent the first and third quartile, respectively. The lower and upper edges of the whiskers represent the minimum and maximum value, respectively. The outliers are indicated by circles and the extreme outliers by asterisks.

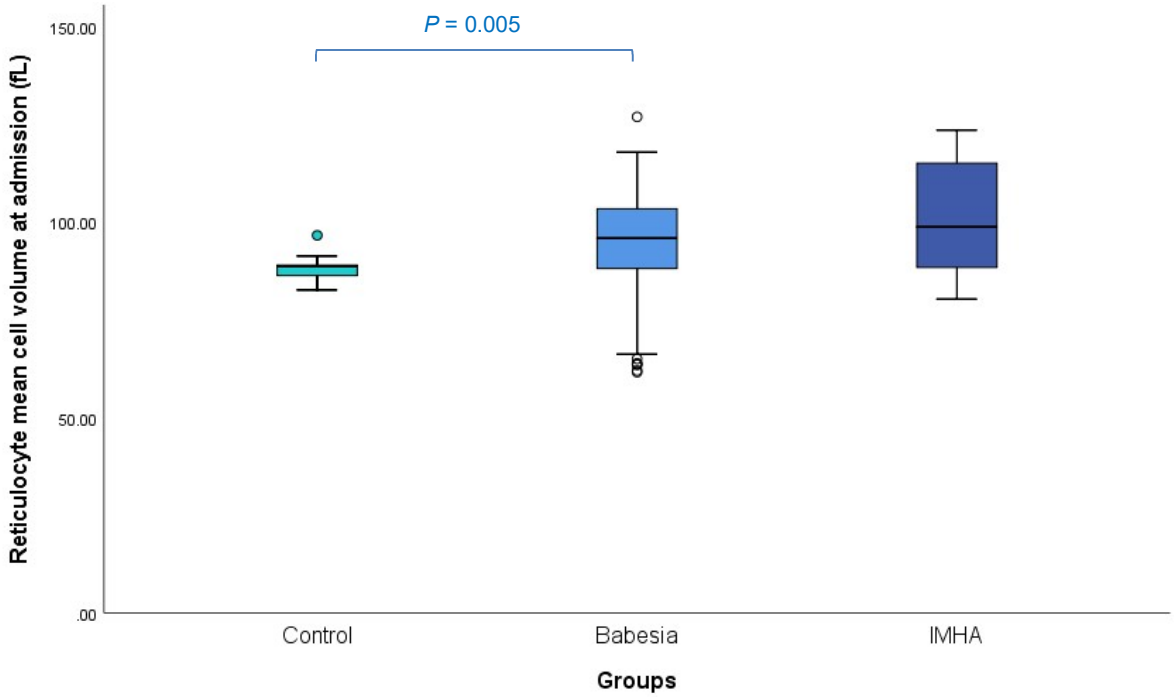

Figure 4 Boxplot demonstrating the admission MCVr results for control, *Babesia* and IMHA groups. The median is represented by the horizontal line that runs through the box. The lower and upper edges of the box represent the first and third quartile, respectively. The lower and upper edges of the whiskers represent the minimum and maximum value, respectively. The outliers are indicated by circles.

4.4 Haematological and reticulocyte variables in experimentally infected *B. rossi* dogs; and change over time

Of the five experimentally infected dogs, an unexpected mortality was recorded on day four of the experimental procedure for one of the dogs that received the high dose parasite inoculum. Experimental end points (i.e. typical clinical signs such as pyrexia, lethargy, anorexia, anaemia and haemoglobinuria) for the remaining four dogs were reached on the fourth day after infection. Parasitaemia was seen on peripheral blood smears on days two (high dose) and three (low dose); and treatment ensued on day four of infection. Blood was collected for an additional two days after treatment. Results for each variable on each day are displayed in Table 2. There were no significant differences between the high dose and low dose dogs regarding the

haematological and reticulocyte variables. Therefore, these variables were evaluated for the group as a whole.

The HGB concentration was significantly lower on day three compared to day one of infection ($P = 0.042$). No significant differences were seen for HCT, MCV, MCHC and CHCM between day one of infection and any of the other five days of the study. However, the HCT continued to decrease from day two to five of infection (Fig. 5). The RET% and ARC (Fig. 6) were significantly increased from day one to day two of infection ($P = 0.043$ and $P = 0.043$, respectively), as well as from day one to day three of infection ($P = 0.043$ and $P = 0.043$, respectively). However, no significant differences were seen between day one and days four, five and six (Fig. 6) The MCVr ($P = 0.043$), CHCMr ($P = 0.042$), CHr ($P = 0.043$) and CH delta ($P = 0.043$) were significantly decreased on day four compared to day one, whereas CHDWr ($P = 0.042$) and RDWr ($P = 0.043$) were significantly increased.

Table 2: Daily changes for haematological and reticulocyte variables in dogs experimentally infected with *B. rossi*.

Variable	**Unit**	**Experimental group median values**					
		Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
Haemoglobin concentration (HGB)	g/L	134.0 (133.0 – 139.0)	135.0 (131.0 – 138.0)	121.0* (116.0 – 129.5)	95.0 (79.5 – 135.5)	78.0 (53.5 – 95.0)	76.5 (59.5 – 80.75)
Haematocrit (HCT)	L/L	0.39 (0.35 – 0.41)	0.40 (0.39 – 0.42)	0.36 (0.33 – 0.38)	0.28 (0.20 – 0.39)	0.23 (0.15 – 0.28)	0.24 (0.19 – 0.25)
Mean cell volume (MCV)	fL	67.8 (66.5 – 67.8)	67.3 (66.7 – 67.9)	68.1 (67.2 – 68.7)	70.8 (68.3 – 75.1)	68.6 (68.0 – 70.7)	67.7 (67.5 – 69.1)
Mean cell haemoglobin concentration (MCHC)	g/L	341 (334 – 388)	337 (335 – 340)	333 (331 – 372)	337 (330 – 483)	343 (335 – 353)	325 (321 – 328)
Cell haemoglobin concentration mean (CHCM)	g/L	337 (335 – 340)	338 (333 – 339)	330 (326 – 339)	299 (288 – 335)	322 (315 – 328)	326 (324 – 334)
Reticulocyte percentage (RET%)	%	1.22 (0.94 – 1.26)	1.89* (1.01 – 2.55)	2.87* (1.25 – 3.66)	1.89 (1.10 – 3.81)	1.94 (1.15 – 2.36)	1.16 (1.03 – 1.29)

Absolute reticulocyte count (ARC)	x 10^9/L	60.1 (52.1 – 77.5)	107.6* (59.6 – 155.0)	134.1* (69.8 – 185.8)	57.1 (28.7 – 215.4)	62.4 (29.4 – 92.7)	36.5 (32.3 – 43.6)
Reticulocyte mean cell volume (MCVr)	fL	88.3 (87.4 – 89.5)	87.9 (85.5 – 90.5)	83.7 (77.7 – 91.4)	83.7* (80.6 – 86.6)	80.65 (79.2 – 81.8)	75.8 (70.7 – 84.9)
Reticulocyte cell haemoglobin concentration mean (CHCMr)	g/L	286 (285 – 288)	283 (282 – 286)	285 (284 – 290)	270* (267 – 282)	276 (266 – 281)	271 (268 – 286)
Reticulocyte haemoglobin content (CHr)	pg/cell	25.1 (24.9 – 25.5)	24.7 (24.1 – 25.6)	23.8 (22.8 – 26.1)	22.6* (21.5 – 24.2)	21.9 (21.5 – 22.7)	21.1 (19.2 – 23.0)
Distribution width (variability) of CHr (CHDWr)	pg	2.81 (2.62 – 2.91)	2.83 (2.69 – 2.93)	3.67 (2.69 – 4.09)	4.85* (3.18 – 5.50)	3.93 (3.86 – 4.36)	3.88 (3.71 – 4.31)
Distribution width (variability) of reticulocyte cell size (RDWr)	%	11.9 (11.8 – 12.5)	12.1 (11.9 – 12.5)	12.4 (10.9 – 13.8)	19.9* (12.4 – 20.6)	16.3 (13.8 – 20.0)	17.5 (15.6 – 22.8)
CH delta	pg	2.2 (2.0 – 2.7)	1.9 (1.6 – 2.5)	1.0 (0.3 – 2.9)	-0.2* (-0.6 – 1.2)	-6.5 (-1.1 – -0.2)	-1.6 (-3.8 – 0.2)

The * indicates a significant difference from the value on day 1.

