UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

A comparison between manual count, flow cytometry and qPCR as a means of determining *Babesia rossi* parasitaemia in naturally infected dogs

By

Lourens de Villiers

Dissertation presented for the degree of
Master of Veterinary Science,
Department of Companion Animal Clinical Studies,
Faculty of Veterinary Sciences, University of Pretoria

Pretoria, June 2018

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Supervisor

Prof Andrew L. Leisewitz

BVSc (Hons), MMedVet (Med), ECVIM-CA, PhD

Section of Small Animal Medicine

Department of Companion Animal Clinical Studies

Faculty of Veterinary Science

University of Pretoria

Co-supervisor

Prof Melvyn Quan

BVSc, MSc, PhD

Vector and Vector-borne Diseases Research Programme

Department of Veterinary Tropical Diseases

Faculty of Veterinary Science

University of Pretoria

Dedication

This dissertation is dedicated to my beautiful wife, Dr Mari de Villiers, whose unwavering love and compassion throughout the writing process inspired me to fulfil my dream of writing this dissertation, and to both my parents, Pieter and Charlotte de Villiers, who have been the pillars of support throughout my long years of study.

This dissertation is in honour of all of you.

Declaration of originality/plagiarism

By submitting this dissertation for the degree MSc (Veterinary Science) at the University of Pretoria, I declare that the entirety of the work contained therein is my own original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Pretoria University will not infringe any third party rights, and that I have not previously in its entirety or in part submitted it for obtaining any qualification at this or any other tertiary institution. Declaration of originality attached as appendix A.

June 2018

Ethical and research clearance

The author, whose name appears on the title page of this dissertation, has obtained, for research described in this work, the applicable research ethics approval. The author declares that he 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*. Ethical clearance certificate attached as appendix B.
Reference: V060-16

The author declares that the protocol used in this study was registered and approved by the Research Committee of the Faculty of Veterinary Science, Onderstepoort, at the University of Pretoria, and was written in accordance with the guidelines set by the Research Committee. Research committee clearance certificate attached as appendix C.
Reference: V060-16

The author declares that the relevant Department of Agriculture, Forestry and Fisheries approval was obtained for permission to do research in terms of section 20 of the Animal Diseases Act, 1984 (Act no.35 of 1984).
Reference: 12/11/1/19

Details of presentations

Portions of the work included in this dissertation have been presented at the following events:

- Protocol Defence - slide presentation and discussion
  Faculty of Veterinary Science
  University of Pretoria
  21/06/2016
- Grand Rounds Presentation - slide presentation and discussion
  Faculty of Veterinary Science
  University of Pretoria
  29/08/2017
- Faculty Day 2017 - poster presentation
  Faculty of Veterinary Science
  University of Pretoria
  07/09/2017

Acknowledgements

A sincere and special thank you to the following individuals:

- Prof Andrew Leisewitz (supervisor) - for his mentorship, wisdom and support throughout this study, for navigating me through the turbulent waters of post-graduate research, and for remaining with me throughout the project even when the going got tough.
- Prof Melvyn Quan (co-supervisor) - for his guidance and intellectual inputs on the project and for sharing his wealth of knowledge on qPCR.
- Mrs Milana Troskie (co-worker) - for her dedicated laboratory work in conducting the RLB and qPCR runs for the study.
- Mrs Joyce Jordaan (co-worker) - for conducting the statistical analysis of the project and for her positive teamwork and determined and persevering spirit.

A heartfelt thank you also to the following individuals:

- Dr Jeanne Rudman - for her selfless assistance in sampling blood from *Babesia*-infected dogs at Mamelodi Animal Health Clinic.
- Mrs Carien Muller - for her expertise and guidance in the laboratory, her availability to always assist, and her unflinching kindness and care.
- Sr Marizelle de Clercq - for her assistance in sampling the healthy control dogs.
- Mr Pieter de Villiers - for the contribution he made and time sacrificed in assisting with processing the raw data for the light microscopic manual counts.
- Dr Mari de Villiers - for her assistance in sampling *Babesia*-infected blood for the study at Doornpoort Animal Clinic and her support during my late-night laboratory work.
- Dr James Davies - for allowing me to do sampling at his clinic and for his support and compassion regarding my work and study obligations.
- Dr Marco Romito - for his insights and constructive arguments that helped me construct my study protocol and for his invaluable knowledge of qPCR.
- Dr Alri Pretorius - for her willingness to initiate me in flow cytometric analysis and her informal review of my flow cytometric methodology.
- Prof Geoffrey Fosgate - for his opinions on the preliminary statistical analysis of the project.
- Prof Marinda Oosthuizen - for her inputs in the planning phase of the project.

- Prof Amelia Goddard - for her oversight of my laboratory work administration and guidance committee requirements.
- Dr Yolandi Rautenbach - for her insights and lending an ear to my debates and ideas on flow cytometric analysis and methodology.
- Prof Robert Kirberger - for his guidance in constructing my research protocol.
- Mrs Esther Visser, Mrs Zelda Coetzer and Mrs Leonie Johnson - for their assistance on matters of post-graduate administration.
- Mrs Antoinette Lourens - for her helpfulness in obtaining hard-to-find research articles at the library.
- The 2016/17 final year students and outpatients panel of clinicians - for their assistance in sampling *Babesia*-infected dogs at the Onderstepoort Veterinary Animal Hospital.

I would briefly like to thank the following departments/institutions:

- National Research Foundation - as source of primary funding for the project (Grant CPRR13080726333 held by Prof A Leisewitz).
- Department of Companion Animal Clinical Studies (CACS), University of Pretoria - as source of additional funding for the project (A0Z388).
- Department of Veterinary Tropical Diseases (DVTD), University of Pretoria - for their collaboration on the project.

Most importantly and above all else, I thank my heavenly Father and Saviour for His love and guidance throughout this study. “He will cover you with His feathers, and under His wings you will find refuge” - Psalms 91:4.

Table of contents

List of tables

List of figures

List of abbreviations

%	percent
µL	microlitre(s)
µm	micrometre(s)
µM	micromole(s)
ARDS	acute respiratory distress syndrome
A-Sq	Anderson-Darling test
AUC	area under the curve
B	*Babesia*
BD	Becton Dickinson
BrEMA1	*Babesia rossi* erythrocyte membrane antigen gene
℃	degrees Celsius
CI	confidence interval
cm	centimetre(s)
COV	coefficient of variation
C_p	crossing point
C_q	quantification cycle
C_t	threshold cycle
DAC	Doornpoort Animal Clinic
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DVTD	Department of Veterinary Tropical Diseases
E	*Ehrlichia*
EDTA	ethylenediamine tetra-acetic acid
et al	*et alia* / and others
ex vivo	out of the living
FITC	fluorescein isothiocyanate
FL	light scattering / fluorescence
FRET	fluorescence resonance energy transfer
FSC	forward scatter
H	*Haemaphysalis*

H_0	null hypothesis
HRM	high-resolution melting
Ht	haematocrit
IMHA	immune-mediated haemolytic anaemia
in vivo	within the living
IQR	inter-quartile range
iRBC(s)	immature red blood cell(s)
ITS	intergenic spacer region
MAFFT	multiple alignment using fast fourier transform
MAHC	Mamelodi Animal Health Clinic
Max	maximum
MBC	minimum blood count
MGB	minor groove binder
Min	minimum
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
MSc	Master of Science
n	population size
NA	not applicable
nm	nanometre
no	number
NR	not recorded
OVAH	Onderstepoort Veterinary Academic Hospital
P	*Plasmodium*
PABAK	prevalence-adjusted bias-adjusted kappa
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDF	portable document format
PfEMP-1	*Plasmodium falciparum* erythrocyte membrane protein 1
pH	potential of hydrogen
pmol	picomole
Pr > A-Sq	Anderson-Darling test significance
pRBC(s)	parasitized red blood cell(s)

***P*-value**	probability value
qc	quality control
qPCR	quantitative real-time polymerase chain reaction
qs	*quantum satis*
R	radius
R	*Rhipicephalus*
RBC(s)	red blood cell(s)
rcf	relative centrifugal force
RLB	reverse line blot
RNA	ribonucleic acid (singular)
RNase	ribonucleic acid (plural)
ROC	receiver operating characteristic
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
r_s	Spearman rank-order correlation coefficient
RT-qPCR	reverse transcription quantitative polymerase chain reaction
s	second(s)
SD	standard deviation
SE	standard error
sp	species (singular)
spp	species (plural)
SSC	side scatter
T	*Theileria*
TAE	tris base, acetic acid and EDTA
TBE	tris base, boric acid and EDTA
TCM	tri-colour method
TE	tris base and EDTA
VESA	variant erythrocyte surface antigen
vs	versus
WBC(s)	white blood cell(s)
WSRT Sig	Wilcoxon signed-rank test significance
x	times/multiplied by