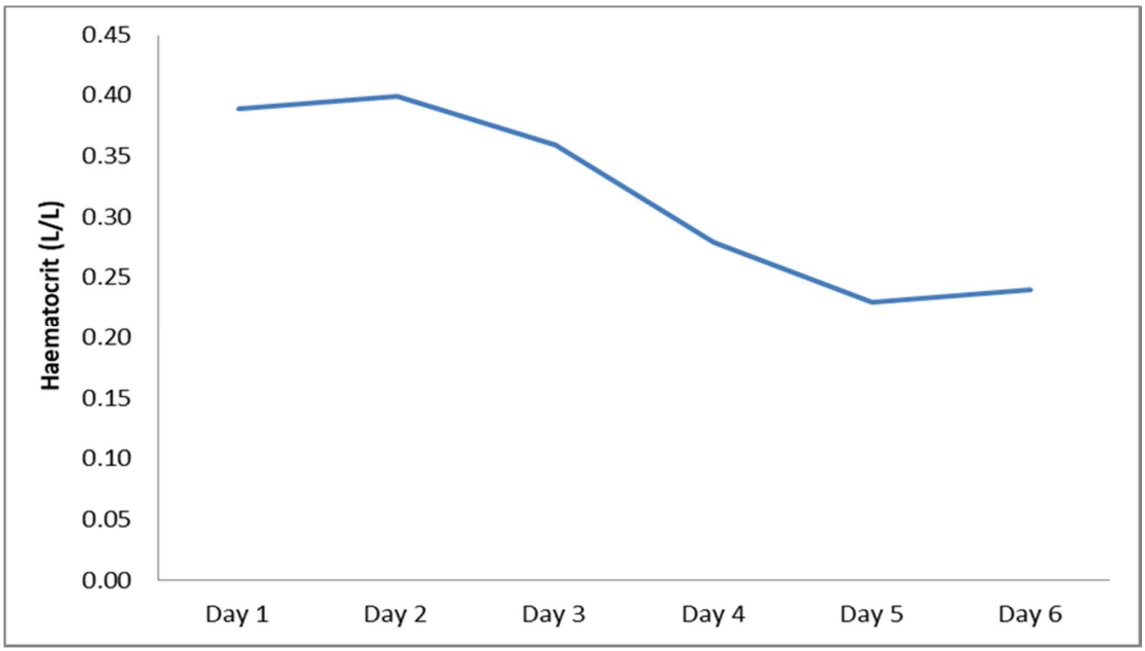

Figure 5: Line graph displaying the trend of the HCT over six days of experimentally *Babesia*-infected dogs

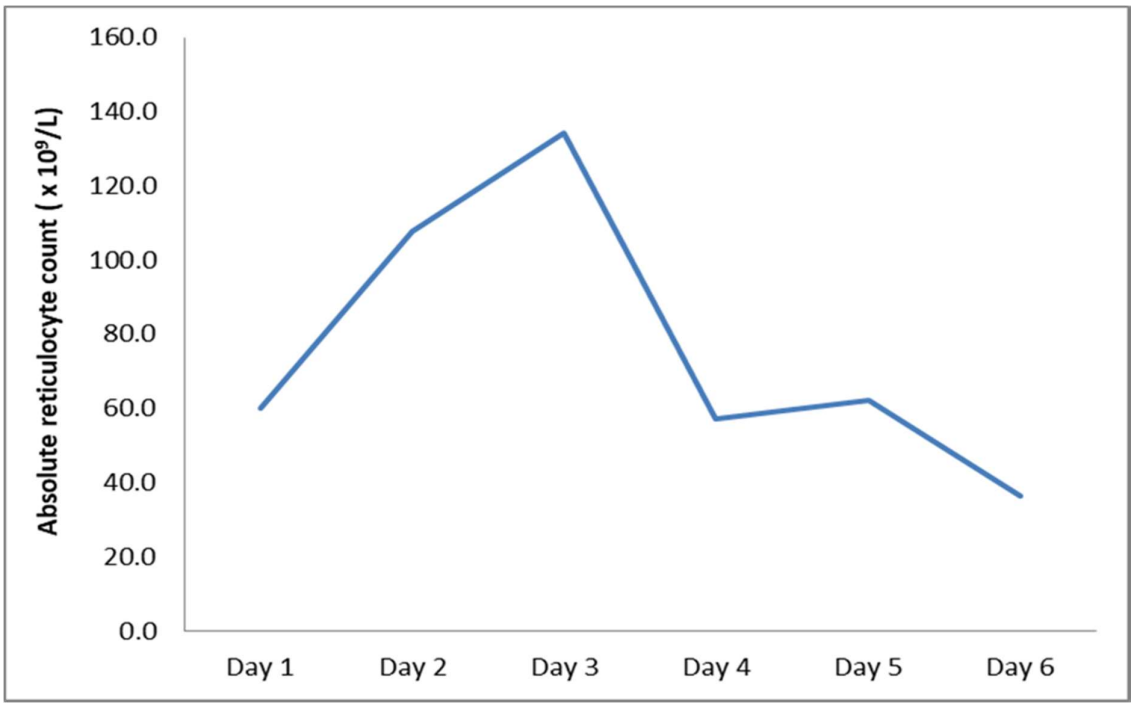

Figure 6: Line graph displaying the trend of the ARC over six days of experimentally *Babesia*-infected dogs

Chapter 5: Discussion

This study reported on the regenerative response and reticulocyte indices of dogs naturally and experimentally infected with *B. rossi*. The study showed that the regenerative response in dogs naturally and experimentally infected with *B. rossi* is inappropriate, despite the observed severity of the anaemia, compared to dogs with IMHA.

The ARC, as measured by the ADVIA 2120, is a good indicator of regeneration as it takes into account the degree of anaemia and is calculated by multiplying the RET% by the patient's total erythrocyte count (Cowgill et al., 2003; Latimer and Duncan, 2011). It stands to reason that a lower HCT should have a higher ARC because it is expected for severe anaemia to evoke a greater stimulus for increased erythrocyte production (Harvey, 2012). In our study, the HCT for the naturally infected *Babesia* and IMHA groups were significantly lower than the control group, whereas the ARC was significantly higher in the *Babesia* and IMHA groups compared to the control group. This finding was expected as a normal response to anaemia secondary to extramedullary causes; as in dogs with an acute moderate to marked haemolytic or haemorrhagic anaemia and functional bone marrow, a strong regenerative response with aggregate reticulocytes is expected (Cowgill et al., 2003). However, the admission ARC was significantly lower in the *Babesia* group compared to the IMHA group, despite no significant difference for the HCT between the two groups. The anaemia caused by *B. rossi* is due to both intra- and extravascular haemolysis (Schoeman, 2009) and, similar to dogs with IMHA, an appropriately marked regenerative response is expected. This inadequate regenerative response was previously noted in a study that investigated the progression of haematological changes in 32 transfused and 54 non-transfused dogs naturally infected with *B. rossi* (Scheepers et al., 2011). Dogs in the study showed a mild to moderate regenerative response throughout the study period of one week, despite having a severe anaemia and regardless of blood transfusion status. Additionally, a retrospective study on admission blood samples of dogs with babesiosis reported an absence of regeneration for 212 moderately anaemic

dogs (HCT 0.15 – 0.29 L/L) and only slight regeneration for 330 severely anaemic dogs (HCT < 0.15 L/L) (Reyers et al., 1998).