Glossary

Adjusted flow cytometry	Flow cytometric results that have been adjusted using a correction factor to account for both background and reticulocyte interference.
Autofluorescence	Self-fluorescence or inherent fluorescence of a cell or dye.
Background fluorescence	An undesirable fluorescent signal that could be confused with the parameter measured. In this study reference is made in particular to autofluorescence and non-specific fluorescence related to reticulocyte interference.
Capillary blood	Blood sampled from the peripheral blood circulation i.e. the ear pinna. A term used interchangeably with 'peripheral blood'.
Correction factor	A formulated calculation used to adjust raw flow cytometric results for both background and reticulocytes interference.
Events	A single cell or particle detected by a flow cytometer.
Feather edge	The thinnest area on a blood smear where the differential count can be performed.
Flow cytometric	In reference to flow cytometry and the measurement of cells.
Flow cytometry	A diagnostic tool used to detect and sort cells or particles and their components by staining the cells with a fluorescent dye and measuring the light scatter / fluorescence emitted by the cells as they flow past an excitation light source.
Gate/gating	Delineation placed around a population of cells with similar characteristics and used as a tool to investigate cells of interest.
Gold standard	A diagnostic tool of superior quality which serves as a point of reference against which other diagnostic tools may be compared.
Howell-Jolly body	Basophilic nuclear remnant clusters of DNA found in erythrocytes.
Light microscopy	A diagnostic tool used to magnify a specimen or cell by use of focused light and a system of lenses.
Manual count	A method of cell quantification that implies individual counting of cells through blood smear evaluation under a light microscope.

Minimum blood count	An automated differential count without a manual count, also referred to as a machine blood count.
Normoblast	Refers to an immature red blood cell containing haemoglobin and a pyknotic nucleus.
Pathogenesis	Entails the origin, manner of development or biological mechanism that results in a diseased state.
Photobleaching	The photochemical alteration of a dye or pigment when exposed to a light source which results in the loss of fluorescent ability.
qPCR	A diagnostic tool used to amplify and simultaneously quantify a target DNA molecule.
Red cell area	Monolayer of red cells used to evaluate red cell morphology, where the central pallor of each cell is still visible.
Reticulocyte interference	Relates to non-specific fluorescence associated with undesirable reticulocyte staining.
Sequestration	The adherence of parasite infected erythrocytes to the endothelium of capillaries or venules.
Unadjusted flow cytometry	Results that have not been adjusted by a correction factor to account for background or reticulocyte interference.
Venous blood	Blood sampled from the central blood circulation i.e. the jugular or cephalic vein. A term used interchangeably with ‘central blood’.
α (alpha)	Volume healthy blood needed as diluent for each dilution.
β (beta)	Volume PBS added to healthy blood for each dilution to compensate the Ht to the same level as the infected blood Ht.
γ (gamma)	% SYBR Green I positive events, which is the unadjusted flow cytometric estimate of parasitaemia.
δ (delta)	Number of SYBR Green I positive events estimated to be reticulocytes.
ε (epsilon)	Number of SYBR Green I positive events that were not estimated as reticulocytes.
ζ (zeta)	Percentage actual SYBR Green I positive events related to parasitaemia and not reticulocyte interference.
η (eta)	Adjusted flow cytometric estimate of parasitaemia which is a truer estimate of % parasitaemia after having been compensated for both background and reticulocyte interference.

Abstract

Light microscopic manual count is the current gold standard for parasite quantification. The ability to determine parasite density in whole blood is crucial to understanding disease pathogenesis and finding a suitable automated method of *B. rossi* parasite quantification would facilitate higher throughput and provide results that are more objective. This study investigated both peripheral capillary and central venous whole blood to estimate the correlations between light microscopy, flow cytometry and quantitative real-time PCR (qPCR). Furthermore, the use of SYBR Green I as a DNA marker for the detection and quantification of *B. rossi* by flow cytometric analysis was explored.

The study objectives included: a) validating the use of SYBR Green I as DNA marker to detect and quantify *Babesia rossi* nucleic acid by flow cytometric analysis; b) correlate *B. rossi* parasite density in venous blood quantified by manual count, flow cytometry and qPCR in the same dog; and c) correlate the parasite density of *B. rossi* in capillary blood with the parasite density in venous blood as determined by manual count, flow cytometry and qPCR in the same dog.

Peripheral capillary and central venous blood were sampled from forty naturally *B. rossi*-infected dogs and ten healthy control dogs. Samples were analysed by reverse line blot hybridization to confirm a mono-*B. rossi* infection. Capillary blood parasite density was quantified using light microscopic manual counting and venous blood parasitaemia quantified by manual counts, flow cytometry and qPCR.

Flow cytometry, using SYBR Green I staining, showed promise in quantifying *B. rossi* nucleic acid in venous blood. A significant correlation was found between the venous manual counts and adjusted flow cytometric results (r_s = 0.465; P < 0.001), as well as qPCR (r_s = -0.500; P < 0.001). A significant correlation was also observed between the capillary manual counts compared to venous manual counts (r_s = 0.793; P < 0.001), adjusted flow cytometric results (r_s = 0.399; P = 0.004), and qPCR (r_s = -0.526; P < 0.001).

The study results suggest that qPCR is of value as an alternative to the gold standard manual count for quantifying *B. rossi* parasitaemia in canine whole blood and that flow cytometry may be useful with further refinement of issues such as background fluorescence and the influence of reticulocytes.

Chapter 1: Introduction

1.1 Background

Babesia rossi is the most prevalent *Babesia* species in South Africa [1] where acute *B. rossi* infections can be diagnosed accurately by light microscopic demonstration of parasitaemia on a thin stained capillary blood smear [2]. Because central and peripheral parasitaemia differ it is necessary to quantify both when quantifying *B. rossi* parasitaemia [2]. Light microscopy is also utilized traditionally as the gold standard in diagnosis of malaria infections, but the limitations of this diagnostic tool have been reported for both malaria [3, 4] and *Babesia* [5]. Subsequently, alternative techniques for parasite quantification have been suggested [6]. Flow cytometry [7, 8] and quantitative real-time polymerase chain reaction (qPCR) [9, 10, 11 in preparation] have proven efficient in detecting and quantifying *Babesia* infections. Furthermore, the SYBR Green I nucleic acid stain has previously been used to study the *Babesia divergens* parasite [12]. Our study investigated the use of SYBR Green I in flow cytometry for the detection and quantification of *B. rossi* parasitaemia. Light microscopy has been compared to flow cytometry and qPCR for malaria [13], but the novelty of our study investigated the correlation between the quantitative abilities of these three tools for the canine *Babesia* parasite. The similarities shared between *Babesia* and malaria [14, 15, 16] are well-documented and studies that investigated the correlation between parasite density and disease severity in malaria [17, 18] and *Babesia* [2] have highlighted the essential role parasite quantification plays in understanding disease pathogenesis. Investigations into the pathogenesis of malaria [19, 20] and *Babesia* [21, 22] suggest that virulence is attributed to certain parasites, because of their unique ability to cytoadhere and sequester in the microvasculature [23, 24]. The hypothesis that *B. rossi* sequesters in the capillary endothelium of its host and that parasite density and disease severity may be related to the parasite's pathogenesis [2] deserves further consideration. Our study compared manual light microscopic, flow cytometric and qPCR methods for detecting and quantifying *B. rossi* parasites in fresh whole blood from naturally infected dogs.

1.2 Problem statement

Manual counting by light microscopy (the current gold standard) of *Babesia* parasites is very time and labour intensive. An automated method, such as flow cytometry or qPCR, would facilitate higher throughput and ultimately provide a more objective measure of determining parasite density in blood and tissues. Comparing flow cytometry and qPCR to the gold standard modality would enable an objective view on whether an automated method of quantification would be suitable to detect and quantify *B. rossi* parasitaemia.