An interesting phenomenon was observed in the experimentally infected dogs. The ARC gradually increased from day one reaching its highest on day three post infection. A clinically significant anaemia was observed on day four; however, no anaemia was noted on days one and two. It usually takes three to four days after an anaemic insult for a substantial reticulocyte response to occur (Ettinger et al., 2017; Harvey, 2012; Latimer and Duncan, 2011). In some instances, high erythropoietin concentrations secondary to acute, severe anaemia may result in the early release of immature reticulocytes from the bone marrow into the blood stream. These larger basophilic macroreticulocytes, known as shift reticulocytes, then mature in circulation (Harvey, 2012; Latimer and Duncan, 2011). This may be responsible for the ARC changes noted on day two after infection and would indicate enhanced bone marrow erythropoiesis, thus pointing towards a functional bone marrow. The MCVr was also at its highest on days one and two compared to the succeeding days of experimental infection which indicates a greater average volume of the reticulocyte population and supports the early release of shift reticulocytes into circulation. However, this release of shift reticulocytes usually only occurs with a severe degree of anaemia (Harvey, 2012) and a severe anaemia was not present on any of the six days of experimental infection. This suggests the involvement of other factors, either in the production of erythropoietin, such as inflammatory cytokines or inflammatory proteins or factors that influence the release of reticulocytes. Previous studies report that AID causes impaired erythropoietin secretion and/or poor response of erythroid progenitor cells to the hormone (Chikazawa and Dunning, 2016), however, one particular study found that the level of erythropoietin in cats with experimentally induced anaemia of inflammation was normal or slightly increased (Weiss et al., 1983). To our knowledge, there are no previous reports on inflammation and its effect on erythropoietin levels in canines. In humans, intense exercise may result in the release of haematopoietic progenitors and immature reticulocytes, as noted in a study on 20 athletes that performed at supramaximal levels of exercise (Morici et al., 2005). Similar findings were noted in Greyhound dogs immediately after performing in a race (Horvath et al., 2014). However, splenic contraction was highly unlikely in the experimentally infected

dogs since they were well adapted to their environment and never underwent any physical exertion.

On day four, there was a sudden decrease in the ARC to less than its value on day one (no regenerative response). This lack of regeneration was inappropriate for the degree of anaemia observed. In general, the lack of a reticulocyte response in dogs three to four days after an anaemic insult indicates that the anaemia may be exacerbated by insufficient erythrocyte production in the bone marrow (Harvey, 2012). Furthermore, the sudden decrease in the ARC occurred 24 – 48 hours after a parasitaemia was visible on a peripheral blood smear. This finding may indicate a possible suppressive action of the *Babesia* parasite on the bone marrow during the time of parasitaemia, resulting in insufficient erythropoiesis.

Based on the findings of our study, the anaemia caused by *B. rossi* may be attributed to two mechanisms. The first is largely due to processes that result in the destruction of parasitised and non-parasitised erythrocytes (Solano-Gallego and Baneth, 2011). The second mechanism, based on the inadequate production of reticulocytes, could indicate a disturbance along any of the erythrocyte production lines prior to reticulocyte release which include erythropoietin production, growth factor and cytokine function (Interleukin (IL)-3, IL-9, IL-11), colony stimulating factors (GM-CSF or G-CSF), progenitor cell division and maturation (BFU-E and CFU-E) or erythroid precursor development (rubriblasts, prorubricytes, rubricytes, metarubricytes) and their maturation into reticulocytes. To our knowledge, there is no previous literature that evaluates the above possible causes of the inadequate regenerative response.

Multiple similarities exist between the disease process of canine babesiosis and human malaria (Clark and Jacobson, 1998; Lau, 2009). Correspondingly, previous studies on *P. falciparum* have noted that untreated cases of malaria show suboptimal reticulocyte counts for the degree of anaemia (Abdalla et al., 1980; Casals-Pascual and Roberts, 2006; Wickramasinghe and Abdalla, 2000). The anaemia caused by malaria is multifactorial and not only attributed to haemolysis of infected and uninfected red blood cells, but also to an inability to replenish the lost erythrocytes due to an inadequate erythroid response (Casals-Pascual and Roberts, 2006; Ghosh

Kanjaksha and Ghosh; Hassan et al., 1997; Pathak and Ghosh, 2016; Wickramasinghe and Abdalla, 2000).

The causes of impaired erythropoiesis in *P. falciparum* malaria may be categorised into direct and indirect effects. Direct effects suggest that the parasite produces specific factors that have a direct toxic effect on erythroid progenitor cells, erythroid precursor cells, or both. A more substantiated explanation may be the indirect effects which postulates that erythropoiesis is indirectly disturbed by parasite products, which may be directed at T-lymphocytes or haemopoietic microenvironmental cells (Wickramasinghe and Abdalla, 2000). The parasite product mentioned is haemozoin, which is a malarial pigment that remains in macrophages for a considerable time, that in turn produce potentially inhibitory cytokines (Giribaldi et al., 2004). Malaria is also associated with the release of lymphokines such as IFN-ɣ, TNF-α, IL-2, IL-12 and IL-10 which impair or suppress erythropoiesis by inhibiting erythroid progenitor cells or blunting their response to erythropoietin (Wickramasinghe and Abdalla, 2000). Due to the multiple similarities in pathophysiological and clinical course of the two diseases, it is suggestive that the factors that suppress regeneration in falciparum malaria may also inhibit regeneration in canine babesiosis infections. To date it has not been established whether the *Babesia* parasite produces a similar product such as the malarial pigment, haemozoin, and therefore further research is required to determine whether *Babesia* organisms have any specific direct or indirect toxic effect.

Reticulocyte indices may be better than erythrocyte indices at providing information regarding current or real-time bone marrow function because reticulocytes are recently produced and released into blood (Brugnara, 1998). Admission MCVr and RDWr were significantly increased in the *Babesia* group compared to the control group. This indicates a higher average volume of reticulocytes and greater variation in cellular volume within the *Babesia* reticulocyte population, respectively. These results indicate that there is likely the presence of larger reticulocytes within the blood stream that may have been released earlier than normal, such as shift reticulocytes that are larger in size as discussed before.

The CHr and CHCMr are indicators of mean reticulocyte cellular haemoglobin content and mean cellular hemoglobin concentration, respectively, whilst CHDWr describes the variation of the cellular haemoglobin content within the reticulocyte population. The CHDWr results indicate that there is more variation of CHr in *Babesia* and IMHA cases compared to normal healthy dogs. The CHCMr was significantly lower in the *Babesia* and IMHA groups compared to the normal control group, with the reticulocytes of the IMHA group being significantly more hypochromatic than the *Babesia* group. The significantly higher CH delta in the *Babesia* group versus the control group further indicates that the reticulocytes of the infected dogs had lower haemoglobin concentrations than mature erythrocytes. This finding was compounded by the fact that the erythrocyte MCHC and CHCM were significantly lower in the *Babesia* and IMHA groups, compared to the healthy control dogs, indicating a hypochromic anaemia. A common cause of this finding is iron-limited anaemia, which in this case, may be attributed to AID. In addition to hypochromasia, AID is usually associated with microcytic erythrocytes represented by a decreased MCV (Brugnara, 2003). Although the erythrocyte MCV was not significantly different between the *Babesia* and control groups, the median values were slightly higher in the *Babesia* group compared to the controls. This finding is more supportive of the presence of reticulocytes in circulation, and therefore a regenerative anaemia, although inappropriate. The significantly increased MCV observed in the IMHA group was most likely as a result of erythrocyte agglutination. Furthermore, other underlying comorbidities or nutritional deficiencies, which were not picked up during the initial screening process, may have also contributed.

All reticulocyte indices demonstrated significant differences on day four compared to day one of the experimental study. The MCVr on day four was significantly lower than day one of the experimentally infected group along with a significantly higher RDWr. The microcytic reticulocytes were still present two days after treatment and resulted in noticeable variation in cellular volume. This finding was contrary to what was seen in the naturally infected babesiosis dogs. The macroreticulocytosis, together with reticulocyte hypochromasia, seen in the naturally infected group, has been reported in previous studies (Fry and Kirk, 2006; Kotisaari et al., 2002). Reticulocyte size has been evaluated in three anaemia-induced states in humans (Clarkson and Moore, 1976).

This was done by estimating the mean reticulocyte area of subjects, by calculating the ratio of the reticulocyte area and non-reticulocyte area. The first subject group received repeated phlebotomies to produce an iron deficiency anaemia while the second group of patients had a folate or vitamin B12 deficiency. The third state included a human patient and rats receiving methotrexate, an immunosuppressive drug known to produce megaloblastic and aplastic anaemias. In all subjects, the reticulocytes were initially macrocytic and significantly larger than the MCVr of normal subjects, before returning to their normal size or becoming microcytic. The macroreticulocytes were ultimately immature shift reticulocytes that were released in response to acute, severe anaemia (Clarkson and Moore, 1976). The macroreticulocytosis in the naturally infected babesiosis dogs of this study were accompanied by a severe anaemia and significantly lower CHCMr, which therefore supports the above explanation.

In the experimentally infected group, there was a significantly lower CHCMr and CHr with a concurrent increase of the CHDWr on day four of infection. This hypochromasia of the reticulocytes was consistent with the results of the naturally infected group. Based on previous studies, decreased MCVr, CHCMr and CHr have been reported as more sensitive indicators of decreased haemoglobin concentration as seen with iron deficient anaemia (Brugnara, 2000, 2003; Schaefer and Stokol, 2015; Steinberg and Olver, 2005). Furthermore, changes in reticulocyte indices as a result of iron-limited erythropoiesis may be associated with AID, based on previous studies describing the relationship between inflammation and reticulocyte indices (Fuchs et al., 2017; Meléndez-Lazo et al., 2015; Radakovich et al., 2015). A severe inflammatory host response has been associated with dogs with *B. rossi* infection, with both IL-6 and IL-10 significantly raised in the serum (Goddard et al., 2016). Increased serum IL-6 and IL-10, along with other inflammatory cytokines, stimulates hepcidin production, which in turn stimulates the mechanisms resulting in functional and transport pool-related iron-limited anaemia (Meléndez-Lazo et al., 2015). These mechanisms include iron sequestration, decreased production of erythrocyte progenitor cells and a shortened life span of erythrocytes (Radakovich et al., 2015).

Our study had some limitations. Naturally infected babesiosis dogs presented at different stages during the disease process which likely resulted in variation of results

based on the duration of illness prior to presentation. It is likely that many naturally infected dogs had been infected for longer compared to the experimentally infected dogs. This may have resulted in discrepancies of the influences of reticulocyte production, such as differences between the balance of inhibitory and stimulatory cytokines. The severity of infection may also have influenced the intensity of the reticulocyte response in both groups. Despite this, the study concludes an inadequate regenerative response for both groups. Furthermore, any subclinical comorbidities or nutritional deficiencies that were not excluded during the initial screening process may have influenced the results. In the experimental study, the results may have been influenced by the low number of cases. Similarly, the IMHA group had a relatively small sample size. As the IMHA group consisted of clinical cases that previously presented to the OVAH, the presence of other comorbidities was not excluded and could have potentially influenced the results.

Chapter 6: Conclusion

This study aimed to describe the admission ARC and reticulocyte indices in dogs naturally infected with *B. rossi* and compare the results to healthy control dogs, as well as dogs with IMHA. Furthermore, the ARC and reticulocyte indices were evaluated in dogs experimentally infected with *B. rossi* throughout the disease course.

The ARC of the naturally infected group was significantly lower than the IMHA group, despite no significant difference in the HCT between the groups. These findings suggest that the regenerative response in dogs naturally infected with *B. rossi* is inappropriate, despite the severity of anaemia observed, compared to dogs with IMHA.

The ARC on day four of the experimentally infected group was significantly lower compared to day one of infection. These changes occurred around the same time as a peripheral parasitaemia was noted, thus suggesting a direct or indirect effect by the organism on the bone marrow, as reported in falciparum malaria. Other factors would warrant further studies, such as evaluating the bone marrow or erythropoietin concentration in *B. rossi* infected dogs.

Significant changes were noted in all reticulocyte indices of the naturally and experimentally infected groups, suggesting that they may be better indicators of early bone marrow activity and should be evaluated in cases of acute anaemia. Furthermore, the changes observed for the reticulocyte indices in the experimentally infected dogs were consistent with iron-limited anaemia, possibly relating to AID.

References

1. Abdalla S, Weatherall DJ, Wickramasinghe SN, Hughes M. The anaemia of *P. falciparum* malaria. *British journal of haematology* 1980: 46, 171-183.

2. Balch A, Mackin A. Canine immune-mediated hemolytic anemia: pathophysiology, clinical signs, and diagnosis. *The compendium on continuing education for the practicing Veterinarian* 2007: 29, 217-225.

3. Barger AM. The complete blood cell count: a powerful diagnostic tool. *The Veterinary clinics of North America. Small animal practice* 2003: 33, 1207-1222.

4. Böhm M, Leisewitz AL, Thompson PN, Schoeman JP. Capillary and venous *Babesia canis rossi* parasitaemias and their association with outcome of infection and circulatory compromise. *Veterinary parasitology* 2006: 141, 18-29.

5. Brugnara C. Use of reticulocyte cellular indices in the diagnosis and treatment of hematological disorders. *International journal of clinical and laboratory research* 1998: 28, 1-11.

6. Brugnara C. Reticulocyte cellular indices: a new approach in the diagnosis of anemias and monitoring of erythropoietic function. *Critical reviews in clinical laboratory sciences* 2000: 37, 93-130.

7. Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches. *Clinical chemistry* 2003: 49, 1573-1578.

8. Burchard GD, Radloff P, Philipps J, Nkeyi M, Knobloch J, Kremsner PG. Increased erythropoietin production in children with severe malarial anemia. *The American journal of tropical medicine and hygiene* 1995: 53, 547-551.

9. Burgmann H, Looareesuwan S, Kapiotis S, Viravan C, Vanijanonta S, Hollenstein U, Wiesinger E, Prester E, Winkler S, Graninger S. Serum levels of erythropoietin in acute *Plasmodium falciparum* malaria. *The American journal of tropical medicine and hygiene* 1996: 54, 280-283.

10. Car BD, 2010. 'Chapter 5 – The hematopoietic system' in Wiley-Blackwell (ed) Schalm's veterinary hematology, 6th ed. Ames, Iowa.

11. Casals-Pascual C, Roberts DJ. Severe Malarial Anaemia. *Current molecular medicine* 2006: 6, 155-168.

12. Chikazawa S, Dunning MD. A review of anaemia of inflammatory disease in dogs and cats. *Journal of small animal practice* 2016: 57, 348-353.

13. Clark IA, Jacobson LS. Do babesiosis and malaria share a common disease process? *Annals of tropical medicine and parasitology* 1998: 92, 483-488.

14. Clarkson D, Moore E. Reticulocyte size in nutritional anemias. *Blood journal* 1976: 48, 669-677.

15. Cowgill ES, Neel JA, Grindem CB. Clinical application of reticulocyte counts in dogs and cats. *The Veterinary clinics of North America. Small animal practice* 2003: 33, 1223-1244.

16. Ettinger SJ, Feldman EC, Côté, E, 2017. 'Chapter 57 - Anaemia, Erythrocytosis' in Elsevier Saunders (ed), *Textbook of Veterinary internal medicine: diseases of the dog and the cat*, 8th ed. St. Louis, Missouri. pp 231-235

17. Foy DS, Friedrichs KR, Bach JF. Evaluation of iron deficiency using reticulocyte indices in dogs enrolled in a blood donor program. *Journal of Veterinary internal medicine* 2015: 29, 1376-1380.

18. Fry MM, Kirk CA. Reticulocyte indices in a canine model of nutritional iron deficiency. *Veterinary clinical pathology* 2006: 35, 172-181.

19. Fuchs J, Moritz A, Grussendorf E, Lechner J, Neuerer F, Nickel R, Rieker T, Schwedes C, DeNicola DB, Russell J, Bauer N. Canine reticulocyte hemoglobin content (RET-He) in different types of iron-deficient erythropoiesis. *Veterinary clinical pathology* 2017: 46, 422-429.

20. Garden OA, Kidd L, Mexas AM, Chang YM, Jeffery U, Blois SL, Fogle JE, MacNeill AL, Lubas G, Birkenheuer A, Buoncompagni S, Dandrieux JRS, Di Loria A, Fellman CL, Glanemann B, Goggs R, Granick JL, LeVine DN, Sharp CR, Smith-Carr S, Swann JW, Szladovits B. ACVIM consensus statement on the diagnosis of immune-mediated hemolytic anemia in dogs and cats. *Journal of Veterinary internal medicine* 2019: 33, 313-334.

21. Ghosh K, Pathogenesis of anemia in malaria: a concise review. *Parasitology research* 2007: 101, 1463-1469.

22. Giribaldi G, Ulliers D, Schwarzer E, Roberts I, Piacibello W, Arese P. Hemozoin- and 4-hydroxynonenal-mediated inhibition of erythropoiesis. Possible role in malarial dyserythropoiesis and anemia. *Haematologica* 2004: 89, 492-493.

23. Goddard A, Leisewitz AL, Kjelgaard-Hansen M, Kristensen AT, Schoeman JP. Excessive pro-inflammatory serum cytokine concentrations in virulent canine babesiosis. *Plos One* 2016: 0150113.