1.3 Research questions

1 Can *B. rossi* nucleic acid in whole blood sampled from naturally infected dogs be detected and quantified *ex-vivo* by flow cytometry using SYBR Green I staining?

2 Is there a significant statistical correlation between the parasite density of *B. rossi* in venous blood determined by manual count and the estimated parasite density of *B. rossi* in venous blood determined by flow cytometry and qPCR in the same dog?

3a Is there a statistically significant correlation between the parasite density of *B. rossi* in capillary and venous blood determined by manual count in the same dog?

3b Is there a statistically significant correlation between the parasite density of *B. rossi* in capillary blood determined by manual count and the estimated parasite density of *B. rossi* in venous blood determined by flow cytometry and qPCR in the same dog?

1.4 Hypotheses

1 *B. rossi* nucleic acid in whole blood sampled from naturally infected dogs can be detected and quantified *ex-vivo* by flow cytometry using SYBR Green I staining.

2 There is a significant statistical correlation between the parasite density of *B. rossi* in venous blood determined by manual count with the estimated parasite density of *B. rossi* in venous blood determined flow cytometry and qPCR in the same dog.

3a There is a statistically significant correlation between parasite density of *B. rossi* in capillary and venous blood determined by manual count in the same dog.

3b There is a statistically significant correlation between parasite density of *B. rossi* in capillary blood correlated to the estimated parasite density of *B. rossi* in venous blood determined by flow cytometry and qPCR in the same dog.

1.5 Research objectives

1 Validate the use of flow cytometry, using SYBR Green I staining, to detect and quantify *B. rossi* nucleic acid in whole blood *ex-vivo* from naturally infected dogs.

2 Determine the *B. rossi* parasite density in venous blood by manual count and the estimated *B. rossi* parasite density in venous blood by flow cytometry and qPCR in the same dog.

3a Determine the parasite density of *B. rossi* in capillary and venous blood by manual count in the same dog.

3b Determine the parasite density of *B. rossi* in capillary blood by manual count and the estimated parasite density of *B. rossi* in venous blood by flow cytometry and qPCR in the same dog.

1.6 Applications and benefits

- The research was primarily undertaken by the investigator as part of the requirements towards fulfilment of a postgraduate MSc Veterinary Science degree.
- Demonstrating the effective use of flow cytometry, using a SYBR Green I dye, to quantify *B. rossi* parasitaemia from whole blood *ex-vivo* could enable use of the methodology for future investigations into canine babesiosis.
- A significant correlation between manual count, flow cytometry and qPCR would provide evidence that far faster and more objective methods to determine parasitaemia could be employed in future *B. rossi* research.
- The use of a mathematical correction factor to adjust for background fluorescence caused during flow cytometric analysis could assist in alleviating high diagnostic costs of multiple staining techniques, reduce laboratory time spent on preparation and analysis of samples and simplify cytometric analysis post-acquisition.

Chapter 2: Literature review

2.1 Introduction

Canine babesiosis is an important tick-borne disease in South Africa [25], where *Babesia rossi* is most prevalent [1]. Dogs suspected of having acute *Babesia* infection are diagnosed traditionally by demonstration of *Babesia* parasites within the red blood cells (RBCs) of the host [26]. This is done commonly by examination of a thin, stained blood smear under a light microscope; a method which in particular has been utilized effectively in the past to quantify *B. rossi* parasitaemia sampled from both peripheral capillary and central venous blood in dogs [2]. Presently, microscopy is the gold standard for diagnosis of acute *Babesia* infections and is the simplest and most accessible diagnostic modality available to veterinarians in many parts of the world [27]. Unfortunately, light microscopy is fraught with limitations in parasite quantification [3, 4] and the sensitivity of this diagnostic tool is lower than molecular diagnostic modalities [5, 6]. In the past decade, more attention has been directed towards investigating alternative methods to assess *Babesia* parasitaemia, including flow cytometry [7, 8] and quantitative real-time polymerase chain reaction (qPCR) [9, 10, 11 in preparation]. Both flow cytometry and qPCR have been compared to light microscopy for quantifying the parasitaemia of malaria [13]. Furthermore, flow cytometry and polymerase chain reaction (PCR) have also been compared to light microscopy in detecting *Babesia bovis* and *Babesia bigemina* [28]. Various staining methodologies have been used for flow cytometric analysis in *Babesia* research and SYBR Green I has previously been used to study the *Babesia divergens* parasite [12].

Parasite quantification is essential to understanding the disease pathogenesis of *B. rossi* [2]. Research into the pathogeneses of malaria species (spp.), which shares similarities with *Babesia* [14, 15, 16], has helped understand the correlation between parasite density and disease severity [17, 18]. *B. rossi* parasite density has previously been correlated to disease severity when quantifying parasitaemia using light microscopy [2]. The severity of clinical *Babesia* infection can range widely from a mild presentation to severe disease involving multiple organ failure and death [29]. *B. rossi* is the most virulent of the large-sized *Babesia* spp. [30] in dogs and its virulence is likely associated with parasite genotype [31] and other host-parasite interactions. Related studies have explored fundamental questions surrounding disease

pathogenesis in malaria [19, 20] and *Babesia* [21, 22] that suggest that virulence is attributed to certain parasites, because of their unique ability to cause red blood cell adherence to the capillary endothelium, a process known as sequestration [23, 24]. Furthermore, it is a well-established fact that parasite density is associated with disease phenotype [18, 32, 33]. In the case of *B. rossi*, the hypothesis that parasite density and disease severity may be related to the pathogenesis of the parasite [2] deserves more attention. Further investigations into the pathogenesis of *B. rossi* and the link between parasite density and disease severity are essential, hence the need to find suitable alternative, quicker and more objective methods for *Babesia* parasite quantification.

2.2 Canine babesiosis

Babesia is a protozoan parasite of worldwide economic significance and is the second most common parasite in the blood of mammals after trypanosomes [34]. Morphologically, canine *Babesia* can be divided into two distinct forms, namely the large *Babesia* species (measuring approximately 2.5-5.0 µm), and the small *Babesia* species (1.0-2.5 µm) [35]. Originally, a trinomial system for classifying the three most common large *Babesia* species, divided *B. canis* into subspecies, namely *B. canis canis*, *B. canis rossi*, and *B. canis vogeli* [36]. Differences in geographic distribution, vectors, virulence, pathophysiology, and antigenic variation supported this subspecies classification [37, 38, 39, 40]. A subsequent study suggested that, due to a lack of significant genetic overlap between the large *Babesia* spp., a new classification be considered that divided these large *Babesia* spp. into separate species based on genotype [41]. The revised nomenclature was based on differences in molecular phylogenic analysis, tick vectors and epidemiological distribution and is now accepted widely [41, 42, 43]. Additionally, a fourth large *Babesia* species has been detected in the blood and bone marrow of dogs showing abnormalities consistent with babesiosis in North Carolina [44]. Only three small *Babesia* parasites have been described, including *Babesia gibsoni* [45], *Babesia conradae* [46] and *Babesia microti*-like sp. [47], also called *Theileria annae* [48] or “Spanish dog isolate” [49]. A fourth (*Babesia vulpes*) has also been suggested recently [50].

The life cycle of *Babesia* involves the transmission of sporozoites from the salivary glands of a tick vector harbouring the disease [35]. The tick bites the canine host and transfers the parasite into the subcutaneous tissues and bloodstream [29]. The *Babesia* piroplasms then parasitize the RBCs of the host [27]. This is the most common route of infection [29]. *B. gibsoni* infection

has been reported in cases of illegal dog fighting where it is believed that the dogs themselves act as a vector for the parasite and that *B. gibsoni* may be transmitted through a dog bite wound or saliva [49, 51, 52].

Babesia spp. are distributed throughout the world [35]. *B. canis* is transmitted by *Dermacentor reticulatus* and occurs in Southern Europe [30], along with *B. vogeli* [42] and is widespread in Asia [26]. *Haemaphysalis elliptica* (previously *H. leachi*) [53] is the vector of *B. rossi* and geographically restricted to sub-Saharan Africa [54]. *H. elliptica* is the only known vector of *B. rossi* in South Africa and is found commonly on dogs presented for suspected babesiosis to the Onderstepoort Veterinary Animal Hospital (OVAH), located at the Veterinary Faculty in South Africa [55]. The parasite was reported originally in South Africa, but has now been identified in other African regions, including countries like Sudan and Nigeria [56, 57]. *B. vogeli* is carried by *Rhipicephalus sanguineus* and has a cosmopolitan distribution [30]. *B. vogeli* coexists with *B. rossi* in many parts of Africa and *B. vogeli* can be found in both the Americas and Australia [42]. Both *R. sanguineus* and *H. longicornis* have the potential to serve as vectors for *B. gibsoni* [58]. A *B. microti*-like sp. has been reported in several European countries [35]. Babesiosis is more prevalent during the months of summer and spring in regions of temperate climate due to an increased abundance of tick vector activity during these periods [59]. The disease occurs throughout the year in tropical or subtropical climates [60].

Predispositions related to signalment have been reported in several *Babesia* species [61, 62, 63], including variables such as breed [64], sex [64] and age [65] for *B. rossi*. Canine babesiosis may be peracute, acute, or chronic and non-specific clinical signs include fever, lethargy, weakness, and anorexia [29]. *Babesia* can be associated with a variety of clinical signs including fever, anaemia (regenerative or non-regenerative), thrombocytopaenia and jaundice [58]. The pathogenesis and severity of clinical disease can range from mild presentation to severe disease involving widespread organ failure and death [29]. The pathology that *Babesia* causes varies between different species [35] and factors such as age, immune status, concurrent infections and response to illness in the host may also impact on the severity of clinical presentation [26]. The disease severity of *B. canis* infection is milder than *B. rossi*, but more severe compared to *B. vogeli* [26]. *B. rossi* is considered the most virulent *Babesia* species infecting dogs [66]. In dogs the clinical disease caused by *B. rossi* is classified as either complicated or uncomplicated [66]. The least pathogenic of the well-recognized large *Babesia* is *B. vogeli* [67] which in adult dogs often results in a mild or subclinical disease [26], although infection in puppies less than 3-4 months of age may be fatal [27]. *B. conradae* is considered

more pathogenic than *B. gibsoni* [46] and *T. annae* is reported to have a similar, moderate to severe pathogenicity [49].

The generally accepted method for diagnosing *Babesia* involves examination of a thin, stained capillary blood smear by light microscopy and has shown to be an effective modality for diagnosing and quantifying *B. rossi* parasitaemia in South Africa [2]. The commonly used treatment for canine babesiosis resulting from large *Babesia* infection is imidocarb dipropionate [35]. Tick control is the primary focus for prevention, especially since tick populations in areas where *Babesia* is endemic can remain infected for extended periods of time and re-infest potential carriers [58]. A vaccine that contains soluble parasite antigens to protect partially against *B. canis* has been investigated [40, 68, 69] and is currently available on the European market. A previously commercially available vaccine for virulent babesiosis is no longer available in South Africa, due to cost and the short duration of protection it afforded [70, 71, 72].

2.3 *B. rossi* in South Africa

Canine babesiosis is a disease of major economic importance in South Africa and occurs in about 10% of dogs presented to veterinary practices across the country [25]. An estimated 12% of sick dogs presented to the OVAH in South Africa are diagnosed with *Babesia* infection [73]. *B. rossi* is the most common *Babesia* sp. presented to the outpatient clinic at the OVAH [2] and predominates in South Africa [1]. *B. vogeli* infections also occur, but are rare and only constitute a small percentage of *Babesia* cases in South Africa [74]. Mixed infections of *B. rossi* and *B. vogeli* have been reported as a rare occurrence [1]. The presence of both these piroplasms may account for the differences in clinical manifestations of babesiosis observed in South Africa [1, 74]. Considering the severe disease manifestation caused by *B. rossi* [66], it is likely that many of the early scientific reports that predate 1998 that ascribe canine babesiosis to *B. canis* (before the days of molecular diagnosis), were in fact describing the disease caused by *B. rossi* [75, 76]. One of the first descriptions in the literature of *B. rossi* was reported in a side-striped jackal (*Canis adustos*) in East Africa [54]. A recent report confirmed that the black-backed jackal (*Canis mesomelas*) is a natural host of *B. rossi* [77], but the clinical importance of *Babesia* in wild dogs remains of lesser importance when compared to domestic dogs in South Africa [78].

B. rossi results commonly in peracute or acute disease with initial clinical symptoms typically including fever, pale mucous membranes, pigmenturia and splenomegaly [26].

Thrombocytopenia is common and haemolytic anaemia is a hallmark clinical sign [79] that's usually mild to moderately regenerative, normocytic, and normochromic and as a result of haemolysis [80]. Dogs infected with *B. rossi* are categorized loosely as either uncomplicated (i.e. cases treated as outpatients with a good prognosis) or complicated (cases with severe organ involvement, admitted to hospital for care and usually with a poorer prognosis) [2, 15, 66]. Various studies have investigated the complications resulting from *B. rossi* infections, including cerebral pathology [66, 81], immune-mediated haemolytic anaemia (IMHA) [15], acute respiratory distress syndrome (ARDS) [82], hypoglycaemia [65], renal dysfunction [83, 84, 85], hyperlactataemia [86], hemoconcentration [82, 87], pancreatitis [88], collapse at presentation [2], cardiac dysfunction [89, 90] and rhabdomyolysis [91]. Mortality rates in dogs that develop complications have been reported as high as 15 percent (%) [66] and 45 % [87] in respective studies.

The virulence and severity of *B. rossi* has been described in numerous reports [26, 30, 92] and *B. rossi* is considered to be the most virulent of the large *Babesias* [30]. A study investigating circulatory collapse, parasitaemia and outcome found the severity of *B. rossi* infection is correlated with parasite density [2]. Several clinical biomarkers that may assist in determining the severity of *B. rossi* infections have been reviewed [79] and include studies on coagulopathies [93], cytokines [94, 95], acid-base disturbances [96], and endocrine [97] and cardiac predictors [98, 99]. The exact mechanisms that result in the virulence and diversity of clinical manifestations of *B. rossi* are still being investigated at present and genotypic differences among *B. rossi* strains serve as one plausible explanation [31].

2.4 *Babesia* and malaria

The similarities in the pathophysiology of babesiosis and malaria (*Plasmodium* spp.) have been reported in several studies [14, 15, 16, 82, 100, 101]. Both *Babesia and Plasmodium* parasites are transmitted to mammals by arthropod vectors and parasitize RBCs [14]. Similarities between the pathomechanisms of *Babesia bovis* and *Plasmodium falciparum* have been observed [102, 103]. Parasitized red blood cells (pRBCs) adhere to the endothelium of capillaries in both *P. falciparum* [103, 104] and *B. bovis* [23, 103] infections. Both of these parasites alter the erythrocyte membrane which enables them to evade the host's immune response [16]. Once *P. falciparum* infects an erythrocyte the cell's membrane develops elevations called knobs [105, 106] that enable the infected RBC to cytoadhere *in vivo* [107,

108]. *B. bovis*-infected RBCs express 'stellate protrusions' [109] that are similar to knobs in structure and also appear to play a role in the adherence of the parasitized cells to the endothelium [110, 111, 112]. Whether or not *B. rossi* results in similar red cell-endothelial interaction is unclear at this stage.

Some studies have suggested that dogs with complicated *Babesia* share a similar pathophysiological mechanism with *P. falciparum* [2, 14, 15]. Disease severity in *P. falciparum* [17, 18] and *P. vivax* [113] is related to parasite density, which varies by organ system for *P. falciparum* [114]. It is possible that parasite density is similarly related to disease severity in *B. rossi*.

2.5 Parasite sequestration

The concept of sequestration refers to localized accumulation of pRBCs that bind to the endothelium of the microvasculature in the host [102, 115]. Cytoadhesion relates to one possible mechanism through which pRBCs sequester and describes receptor-mediated binding between the pRBCs and the host endothelium [20, 104]. The ability of a RBC to sequester through cytoadherence present numerous advantages for the parasite, including avoiding recognition by the host's immune system [104, 116] and creating a favourable environment for parasite replication [20, 116]. The significance of adhesion to other RBCs through rosetting (binding of pRBCs to uninfected erythrocytes) and autoagglutination (clump formation of pRBCs and non-parasitized red cells adhering to each other) remains controversial [103].