24. Goddard A, Wiinberg B, Schoeman JP, Kristensen AT, Kjelgaard-Hansen M. Mortality in virulent canine babesiosis is associated with a consumptive coagulopathy. *The Veterinary journal* 2013: 213-217.

25. Harpaz R, Edelman R, Wasserman S, Levine M, Davis J, Sztein M. Serum cytokine profiles in experimental human malaria. *The Journal of clinical investigation* 1992: 90, 515-523.

26. Harris N, Kunicka J, Kratz A. The ADVIA 2120 hematology system: Flow cytometry-based analysis of blood and body fluids in the routine hematology

laboratory. *Laboratory hematology: Official publication of the international society for laboratory hematology* 2005: 11, 47-61.

27. Harvey JW, 2012. 'Chapter 4 - Evaluation of Erythrocytes' in: W.B. Saunders (ed), *Veterinary hematology*, Saint Louis, pp. 49-121.

28. Hassan AMAE, Saeed AM, Fandrey J, Jelkmann W. Decreased erythropoietin response in *Plasmodium falciparum* malaria-associated anaemia. *European journal of haematology* 1997: 59, 299-304.

29. Horvath SJ, Couto CG, Yant K, Kontur K, Bohenko L, Iazbik MC, Marín LM, Hudson D, Chase J, Frye M, Denicola DB. Effects of racing on reticulocyte concentrations in Greyhounds. *Veterinary clinical patholology* 2014: 43, 15-23.

30. Irwin PJ. Canine babesiosis. *The Veterinary clinics of North America. Small animal practice* 2010: 40, 1141-1156.

31. Jacobson LS. The South African form of severe and complicated canine babesiosis: Clinical advances 1994–2004. *Veterinary parasitology* 2006: 138, 126-139.

32. Jacobson LS, Clark IA. The pathophysiology of canine babesiosis: new approaches to an old puzzle. *Journal of the South African Veterinary Association* 1994: 65, 134-145.

33. Jacobson LS, Clark IA. The pathophysiology of canine babesiosis: new approaches to an old puzzle. *Journal of the South African Veterinary Association* 1994: 65, 134-145.

34. Joice R, Nilsson SK, Montgomery J, Dankwa S, Egan E, Morahan B, Seydel KB, Bertuccini L, Alano P, Williamson KC, Duraisingh MT, Taylor TE, Milner DA, Marti M. *Plasmodium falciparum* transmission stages accumulate in the human bone marrow. *Science translational medicine* 2014: 6, 244

35. Jootar S, Chaisiripoomkere W, Pholvicha P, Leelasiri A, Prayoonwiwat W, Mongkonsvitragoon W, Srichaikul T. Suppression of erythroid progenitor cells during malarial infection in Thai adults caused by serum inhibitor. *Clinical & laboratory haematology* 2008: 15, 87-92.

36. Klag AR, Giger U, Shofer FS. Idiopathic immune-mediated hemolytic anemia in dogs: 42 cases (1986-1990). *Journal of the American Veterinary Medical Association* 1993: 202, 783-788.

37. Kotisaari S, Romppanen J, Penttilä I, Punnonen K. The ADVIA 120 red blood cell and reticulocyte indices are useful in diagnosis of iron-deficiency anemia. *European Journal of Haematology* 2002: 68, 150-156.

38. Kurtzhals JAL, Rodrigues O, Addae M, Commey JOO, Nkrumah FK, Hviid L. Reversible suppression of bone marrow response to erythropoietin in *Plasmodium falciparum* malaria. *British journal of haematology* 1997: 97, 169-174.

39. Brockus CW, 2011. 'Chapter 1 - Erythrocytes' in Wiley-Blackwell (ed), *Duncan & Prasse's veterinary laboratory medicine: Clinical pathology*, 5th ed. Chichester, West Sussex, UK.

40. Lau AO. An overview of the *Babesia*, *Plasmodium* and *Theileria* genomes: a comparative perspective. *Molecular and biochemical parasitology* 2009: 164, 1-8.

41. Maegraith B, Gilles HM, Devakul K. Pathological processes in *Babesia canis* infections. *Zeitschrift fur Tropenmedizin und Parasitologie* 1957: 8, 485-514.

42. Maggio-Price L, Brookoff D, Weiss L. Changes in hematopoietic stem cells in bone marrow of mice with *Plasmodium berghei* malaria. *Blood journal* 1985: 66, 1080-1085.

43. Malherbe, WD. A clinico-pathological study of *Babesia canis* infection in dogs 1968.

44. Manev I, Marincheva V. Canine immune-mediated hemolytic anemia-brief review 2018.

45. Matjila PT, Penzhorn BL, Bekker CP, Nijhof AM, Jongejan F. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. *Veterinary parasitology* 2004: 122 119-125.

46. Valli VEO, Kiupel M, Bienzle D, Wood RD, 2016. 'Chapter 2 – Hematopoietic System' in Elsevier (ed*), Jubb, Kennedy, and Palmer's pathology of domestic animals*: Volume 3, 6th ed. St. Louis, Missouri, pp. 102-268

47. McCullough S. Immune-mediated hemolytic anemia: understanding the nemesis. *The Veterinary clinics of North America. Small animal practice* 2003: 33, 1295-1315.

48. Meléndez-Lazo A, Tvarijonaviciute A, Cerón JJ, Planellas M, Pastor J. Evaluation of the relationship between selected reticulocyte parameters and inflammation determined by plasma C-reactive protein in dogs. *Journal of comparative pathology* 2015: 152, 304-312.

49. Morici G, Zangla D, Santoro A, Pelosi E, Petrucci E, Gioia M, Bonanno A, Profita M, Bellia V, Testa U, Bonsignore MR. Supramaximal exercise mobilizes hematopoietic progenitors and reticulocytes in athletes. *American journal of physiology-regulatory, integrative and comparative physiology* 2005: 289, R1496-R1503.

50. Nel M, Lobetti RG, Keller N, Thompson PN. Prognostic value of blood lactate, blood glucose, and hematocrit in canine babesiosis. *Journal of Veterinary internal medicine* 2004: 18 471-476.

51. Pathak VA, Ghosh K. Erythropoiesis in malaria infections and factors modifying the erythropoietic response. *Anemia* 2016, 1-8.

52. Phillips RE, Looareesuwan S, Warrell DA, Lee SH, Karbwang J, Warrell MJ, White NJ, Swasdichai C, Weatherall DJ. The importance of anaemia in cerebral and uncomplicated falciparum malaria: role of complications, dyserythropoiesis and iron sequestration. *The quarterly journal of medicine* 1986: 58, 305-323.

53. Radakovich LB, Santangelo KS, Olver CS. Reticulocyte hemoglobin content does not differentiate true from functional iron deficiency in dogs. *Veterinary clinical pathology* 2015: 44, 511-518.

54. Reyers F, Leisewitz AL, Lobetti RG, Milner RJ, Jacobson LS, van Zyl M. Canine babesiosis in South Africa: more than one disease. Does this serve as a model for falciparum malaria? *Annals of tropical medicine & parasitology* 1998: 92 503- 511.

55. Schaefer DM, Stokol T. Retrospective study of reticulocyte indices as indicators of iron-restricted erythropoiesis in dogs with immune-mediated hemolytic anemia. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 2016: 28, 304-308.

56. Schaefer DMW, Stokol T. The utility of reticulocyte indices in distinguishing iron deficiency anemia from anemia of inflammatory disease, portosystemic shunting, and breed-associated microcytosis in dogs. *Veterinary clinical pathology* 2015: 44, 109-119.

57. Scheepers E, Leisewitz AL, Thompson PN, Christopher MM. Serial haematology results in transfused and non-transfused dogs naturally infected with *Babesia rossi. Journal of the South African Veterinary Association* 2011: 82, 136-143.

58. Schoeman JP. Canine babesiosis. *The Onderstepoort journal of veterinary research* 2009: 76, 59-66.

59. Sherry BA, Alava G, Tracey KJ, Martiney J, Cerami A, Slater AF. Malaria-specific metabolite hemozoin mediates the release of several potent endogenous pyrogens (TNF, MIP-1 alpha, and MIP-1 beta) in vitro, and altered thermoregulation in vivo. *Journal of inflammation* 1995: 45, 85-96.

60. Silverman PH, Schooley JC, Mahlmann LJ. Murine malaria decreases hemopoietic stem cells. *Blood journal* 1987: 69, 408-413.

61. Solano-Gallego L, Baneth G. Babesiosis in dogs and cats: expanding parasitological and clinical spectra 2011: 48-60.

62. Steinberg JD, Olver CS. Hematologic and biochemical abnormalities indicating iron deficiency are associated with decreased reticulocyte hemoglobin content (CHr) and reticulocyte volume (rMCV) in dogs. *Veterinary clinical pathology* 2005: 34, 23-27.

63. Udroiu I. Storage of blood in the mammalian spleen: an evolutionary perspective. *Journal of mammalian evolution* 2017: 24, 243-260.

64. Weiss DJ, Krehbiel JD, Lund JE. Studies of the pathogenesis of anemia of inflammation: mechanism of impaired erythropoiesis. *American journal of Veterinary research* 1983: 44, 1832-1835.

65. Welzl C, Leisewitz AL, Jacobson LS, Vaughan-Scott T, Myburgh E. Systemic inflammatory response syndrome and multiple-organ damage/dysfunction in complicated canine babesiosis. *Journal of the South African Veterinary Association* 2001: 72 158-162.

66. White NJ, Ho M. The pathophysiology of malaria. *Advances in parasitology* 1992: 31 83-173.

67. Wickramasinghe SN, Abdalla SH. Blood and bone marrow changes in malaria. *Bailliere's best practice in clinical haematology* 2000: 13 277-299

Appendices

Appendix A: Data collected from the experimentally *Babesia*-infected cases

Case number	HGB Day 1	HGB Day 2	HGB Day 3	HGB Day 4	HGB Day 5	HGB Day 6
1	134	135	131	138	89	77
2	137	134	128	133	97	82
3	133	128	121	95	49	76
4	133	135	112	89	67	54
5	141	141	120	70		

Case number	HCT Day 1	HCT Day 2	HCT Day 3	HCT Day 4	HCT Day 5	HCT Day 6
1	0.39	0.41	0.4	0.37	0.26	0.24
2	0.36	0.4	0.36	0.4	0.29	0.25
3	0.4	0.38	0.31	0.28	0.14	0.23
4	0.33	0.4	0.34	0.27	0.19	0.17
5	0.42	0.42	0.36	0.12		

Case number	MCHC Day 1	MCHC Day 2	MCHC Day 3	MCHC Day 4	MCHC Day 5	MCHC Day 6
1	341	332	329	370	337	323
2	376	337	356	332	334	328
3	331	341	387	337	355	327
4	399	338	333	328	348	320
5	336	337	333	595		

Case number	CHCM Day 1	CHCM Day 2	CHCM Day 3	CHCM Day 4	CHCM Day 5	CHCM Day 6
1	334	335	338	334	327	326
2	341	338	339	336	328	337
3	337	331	326	299	316	326
4	336	340	330	277	315	323
5	338	338	326	298		

Case number	Ret% Day 1	Ret% Day 2	Ret% Day 3	Ret% Day 4	Ret% Day 5	Ret% Day 6
1	0.9	0.92	1.17	3.92	1.7	1.09
2	0.98	1.1	1.33	3.7	2.42	1.22
3	1.28	1.89	2.87	1.44	0.96	1.01
4	1.22	2.28	3.5	0.75	2.18	1.31
5	1.23	2.81	3.81	1.89		

Case number	ARC Day 1	ARC Day 2	ARC Day 3	ARC Day 4	ARC Day 5	ARC Day 6
1	51.8	55.1	69.3	216.1	65.8	38.2
2	52.3	64.1	70.3	214.6	101.7	45.4
3	78.1	107.6	134.1	57.1	19.6	34.7
4	60.1	135.5	172.2	25.8	58.9	31.5
5	76.8	174.5	199.3	31.6		

Case number	MCVr Day 1	MCVr Day 2	MCVr Day 3	MCVr Day 4	MCVr Day 5	MCVr Day 6
1	87.9	89.4	91.2	86.7	79.7	72.4
2	90	91.6	91.6	86.5	79	70.1
3	88.3	86	78.8	81.1	81.6	79.1
4	86.9	85	76.5	83.7	81.8	86.8
5	89	87.9	83.7	80		

Case number	CHCMr Day 1	CHCMr Day 2	CHCMr Day 3	CHCMr Day 4	CHCMr Day 5	CHCMr Day 6
1	286	283	285	280	275	268
2	289	284	287	283	277	272
3	286	281	283	264	263	290
4	287	287	293	270	282	269
5	284	283	285	270		

Case number	CHr Day 1	CHr Day 2	CHr Day 3	CHr Day 4	CHr Day 5	CHr Day 6
1	25	25.2	25.9	24.1	21.9	19.4
2	25.8	25.9	26.2	24.3	21.8	19.1
3	25.1	24	22.3	21.4	21.4	22.7
4	24.8	24.2	23.3	22.6	22.9	23
5	25.1	24.7	23.8	21.5		

Case number	CHDWr Day 1	CHDWr Day 2	CHDWr Day 3	CHDWr Day 4	CHDWr Day 5	CHDWr Day 6
1	2.81	2.83	2.7	3.3	3.84	3.67
2	2.87	2.75	2.68	3.06	3.95	3.82
3	2.95	2.99	4.06	5.29	4.49	3.94
4	2.65	2.86	4.11	5.71	3.91	4.43
5	2.58	2.62	3.67	4.85		

Case number	RDWr Day 1	RDWr Day 2	RDWr Day 3	RDWr Day 4	RDWr Day 5	RDWr Day 6
1	12	12.5	11	12.5	13.5	15.9
2	11.8	11.9	10.7	12.2	14.7	15.5
3	12.9	11.9	13.7	21.2	20.7	19
4	11.9	12.5	13.9	19.9	17.9	24.1
5	11.8	12.1	12.4	19.9		

Case number	CH Delta Day 1	CH Delta Day 2	CH Delta Day 3	CH Delta Day 4	CH Delta Day 5	CH Delta Day 6
1	2.2	2.4	2.9	1.2	-0.7	-3.2
2	2.5	2.6	2.8	1.1	-1.2	-4
3	2.8	1.9	0.1	-0.6	-0.6	0.3
4	1.8	1.3	0.4	-0.2	-0.1	0
5	2.2	1.8	1	-0.5		

Appendix B: Data collected from the naturally *Babesia*-infected cases of study cohort one

No	Study no	Age (months)	Weight (kg)	Gender	HGB (g/L)	HCT (L/L)	MCHC (g/L)	CHCM (g/L)	Ret% (%)	ARC (x10^9/L)	MCVr (fL)	CHCMr (g/L)	CHr (pg/cell)	CHDWr (pg/cell)	RDWr (%)	CHDelta (pg)
1	BC02	24	14.8	Female	41	0.13	326	323	2.88	54.7			28.6			
2	BC04	15	11	Male	153	0.43	355	359	0.36	22.9	87.3	302	25.7	3.99	22.1	0.6
3	BC06	36	22.8	Male	47	0.13	361	346	7.36	124.6	126.9	332	42.3	8.94	16.8	18
4	BC08	12		Male	110	0.34	324	337	2.87	135	94.6	294	27.5	2.8	13.7	2.8
5	BC09	7		Male	123	0.35	348	348	0.44	22.9	85.1	288	24.3	3.53	18.3	0
6	BC10	24		Female	108	0.31	349	342	1.07	49.9	93.2	284	26.0	3.02	16.7	2.8
7	BC11	4	8.4	Male	28	0.09	316	294	3.74	49.4	109.3	244	26.4	3.06	13.6	6.1
8	BC12	10	26	Female	38	0.13	299	316	4.35	78.6	98.6	261	25.5	3.05	13.9	2.7
9	BC14	6	6.8	Female	28	0.10	292	294	4.92	65.7	102.1	259	26.2	2.9	14.7	4.5
10	BC17	9	29.8	Male	67	0.20	343	343	2.03	61.2	87.2	275	23.8	3.29	15.4	1.2
11	BC18	12	35	Male	59	0.19	311	323	3.72	92.6	102.4	270	27.4	2.96	11.8	2.7
12	BC19	12	4.1	Male	74	0.22	332	332	2.11	75	95.7	272	25.8	2.92	13.6	4.6
13	BC21	6	10.4	Female	86	0.27	321	326	1.98	81.5	96.7	276	26.4	3.1	15.2	4.3
14	BC22	2		Female	49	0.15	325	332	4.76	110			21.5			
15	BC23	10	25	Male	43	0.14	299	312	13.42	267.3	99.9	258	25.6	3.48	13.9	3