The interaction between parasite derived antigens on the pRBC surface and receptors on the host endothelium is an important part of *P. falciparum*'s life cycle and is associated with virulence [18, 19, 117]. *P. falciparum*-induced RBC sequestration is caused by a large family of variant proteins called *Plasmodium falciparum* erythrocyte membrane proteins (PfEMP-1) [20, 118] encoded by a diverse family of parasite genes named *var* [114, 115, 119]. The view held that *P. vivax* does not sequester pRBCs is currently being re-evaluated [33, 120, 121] and new evidence suggest that the disease severity of *P. vivax* was previously underestimated [113, 122]. Sequestered parasite density appears to play an important role as a predictor of disease severity [18], but is likely not the only initiating pathological event [32].

The ability of the virulent *B. bovis* to cause pRBC sequestration is well documented [21, 24, 103, 112, 123] in contrast to the less severe *B. bigemina* [124]. Current evidence suggests that

multiple *ves* genes [125] encode a multigene family of proteins termed variant erythrocyte surface antigen (VESA) [21, 23, 126]. VESA1 [126] is likely responsible for the adhesive properties of *B. bovis*-infected RBCs [22]. A correlation between the disease severities associated with *B. bovis*, as well as *B. bigemina*, and the different pathophysiological mechanisms that govern these species is likely [24]. The complexity of the pathomechanisms associated with disease severity in *B. bovis* suggests that the genomic complexity of the *ves* gene family and differences in parasite-derived proteins such as VESA1 involved in cytoadhesion affect the binding of pRBCs with endothelial cell receptors and that genomic diversity may contribute to phenotypic variability [127].

B. rossi has a gene family that results in the expression of a *Babesia rossi* erythrocyte membrane antigen gene (BrEMA1) that may be similar to the PfEMP1 and VESA1 proteins and may play a role in RBC sequestration [31]. Microvascular plugging with pRBCs have been observed in the histology of cerebral tissues of dogs infected with *B. rossi* that died of cerebral babesiosis, although this observation was inconsistent between brains and even very patchy within the same brain [128, 129]. Interestingly, in Böhm's study of *B. rossi* parasitaemias a small proportion of capillary parasitaemia cases had a parasite density similar or lower than venous parasitaemias suggesting that if *B. rossi* does indeed induce RBC sequestration it only occurs in a proportion of *B. rossi*-infected dogs [2]. The existence of at least 12 *B. rossi* genotypes based on polymorphisms in the BrEMA gene have been identified and a significant suggestion that parasite BrEMA genotype and disease severity may be related merits further investigation [31]. Current evidence suggests that if *B. rossi* does induce sequestration akin to what is seen with *P. falciparum* and *B. bovis*, it is weaker than what is seen in these diseases. The role of sequestration in disease pathogenesis does however seem more complex than the simple idea that sequestering parasites induce severe disease and non-sequestering parasites induce less severe disease. *B. rossi* causes the most severe *Babesia*-induced disease in dogs with pathology easily as devastating as what is seen in severe *P. falciparum* malaria and *B. bovis* infection. Red cell sequestration induced by *B. rossi* is far from obvious based on work done to date. The role of parasite biomass and sequestration however remain key factors in understanding disease pathogenesis and urges the need for a study to evaluate better ways of quantifying *B. rossi* parasitaemia.

2.6 Parasite detection

Historically, conventional microscopy of stained blood smears is the gold standard diagnostic tool for parasite identification and quantification in most field laboratories [130]. Parasite detection by light microscopy has been employed for both *Babesia* [2, 94, 131] and malaria [3, 132]. Blood is collected and spread on a glass-slide, air-dried, fixed, stained and the blood smear evaluated under a light microscope to visualize and quantify the parasitized cells [5]. Microscopy is inexpensive, but parasite identification and quantification requires expertise, especially when parasitaemia is low [4, 133].

Flow cytometry has been effectively utilized in the detection and quantitative analysis of malaria [134, 135, 136] and various *Babesia* parasites, including *B. canis* [8], *B. vogeli* [137], *B. gibsoni* [7] and *B. divergens* [12]. Particles or cells are scanned by a laser which measures the light scatter and fluorescence emitted by cells as they flow past an excitation light source [138]. Flow cytometry has the advantages of high throughput capabilities and less subjectivity [13], despite the limitations of unwanted background fluorescence [139].

Quantitative real-time polymerase chain reaction is a sensitive tool for detecting and differentiating various *Babesia* spp. including *B. canis*, *B. rossi* and *B. vogeli* [9, 10, 140] and has also been used for several studies on malaria [141, 142, 143]. The technique allows amplification of specific target deoxyribonucleic acid (DNA) and quantifies the DNA as the PCR product is being generated [5]. Although expensive, qPCR is a very sensitive diagnostic tool and offers various advantages over conventional PCR [144].

Reverse line blot (RLB) hybridization is often utilized when multiple species are to be detected simultaneously in a blood sample and has been used to detect several *Babesia s*pp. and related parasites including *B. rossi, B. vogeli*, *E. canis* and *Theileria* spp. [1, 74, 145]. RLB constitutes amplified PCR product exposure to specific oligonucleotides bound to a membrane, hybridization and visualization of positive results through dot formations [5].

Studies comparing conventional microscopy, flow cytometry and PCR have been conducted for malaria in humans [13] and *Babesia* in cattle [28]. Individual studies using light microscopy [2] and qPCR [10] have been used to investigate *B. rossi*. Flow cytometry has not been used to detect or quantify *B. rossi* parasitaemia before, nor has any study correlated results across all three modalities. Methods such as flow cytometry and PCR-based assays may one day replace light microscopy as the gold standard for parasite detection due to the advantages of greater

sensitivity and specificity [13]. The increased use of molecular techniques in clinical diagnosis and epidemiological studies are starting to overcome the limitation of the high costs involved [130].

2.6.1 Light microscopy

Light microscopy has proven effective for parasite identification and quantification in several *Babesia* studies [68, 146, 147]. Limitations include variability between readers and variations between replicate slides [4]. Furthermore, inadequate operator technique and uneven cell distribution across the slide can hamper diagnostic sensitivity imposed by conventional microscopy [3]. Various methodologies of parasite quantification using light microscopy have been described [148, 149] and usually consist of calculating the percentage of infected RBCs, the number of parasites per white blood cell (WBC), or the number of parasites per microscope field [3]. RBCs may be counted in several fields and an average number of cells per field determined; a set number of RBCs to be counted can then be calculated by approximating the number of fields needed to quantify the parasitaemia and be expressed as a percentage of pRBCs [94]. Semi-quantitative methods for parasite counting have been utilized by counting the number of pRBCs in several fields and grading the parasitaemias [131]. Calculating both the total number of RBCs and pRBCs over a set number of fields enables one to determine a percentage pRBCs [149]. A combination of techniques have yielded a semi-quantitative, non-volumetric method of quantifying *B. rossi* parasitaemia in which blood smears were scored at 1000x magnification under oil immersion with a digital analysis program; free parasites were excluded and parasitized and unparasitized RBCs were counted in the red cell area, feather edge and along the straight margins of the smear and expressed as a percentage parasitaemia [2].

Investigations into the relationship between capillary and venous parasitaemias measured by light microscopy have demonstrated that most capillary parasitaemias are higher than venous parasitaemias in dogs infected by *B. rossi* [2], in French large canine *Babesia* isolates (presumably *B. canis*) [150], and in cattle infected by *B. bovis* [151]. Similarly, the number of positive cases from capillary blood was significantly higher than the number of positive cases from venous blood in a study of *P. falciparum* [152]. Furthermore, the parasite detection rate for *P. falciparum* was significantly higher in capillary blood than in venous blood [153]. Conversely, higher venous parasitaemias in relation to capillary parasitaemias were detected by microscopy in North America where a large *Babesia* (presumably *B. vogeli*) was quantified [154]. *B. vogeli* is

a significantly less pathogenic parasite than *B. rossi*. Similarly, higher venous parasitaemias were also observed in asymptomatic subjects in a recent study of *P. falciparum* malaria in Cameroon [155]. The contrast and discrepancies between studies that compare capillary and venous parasitaemia for both *Babesia* and malaria may be inherently related to the unique pathophysiology of the disease caused by these parasites [2, 152] and merits further investigation.

2.6.2 Flow cytometry

Flow cytometric analysis enables multiple parameters of RBCs to be recorded simultaneously [8] and thus is useful as a diagnostic tool that allows qualitative and quantitative characterization of cells within a sample [138]. Cytometric profiling has high throughput capabilities, can be performed relatively quickly and provides the advantages of automation [13]. The ability to assess pRBCs by means of flow cytometry exploits the fact that normal circulating RBCs predominately lack DNA and therefore stained parasite nucleic acid is more readily detected [6]. Parasitaemia may be calculated as a factor of DNA-positive stained pRBCs per the total number of RBC events detected by the flow cytometer [139].

Quantification of pRBCs may present difficulties in anaemic patients with a high count of micronucleated reticulocytes, resulting in higher levels of unwanted background fluorescence [13]. Immature RBCs (iRBCs) called reticulocytes contain low levels of ribonucleic acid (RNA) which could be confused with pRBCs when not using a DNA-based stain [139]. The resultant excess in background fluorescence could result in imprecisions at low but significant parasitaemias [156]. In addition to reticulocytes other confounders to staining methodology include normoblasts and DNA remnants called Howell-Jolly bodies [6], but the extent of their impact remains controversial. Reticulocyte interference is likely the most important confounder causing issues of non-specific staining in cytometric RBC analysis and has been noted in several studies [139, 156, 157]. To overcome the problem of non-specific staining, endeavours to improve staining efficiency have been proposed, including antibody-based staining strategies [136] and the use of a tri-colour method (TCM) in the quantification of whole blood [135]. The question as to what extent reticulocytes are parasitized by *Babesia* [158] or malaria [159] adds further insult to the problem of cytometric quantification. The majority of malaria parasites, notably *P. vivax*, prefer to invade reticulocytes [160, 161]. A similar predilection for reticulocytes has been observed for *B. gibsoni* [162, 163]. No information is available on the RBC predilection

of *B. rossi* or the impact it would have on parasite quantification when reticulocyte interference is accounted for in *B. rossi* cytometric analysis.

Various nucleic acid stains have been investigated in the flow cytometric analysis of several *Babesia* spp., including Hydroethidine for *B. canis* [8]; SYTO16 [7] and thiazole orange [162] for *B. gibsoni*; and SYBR Green I for *B. divergens* [12]. Different staining techniques and dyes have also been used in malaria research, including Hoechst [160], SYBR Green I [164], and YOYO-1 [156]. SYBR Green I is a membrane-permeable DNA-based dye with an excitation wavelength maximum of 488 nanometres (nm) and emission maximum of 522 nm [139]. Its excitation and emission spectra is broad, lending flexibility to the choice of detection wavelengths [165]. The stain primarily enables detection of double-stranded DNA, as well as RNA [139] and versatility is evident due to its compatibility with most standard cytometers [139], including the Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA). SYBR Green I is detected in the fluorescein isothiocyanate (FITC) channel [166] and due to the nature of the DNA nucleic acid interaction with SYBR Green I marked fluorescence enhancement is seen with its use [167]. The dye is susceptible to light exposure and subsequent photobleaching [168], so restriction to a dark environment when handling SYBR Green I is recommended. SYBR Green I has been utilized extensively by flow cytometry to evaluate *P. falciparum* malaria [134, 169, 170] and in assays dealing with antimalarial drug sensitivity [171, 172, 173]), but its use in *Babesia* research is limited currently to *B. divergens* [12].

The use of flow cytometry for recent investigations into *B. rossi*-infected blood are limited to a study that correlated the severity of clinical signs in dogs infected with *B. rossi* with a suggested functional immune suppression secondary to immune modulatory mechanisms induced by *B. rossi* [174]. Flow cytometry has also been used previously in a *B. rossi* investigation exploring platelet-activation and the platelet-leukocyte interaction in *B. rossi*-infected dogs [175]. A study to investigate the detection and quantification of *B. rossi* parasitaemia using flow cytometry has not been conducted to date. The use of SYBR Green I staining for cytometric studies on babesiosis is limited to characterization of stage-specific parasite development by cytometric profiling of *B. divergens* [12]. An investigation into the use of SYBR Green I as a suitable staining technique for flow cytometric detection and quantification of canine babesiosis and *B. rossi* merits consideration.

2.6.3 Conventional and real-time PCR

Molecular diagnostics based on nucleic acid detection are utilized widely [5] and are capable of detecting very low parasite densities in samples [143]. Real-time PCR was developed originally to detect and quantify PCR products during the amplification process [176], providing quantitative information as opposed to the mere qualitative presence or absence of specific DNA sequences detected by conventional PCR [177]. Traditional PCR has been described in several studies investigating *Babesia*, including discriminating between *B. canis*, *B. vogeli* and *B. rossi* [178]; differentiating *B. gibsoni* from *B. canis*, *B. vogeli* and *B. rossi* [179]; and differentiating *B. canis* from *B. vogeli* [180]. Real-time PCR has several advantages over conventional PCR [144], as it does not require post-PCR electrophoretic analysis, enabling a faster and simpler automated amplification system [5].

A standard nomenclature has been suggested when reporting on qPCR experiments, including discriminating between the abbreviations qPCR for quantitative real-time PCR and RT-qPCR for reverse transcription-qPCR; the use of C_q (quantification cycle) instead of the commonly used C_t (threshold cycle) or C_p (crossing point) to describe the fractional PCR cycle used for quantification; and the term hydrolysis probes used in referral to TaqMan probes [181]. The design and optimization of specific primers and probes are essential to ensuring the sensitivity and specificity of a qPCR assay [176]. Direct detection of target sequences is facilitated by the use of fluorescent agents [130]. Examples of fluorescent agents used in *Babesia* studies include non-specific probes like SYBR Green I [182], specific hydrolysis probes [183], or molecular beacons [10]. In multiplex qPCR assays the use of dual-labelled probes [184] or minor groove binder (MGB) hydrolysis probes [185] are preferred [186]. Historically, the 18S ribosomal ribonucleic acid (rRNA) gene and intergenic spacer region (ITS) have been utilized to elucidate phylogenic data [34]. Both the 18S rRNA gene [9, 185, 187] and ITS region [10, 41, 188] have been utilized in *Babesia* research. Recently, a novel qPCR diagnostic assay targeting mitochondrial DNA spanning the *Isu5-Isu4* region was developed which differentiates a more expansive range of *Babesia* spp. compared to the established 18S qPCR [140].

Real-time PCR assays provide an unique versatility in methodologies used for *Babesia* research, including a multiplex qPCR assay for detection of *E. canis* and *B. vogeli* [186]; a quantitative fluorescence resonance energy transfer (FRET)-PCR with a melting curve analysis that differentiates *B. gibsoni*, *B. canis*, *B. vogeli*, and *B. rossi* by dissociation temperature [9]; a qPCR high-resolution melting (HRM) method for differentiation of *B. canis* isolates [189]; and the use of chimeric DNA-RNA primers in a qPCR for detection of *E. canis* and *B. vogeli* [184]. A

recent study on the optimizations and validation of a qPCR assay for the detection of *B. rossi* and *B. vogeli* provided a valuable contribution to diagnostic availability for investigations into *B. rossi* in South Africa [11 in preparation]. Regardless of the methodology, the use of qPCR assays in various studies investigating *B. rossi* and other *Babesia* parasites [9, 10, 11 in preparation] attests to the growing preference for this modality as a diagnostic tool for parasite quantification [130].

2.7 Conclusion

The similarities between babesiosis and malaria have resulted in a collaborative research platform that enables a mutually beneficial investigative relationship for both parasites. Elucidating the pathogenesis of *B. rossi* requires defining the link between parasitaemia and disease severity. Alternative tools for *B. rossi* parasite diagnosis and quantification that facilitate higher throughput will likely facilitate more efficient and objective investigations into *B. rossi* pathogenesis. The well-documented differences between peripheral capillary and central venous parasitaemias resulting from *B. rossi* infection may be related to the inherent pathogenesis of this parasite and encourages evaluation of both capillary and venous parasitaemias when investigating parasite densities. Flow cytometry and qPCR may serve as viable candidates to replace conventional light microscopy as the gold standard in parasite quantification of *B. rossi* and the need for a study correlating results between these three modalities has been established. Furthermore, the opportunity to establish the validity of a SYBR Green I nucleic acid dye as a staining tool in flow cytometric analysis of *B. rossi* is worth exploring.