16	BC24	32	10.8	Male	147	0.43	339	350	6.45	426.7	62	385	23.5	1	1.6	5.7
17	BC25	84	65	Male	48	0.16	302	312	11.27	226.2	108.9	262	28.3	3	12.4	3.9
18	BC26	3	10.4	Female	45	0.16	292	302	16.01	349.2	94.8	242	22.8	3.36	15.4	1.1
19	BC27	3	11.4	Male	32	0.12	264	284	20.88	307	110.3	237	26.0	3.33	14.1	3.3
20	BC28	7	14	Male	75	0.23	322	332	2.22	82.5	90.9	274	24.7	2.86	15.4	3.4
21	BC31	48	49.6	Male	124	0.35	356	371	0.36	19.2	70	303	21.1	3.38	14.8	-3.3
22	BC32	7	16.6	Male	47	0.15	320	337	6.15	136.7	104.8	265	27.5	2.81	11.8	5.1
23	BC33	48	20	Female	60	0.20	306	318	6.26	167	98	270	26.4	3.14	13.2	2.4
24	BC36	4	18.4	Male	28	0.08	327	296	2.07	25.7	98.9	254	24.8	2.29	14.3	3.9
25	BC37	32	33	Male	34	0.12	276	295	31.76	438.4	114.9	248	28.3	4.32	14.8	4.7
26	BC38	28	9	Male	64	0.19	334	347	1.96	55.9	96.3	266	25.3	3.4	16.1	1.5
27	BC39	35	13	Male	25	0.09	277	261	24.4	241.5	113.8	235	26.5	3.1	13.7	3.5
28	BC40	24	25.8	Male	50	0.16	325	333	7.12	160.3	97.8	274	26.7	3.17	12.8	3.4
29	BC41	96	37	Female	177	0.51	344	364	0.82	67	65	294	19.1	3.24	12.1	-4.2
30	BC42	23	8	Male	39	0.13	304	334	17.25	294.9	103	272	27.8	3.3	12.7	3.6
31	BC44	24	7.2	Female	86	0.26	325	347	1.26	562.9	109.9	259	28.1	3.47	15.4	3.5
32	BC45	144	18.2	Male	152	0.45	341	358	0.22	17.4	63.9	317	20.1	2.79	14.6	-0.7

33	BC46	8	22	Male	23	0.08	275	274	3.79	40.9	102.7	255	25.9	4.14	15.3	4.8
34	BC47	96	3	Female	95	0.28	338	360	1.02	42.9	86	300	25.5	3.33	15.7	1.3
35	BC48	120	54	Male	84	0.26	326	342	1.94	70.9	73.9	297	21.8	4.49	22.4	-3.3
36	BC49	9	28	Male	76	0.23	326	340	2.85	99.3	85.1	291	24.6	3.18	15.1	0.8
37	BC50	78	9.2	Male	175	0.50	348	368	0.56	42.5	61.6	313	19.3	4.71	21.4	-6
38	BC51	11	19.8	Male	100	0.30	337	356	1.45	63.9	86.9	303	26.1	3.12	14.1	1.4
39	BC52	20	15.6	Male	73	0.23	321	338	1.8	64	93.7	291	27.0	3.31	14.4	4.6
40	BC53	35	33.2	Male	21	0.07	304	299	10.63	91.6	110.9	269	29.5	4.2	15.2	7.4
41	BC54	21	4.4	Female	47	0.15	309	335	0.97	21.1	94.1	302	28.3	3.12	14.1	3.8
42	BC55	18	36	Female	100	0.30	330	332	1.69	73.9	92	287	26.2	3.46	14.6	2.3
43	BC56	54	14.2	Male	44	0.14	312	330	7.64	157.3	103.1	280	28.7	3.33	14	5.8
44	BC58	10		Male	64	0.20	319	325	3.03	80.1	93.5	294	27.3	4.32	16.6	3.7
45	BC59	6	3	Female	32	0.11	302	299	6.13	90.9	95.9	268	25.4	3.52	17.6	3
46	BC61		7	Female	52	0.16	322	339	4.28	101.6	102.4	282	28.6	2.77	12.1	4.8
47	BC62	4	13.8	Male	45	0.14	321	320	4.24	93.2	89.5	285	25.3	3.18	15.6	4.1
48	BC63	12	21.6	Male	27	0.08	332	294	5.13	63.1	98.6	267	26.0	3.29	15.9	6.1
49	BC64	4	8	Male	117	0.37	317	335	1.7	93.5	87.9	297	25.9	3.06	13.4	2.7

50	BC65	40	7.5	Male	166	0.51	328	348	0.42	31.7	63.5	295	18.9	5.41	21.2	-5.7
51	BC66	37	26.2	Male	32	0.10	327	291	5.3	82.1	101.9	267	27.0	3.93	15.4	7.8
52	BC67	5	17.8	Female	47	0.16	296	292	9.13	188.6	94.5	272	25.5	3.07	14.5	2
53	BC68	38	21.6	Female	109	0.33	328	344	1.71	82.8	88.4	288	25.1	3.75	18.5	0.8
54	BC69	56	28.2	Female	49	0.15	314	334	3.15	66.9	104.3	291	30.1	3.32	13.5	5.8
55	BC70	72	27	Female	41	0.14	299	294	12.56	240	103.4	260	26.7	3.85	15.9	5
56	BC71	132	5.8	Female	169	0.50	337	359	0.93	70.9	63.6	297	18.9	4.68	18.4	-5.9
57	BC72	3	4.6	Female	28	0.10	284	275	6.65	87.3	94.4	265	24.8	4.43	16	2.7
58	BC74	9	24.2	Female	39	0.13	290	297	24.41	400.1	106.2	272	28.7	3.2	12.8	4.7
59	BC76	9	5.2	Male	38	0.12	320	321	10.33	174.9	95.1	271	25.6	3.12	15.1	2.8
60	BC77	11	12	Female	67	0.19	342	337	3.55	99.7	100.4	287	28.6	3.11	12.4	4.4
61	BC78	4	12.6	Male	47	0.17	271	294	30.3	711.4	97.4	255	24.7	3.58	14.5	3.5
62	BC79	42	23.2	Male	61	0.19	328	332	4.22	103.1	98	299	29.1	6.12	19.8	5.4
63	BC80	96	40	Male	65	0.20	317	329	2.31	66.9	79.1	279	22.0	4.17	19.3	-2.1
64	BC81	36	20	Male	103	0.32	323	341	1.46	63.9	77	307	23.5	3.66	17.6	-2.3
65	BC82	18	50	Male	59	0.20	293	323	10.05	255	109.4	273	29.7	3.46	12	3.8
66	BC83	24	12	Male	71	0.26	270	294	4.25	138.8	97.3	273	26.4	3.18	13.9	1.4

67	BC86	7	31.6	Female	45	0.15	303	326	10.59	224	94.1	272	25.4	2.79	13.4	2.8
68	BC87	108	35.6	Male	158	0.44	356	362	0.71	48.6	82.5	296	24.2	2.74	14.1	1.5
69	BC88	8	28.2	Female	44	0.14	327	321	0.76	15.2	88.9	277	24.5	3.05	15.2	2.9
70	BC89	29	12.8	Female	33	0.11	300	320	14.78	211.1	101.2	262	26.4	3.29	11.7	3.1
71	BC90	24	38.0	Male	100	0.30	330	346	3.6	152.1	89	280	24.7	3.45	14.6	0.8
72	BC91	96	19.0	Male	32	0.12	277	287	27.71	418.7	104.4	230	23.8	3.45	15.1	3.9
73	BC93	9		Female	46	0.11	403	316	0.97	14.9	89.8	291	25.9	3.91	16.3	2.5
74	BC94	36	18.6	Male	85	0.24	349	348	0.56	18.8	90.7	278	25.2	5.01	17.6	1.1
75	BC95	96	38.0	Male	172	0.48	357	363	0.25	19.3	84	279	23.3	3.7	15.2	1.3
76	BC96	12	20.0	Male	34	0.11	316	312	6.54	90.5	109.7	254	27.7	3.56	13.1	4.6
77	BC97	36	9.0	Male	29	0.09	318	325	2.51	34.4	97.7	267	25.9	2.88	12.3	4.6
78	BC98	24	25.6	Female	146	0.41	353	357	0.19	11.8	66.2	274	18.4	5.04	18.9	-4.9
79	BC99	12	14.2	Male	88	0.28	314	314	0.4	18.3	75.2	263	19.8	3.5	16.3	0.7
80	BC100	36	6.6	Female	119	0.35	335	333	0.75	42.3	67.9	256	17.4	2.98	14.4	-3.4
81	BC101	3	8.6	Male	59	0.18	329	314	2.5	69.6	76.3	263	20.1	3.37	13.1	-0.4
82	BC102	6	20.2	Male	40	0.12	323	332	4.93	92.2	102.3	258	26.2	3.8	14.9	4.7
83	BC103	11	55.0	Male	67	0.17	386	343	0.98	22.1	91.4	284	25.9	3.43	14.1	2.9