Chapter 3: Methodology

3.1 Study population and sampling

The study population comprised of dogs that were presented for consultation at the OVAH, Doornpoort Animal Clinic (DAC), and Mamelodi Animal Health Clinic (MAHC). These study sites are within 35 km of one another in the Pretoria region of South Africa. Samples collected were those taken routinely in the course of diagnosis of babesiosis in pet dogs. Dogs were selected for the study based on clinical findings classically suggestive of babesiosis and confirmation of the disease on a thin, stained capillary blood smear. Samples were scrutinized further by means of RLB to ensure that dogs included in the data set contained a mono-infection of *B. rossi*. A babesiosis information sheet (appendix E) was presented to each owner prior to sampling and written consent (appendix F) obtained to facilitate ethically informed consent. Sixty animals in total were sampled during the course of the study, of which ten animals were excluded from the data set. The study population size thus consisted of fifty dogs of which forty dogs were confirmed to be infected with babesiosis and ten healthy dogs were sampled as controls. An additional replicate sample was taken from each of fifteen dogs from the infected study population for the purpose of repeatability analysis.

3.2 Inclusion and exclusion criteria

Dogs were selected for the study based on clinical findings classically suggestive of babesiosis and confirmed on a thin, stained capillary blood smear. Samples were scrutinized further by means of RLB to ensure a mono-infection of *B. rossi*. Very few samples were expected to contain a multiplicity of infections. The cut-off value for classification of qPCR positive cases was based at a C_q of 36.12 with a 95% confidence interval of 34.44 - 37.16 [11 in preparation]. Control dogs (blood donors) were considered healthy based on history, clinical examination, peripheral blood smear evaluation and RLB to rule out parasitaemia. Dogs were sampled until an infected population of forty dogs with confirmed *B. rossi* mono-infection and ten healthy dogs confirmed to be devoid of parasites were collected.

Dogs suspected upon clinical examination to have any significant concurrent medical disease or having received any anti-babesial treatment within six weeks prior to examination were not sampled. Dogs with a history of splenectomy were not sampled. Data from animals not conforming to the inclusion criteria subsequent to RLB analysis were excluded from the data set. Samples that had broken or damaged blood smears, or blood smears of poor quality to quantify, were excluded from the data set.

3.3 Patient data capture

A data capture sheet (appendix E) was completed for each patient sampled and all data captured manually was scanned and stored electronically in a portable document format (PDF). All data was ultimately transposed into a spread-sheet and analysed. Patient data recorded included pertinent medical history, clinical examination findings and sampling data. A laboratory sample submission form was completed for each patient for laboratory record purposes prior to haematological analysis of venous ethylenediamine tetra-acetic acid (EDTA) blood.

3.4 Reverse line blotting

RLB was conducted by the Department of Veterinary Tropical Diseases (DVTD) at the Faculty of Veterinary Science in Onderstepoort as described previously [1] to ensure that the infected samples included in the data set contained a mono-infection of *B. rossi* and that the control samples were free of *Babesia* or other parasites. All sampling was done by the primary investigator. Five hundred microlitres (µL) venous blood from each sample was aliquoted with a pipette into a cryovial and archived at -80 degrees Celsius (℃) until RLB was performed. Each cryovial was labelled with a patient name, sample number, medium type (EDTA), the date of collection, and study supervisor details. RLB extractions were done concurrently with qPCR extractions and extracted DNA was kept at -20 ℃ until RLB and qPCR were conducted. The RLB was conducted with a set of primers that amplified a 460-540 base pair fragment of the 18S SSU rRNA spanning the V4 region, a region conserved for *Babesia* and *Theileria*. The *Ehrlichia* PCR amplified the V1 hypervariable region of the 16S SSU rRNA. The membrane used for RLB included probes for *B. vogeli, B. rossi, B. canis,* and *E. canis*.

3.5 Minimum blood and reticulocyte count

Minimum blood count (MBC) and a reticulocyte count were performed on all the samples using the ADVIA 2120 automated cell counter (Siemens, Munich, Germany). MBC and reticulocyte count analysis was conducted on the same day of sample collection for all samples respectively. A report was issued by the laboratory for each sample and all reports were scanned and stored electronically in a PDF. All MBC and reticulocyte count assays were performed by the primary investigator.

3.6 Dilution series

A dilution series was performed by the primary investigator in order to gain insight into the diagnostic ability of each modality to detect a positive sample at different dilutions. The goal of the dilution series was to establish at what dilution each modality would be unable to detect a positive sample. A five-fold serial dilution series of parasitized venous blood was analysed in triplicate and prepared using an infected sample confirmed by light microscopy and RLB to have both a high parasitaemia and mono-infection of *B. rossi* respectively. The parasitized blood was diluted during the serial dilutions with blood from a universal blood donor dog confirmed by RLB to be uninfected with *B. rossi* or any other parasites.

The haematocrit (Ht) of both the infected and healthy dogs were analysed by MBC prior to dilution. The Ht of the healthy sample was decreased to the same Ht as the infected sample by way of dilution with phosphate-buffered saline (PBS), in order to allow for compatibility as the diluent used for diluting the infected blood. If the Ht of the blood used as diluent differed from the Ht of the infected blood to be diluted, bias could be introduced during parasite quantification. The following formulas were used to calculate and compensate for the aforementioned:

(Infected dog Ht / healthy dog Ht) x 1.2 millilitres (mL) = α mL (where α is the volume healthy blood needed as diluent for each dilution).

1.2 mL - α = β mL (where β is the volume PBS added to the healthy blood for each dilution to compensate the Ht to the same level as the infected blood Ht).

Total volumes of healthy blood and PBS needed as diluent for the complete dilution series were calculated and mixed prior to starting the serial dilution. The Ht of the prepared diluent was

analysed by MBC prior to the serial dilution to confirm compatibility with the predetermined infected blood Ht. The infected blood was diluted in a series of six (5^0 to 5^{-6}) subsequent dilutions run in triplicate (5_1, 5_2 and 5_3), using the healthy blood (diluted with PBS) as diluent. The initial input volume of the infected blood was 1.5 mL for the dilution series (5^0, undiluted) and 300 µL were transferred during each subsequent dilution, using a new pipette tip with each transfer. After 300 µL was transferred, 1.2 mL of the diluent was added and gently mixed by use of pipetting. A control sample preparation was made using whole blood from a healthy uninfected dog (5^c, undiluted). A dilution series was only conducted on parasitized venous blood and parasitaemia analysed using light microscopy, flow cytometry and qPCR. For practical reasons pertaining to adequate volume blood needed for a dilution series, such a serial dilution could not be performed on parasitized capillary blood.

3.7 Parasite determination by light microscopy

Manual parasite sampling and quantification was performed in a similar way to what has previously been described [2]. All manual count quantifications were performed by the primary investigator.

3.7.1 Peripheral capillary blood sampling

A thin capillary blood smear was made from the first drop of blood that formed after the clipped inner surface of an ear was pricked with a 21-gauge needle 5-15 millimetres (mm) away from the ear margin and away from the marginal ear vein. Occasionally it was necessary to squeeze the ear to encourage a sufficiently large droplet to form. The blood was transferred to a glass slide with a heparinised micro-haematocrit tube in order to prevent ear debris affecting the integrity of the feather edge. A spreader with a leading edge that was narrower than the normal slide was used to make a thin smear. Slide quality was judged according to the following criteria: all smears should have originated at the frosted edge of the slide; the smear needed to be more than 1 centimetre (cm) long; not more than two streaks extending more than 5 mm from the feather edge were present. If smear quality was unacceptable, a new smear was made using the same prick site. For fifteen of the infected dogs sampled, a replicate capillary smear was made from the opposite ear within five minutes (min) of the first smear for the purposes of

sample replication. Each slide was labelled on the frosted edge with a patient name, sample number, the date of collection and study supervisor details.

3.7.2 Central venous blood sampling

Venous blood (4 mL) was collected into EDTA within 10 min of making the capillary smear. Samples were collected from the jugular vein. Cephalic vein samples were only collected if access to the jugular vein was hindered, if the dog was fractious or if a cephalic catheter needed to be placed for a therapeutic reason. In case of the latter, a sample was withdrawn immediately after the catheter was placed. The blood was stored at 4 ℃ until processed within 12 hours. Venous smears were made from each EDTA sample on the same day as sample collection. Blood was transferred to a glass slide and a spreader with a leading edge that was narrower than the normal slide was used to make a thin smear. Slide quality was judged according to the same criteria as the capillary smears (above). If smear quality was unacceptable, a new smear was made from the EDTA blood. For fifteen of the infected dogs sampled, a replicate venous smear was made within five minutes (usually within 1 min) of the first smear, for purposes of sample replication. Each slide was labelled on the frost edge with a patient name, sample number, the date of collection and study supervisor details.