84	BC106	36	10.0	Male	82	0.25	325	339	0.83	29.7	92.7	270	24.8	2.88	13.8	1.3
85	BC107	12	28.8	Male	29	0.10	291	301	7.15	88.3	105.3	277	29.0	3.53	14.5	5

Appendix C: Data collected from the naturally *Babesia*-infected cases of study cohort two

No	Study no	Age (months)	Weight (kg)	Gender	HGB (g/L)	HCT (L/L)	MCHC (g/L)	CHCM (g/L)	Ret% (%)	ARC (x109/L)	MCVr (fL)	CHCMr (g/L)	CHr (pg/cell)	CHDWr (pg/cell)	RDWr (%)	CHDelta (pg)
1	BLSC1	23	19.3	Male	58	0.18	331	321	2.51	61.4	96.2	287	27.4	3.07	12.9	4.4
2	BLSC2	11	28.6	Male	72	0.22	321	315	5.8	183	94.6	278	26.1	3.62	14.6	3
3	BLSC3	9	11.8	Female	42	0.15	287	292	25.87	427.8	117.9	265	31.1	4.34	13.8	6.9
4	BLSC4	9	4.7	Male	22	0.08	290	253	14.89	135.1	113.9	238	27.0	3.97	13.2	6.5
5	BLSC7	60	7.4	Male	40	0.14	290	293	15.13	262	107.4	257	27.4	3.55	12	4
6	BLSC9				95	0.28	346	337	0.79	31.9	74.7	289	21.6	4.92	20	-2.3
7	BLSC10	36	32	Male	57	0.18	311	310	8.34	197.5	107.9	277	29.7	3.19	11.5	5.1
8	BLSC11	6	23	Female	50	0.16	323	313	2.74	57.8	88.1	272	23.8	3.77	16.3	-0.5
9	BLSC12	9	18.6	Male	25	0.10	261	247	34.56	390.3	106.1	243	25.7	3.46	12.2	5.4
10	BLSC13	3	8.8	Female	22	0.08	286	264	6.41	63.6	112.5	255	28.6	4	14.4	7.6
11	BLSC14	18	6.8	Male	144	0.43	333	325	0.36	22.8	72.5	290	21.1	5.57	21.3	-2.2
12	BLSC15	19	23.8	Male	45	0.13	331	307	3.54	75.9	93.7	257	23.9	3.32	14.4	3.7
13	BLSC18	36	14.6	Male	35	0.12	294	289	21.61	323.3	103.6	254	26.2	3.44	12.5	2.8
14	BLSC19	24	14.7	Male	41	0.12	350	314	2.64	36.4	103.9	286	29.5	3.57	12.8	5.7

15	BLSC22	10	3.4	Female	19	0.07	264	248	27.25	226.4	109.7	239	26.0	3.84	14.6	4.7
16	BLSC29	9	45.6	Male	28	0.07	373	291	3.17	30.6	106.4	271	28.6	4.46	14.7	5.6
17	BLSC40	42	5.6	Male	48	0.16	298	310	8.58	185.4	102.4	256	26.1	3.05	12.1	3
18	BLSC42	10	2.6	Male	40	0.13	310	325	2.16	40.4	93.5	288	26.7	3.22	14.6	3.7

Appendix D: Data collected from the control cases

No	Study no	Age (months)	Weight (kg)	Gender	HGB (g/L)	HCT (L/L)	MCHC (g/L)	CHCM (g/L)	Ret% (%)	ARC (x109/L)	MCVr (fL)	CHCMr (g/L)	CHr (pg/cell)	CHDWr (pg/cell)	RDWr (%)	CHDelta (pg)
1	CON2	84	34	Female	198	0.59	336	354	0.77	67.7	85.6	304	25.7	3.01	14.9	1
2	CON3	72	31	Female	184	0.54	339	353	0.53	41.4	86.7	309	26.6	3.01	14.1	1.1
3	CON6	84	23.6	Male	189	0.57	332	348	0.2	16.2	86.3	317	27.2	3.34	14.7	2.3
4	CON7	24	30	Female	167	0.49	340	337	0.57	42.7	87.2	279	24.2	3.06	13.6	2.2
5	CON8	84		Female	151	0.45	340	332	0.64	41	88.8	267	23.6	3.19	12.1	0.8
6	CON9	48	15	Female	155	0.45	345	340	0.48	31.5	88.8	303	26.7	3.01	12.3	3.6
7	CON11	64	8.8	Female	198	0.59	336	343	0.41	34.5	96.6	281	26.9	2.83	10.5	3.2
8	CON14	78	35	Male	175	0.50	351	358	0.2	15	88.6	303	26.7	3.19	13.3	2.9
9	CON15	84	27	Female	171	0.50	341	354	0.7	53.2	82.6	288	23.6	3.19	15.3	1
10	CON18	18	58	Male	185	0.55	340	346	0.74	60.9	90.3	284	25.5	3.32	13.1	2.9
11	CON19	18	65	Male	187	0.55	339	349	0.45	36.4	89	282	25.0	3.27	13	1.8
12	CON20	3	9	Male	127	0.38	334	340	1.83	102.5	86.1	273	23.4	2.81	12.3	0.9
13	CON21	3	8	Female	128	0.39	325	334	1.92	112.7	88.6	267	23.5	2.79	12.1	1.6
14	CON22	27	29	Female	196	0.57	342	345	0.7	55.8	91.3	297	27.0	2.93	11	2.6

Appendix E: Data collected from the IMHA cases

No	Study no	Age (months)	Weight (kg)	Gender	HGB (g/L)	HCT (L/L)	MCHC (g/L)	CHCM (g/L)	Ret% (%)	ARC (x10^9/L)	MCVr (fL)	CHCMr (g/L)	CHr (pg/cell)	CHDWr (pg/cell)	RDWr (%)	CHDelta (pg)
1	H01	72	6.2	Female	17	0.12	478	273	41.2	227.8	123.2	282	35.0	9.51	16.3	9.2
2	H02	72	15	Female	35	0.12	434	289	2.46	34.2	83.5	254	20.9	3.78	20.9	2.6
3	H03	84	12.6	Female	49	0.16	373	310	0.23	5	80.3	292	23.0	3.86	21.2	0.2
4	H04	60	29.6	Female	40	0.15	298	266	39.4	543.7						
5	H05	120	20.4	Male	64	0.22	309	292	6.91	163.8						
6	H06	60	9.8	Male	42	0.14	283	289	0.56	10.2	98.8	255	24.7	5.86	25.5	2.3
7	H07	24		Female	28	0.10	382	293	29.02	281.8	119.3	246	29.1	3.79	13.2	7.3
8	H08	120	28.6	Male	73	0.27	308	272	30.67	1050.1	97.6	261	25.4	4	13.8	4.5
9	H09	84	37.2	Male	40	0.16	314	267	16.9	231.5						
10	H10	36		Female	45	0.17	269	258	27.38	447.8						
11	H11	60	13.4	Male	48	0.17	310	283	34.39	608.6	123.5	234	28.8	3.7	12.2	4.5
12	H12	120		Male	32	0.11	382	301	3.58	55.4	83.3	233	19.3	3.18	15.4	3
13	H13	96		Female	29	0.12	246	235	11.08	149.8	110.8	205	22.6	3.02	12.4	0.5

14	H14	48		Female	24	0.09	257	259	40	404						
15	H15	72		Male	45	0.15	328	312	19.46	376.5	93.3	266	24.7	3.26	11.8	1.4
16	H16	60		Male	57	0.20	278	280	14.29	330.7	101.8	246	24.9	3.29	13.1	0.3