3.7.3 Capillary and venous blood analysis

Blood smears were stained with Rapidiff (Clinical Sciences Diagnostics, Southdale, South Africa), a Romanowsky stain. Each smear was fixed in fixative solution (Rapidiff fixative) for 5-60 seconds (s), stained with solution I (Rapidiff 1) for 15-45 s and stained with solution II (Rapidiff 2) for 20-45 s. During fixation and staining the slides were agitated in the solution and the excess fluid tapped off in-between each step. Finally, the smears were washed carefully under cold water and allowed to dry. After staining each smear was photographed under oil at 1000x magnification with the use of a light microscope and the aid of a digital analysis program (AxioVision Rel. 4.8, Carl Zeiss Imaging Solutions). Digital images of various portions of the smear were transferred to a computer screen, magnified and captured. The photographs were inserted into an electronic text document, re-sized, the brightness and contrast adjusted accordingly and then printed. Parasite quantification was done manually on the printed images captured and counted RBCs were marked off to ensure accurate quantification. pRBCs were marked with a red marker and healthy RBCs marked with a pencil. A semi-quantitative non-

volumetric method was used to quantify parasitaemias [94, 148, 149] similar to what has previously been described [2]. Free parasites and indistinct RBCs were ignored. Unparasitized RBCs and pRBCs were counted separately in each field. Cells were counted by starting at the left side of the digital image captured and systematically moving to the right during counting. On each smear 650 RBCs were examined in the red cell area, the feather edge and along the sides (straight margins) respectively. A total of 1950 RBCs were examined per smear. Only complete oil immersion fields were scored, as scoring only proportions of fields might have artificially increased parasitaemias. Identification markings on the frost edge were covered and a randomized slide number allocated to each smear prior to quantification to blind analysis. Results were calculated and for each smear expressed as a percentage (%) parasitaemia, i.e. total amount pRBCs / total amount RBCs x 100 = % parasitaemia. Percentage parasitaemias were recorded for each randomly allocated slide number and the corresponding true sample number for each slide unveiled only after all results were captured for the study in order to ensure the correlation to flow cytometry and qPCR results were unbiased.

3.7.4 Light microscopy dilution series

The methodology for preparation of the dilution series is described above. A venous blood smear was made from each sample in the dilution series run in triplicate and the *B. rossi* parasite density quantified using light microscopy (as described above) in order to evaluate the diagnostic ability of light microscopy to detect a positive sample at different dilutions. A total of 24 venous smears were analysed.

3.8 Parasite determination by flow cytometry

3.8.1 Optimization of the gating strategy

The development and optimization of a flow cytometric protocol for analysing *B. rossi*-infected whole blood using a SYBR Green I nucleic acid gel stain - 10000X concentrate in dimethyl sulfoxide (DMSO) (Invitrogen, catalogue no. S7563) in order to quantify parasitaemia is provided elsewhere (appendix G).

3.8.2 SYBR Green I DNA staining protocol

Sample staining using a SYBR Green I dye was performed similar to what has previously been described [12], but was optimized for *B. rossi*. SYBR Green I working stock was prepared at a 1:1000 concentration by diluting 1 µL SYBR Green I stock with 999 µL PBS. The working stock was stored in Eppendorf tubes wrapped in aluminium foil at -20 ℃ in the dark as 20 µL aliquots to minimize freeze-thawing. Prior to flow cytometric analysis samples were prepared as follows:

1. Two falcon tubes were marked 'unstained' and 'stained' respectively.
2. 50 µL whole blood sample was pipetted into each of the two falcon tubes and each tube was washed with 1.5 mL PBS, vortexed for 5 s, centrifuged at 450 relative centrifugal force (rcf) (1725 revolutions per minute (rpm), where the radius (R) = 135 mm) for 5 min at 23 ℃ and the supernatant pipetted off.
3. The cell pellet in the tube marked 'unstained' was re-suspended in 20 µL PBS, and the pellet in the tube marked 'stained' in 15 µL PBS.
4. 5 µL SYBR Green I dye working stock (1:1000 dilution) was added.
5. Both tubes were vortexed for 5 s and incubated in the dark for 30 min at 37 ℃.
6. Following incubation, the contents of each tube were washed with 1.5 mL PBS, vortexed for 5 s, centrifuged at 450 rcf (1725 rpm, where R = 135 mm) for 5 min at 23 ℃ and the supernatant pipetted off.
7. Each tube's cell pellet was finally re-suspended in 500 µL PBS, vortexed for 5 s and analysed by use of an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA).

3.8.3 Quantification of *B. rossi* parasitaemia

Each sample was prepared, processed and analysed by flow cytometry on the same day of sample collection. A wash cycle with distilled water and calibration using SPHERO^{TM} Rainbow Calibration Particles (6 Peaks and 8 Peaks) for quality control (qc) analysis was done daily prior to sample processing and a wash or backflush was done in-between each sample that was run. Cleaning, decontamination and wash cycles were conducted at the end of each day as per quality control guidelines. Whole blood from an infected dog was prepared according to the SYBR Green I DNA staining protocol, processed on the Accuri C6 flow cytometer interfaced with a computer and analysed using BD CSampler Software (BD Biosciences, Franklin Lakes,

New Jersey, USA). Both a stained and unstained preparation was analysed for each sample. The final optimized gating strategy (as discussed in appendix G) was used as a template to gate for the red cell zone of interest. All samples were analysed on the same gating template, without any adjustments made to the gate prior or subsequent to analysis. Fifty thousand events were recorded per sample. Events captured by the optimized red cell gate differed from sample to sample, because the gating template was not adjusted or gated events predetermined. The number of gated events was recorded on a forward scatter (FSC) vs. side scatter (SSC) dot plot. By use of an optimized quadrant marker on the FL-1 vs. FL-4 dot plot the number of SYBR Green I positive events detected in the FL-1 channel in the upper left quadrant were recorded (see example in Figure 7). Parasitaemia determined by unadjusted flow cytometric results was calculated by the following formula:

(SYBR Green I positive events / total gated events) x 100 = γ % (where γ represents % SYBR Green I positive events, which is the unadjusted flow cytometric estimate of parasitaemia).

3.8.4 Correction factor to compensate for background and reticulocyte interference

A correction factor methodology was designed that allowed for mathematical correction of the parasitized red cell percentage for background fluorescence and reticulocytes counted to calculate an adjusted flow cytometric result, which was a more accurate estimate of the percentage parasitaemia. Reticulocyte interference was estimated by determining what percentage of SYBR Green I event fluorescence could likely be attributed to reticulocytes. The reticulocyte percentage was determined by the ADVIA 2120 automated cell counter (Siemens, Munich, Germany). Both infected and control samples were corrected for reticulocytes (with each sample specifically corrected for that sample's reticulocyte count) by use of the following formulas:

Reticulocyte % x total SYBR Green I positive events = δ (where δ represents the number of SYBR Green I positive events estimated to be reticulocytes).

Total SYBR Green I positive events - δ = ε (where ε represents the number of SYBR Green I positive events that were not estimated as reticulocytes).

(ε / total gated events) x 100 = ζ % (where ζ represents the percentage actual SYBR Green I positive events related to parasitaemia and not reticulocyte interference).

Background due to autofluorescence was determined by averaging the % SYBR Green I positive events across the ten control samples after reticulocyte correction. The average

background percentage was calculated as 0.37 %, after having been corrected for reticulocyte interference as described above. Both infected and control samples were corrected for background due to autofluorescence by use of the following formula:

ζ % - 0.37 % = η % (where η % represents the adjusted flow cytometric estimate of parasitaemia which is a truer estimate of % parasitaemia after having been corrected for both background fluorescence and reticulocyte count).

3.8.5 Flow cytometry dilution series

The methodology for preparation and conduction of the dilution series is described above. One hundred µL was retained from each sample in the dilution series run in triplicate and used to quantify the *B. rossi* parasite density using flow cytometry (as described above) in order to evaluate the diagnostic ability of flow cytometry to detect a positive sample at different dilutions. A total of 48 falcon tubes were prepared and for each sample both a stained and unstained preparation was analysed.

3.9 Parasite determination by qPCR

3.9.1 Sampling

All sample processing was done by the primary investigator. An aliquot of 500 µL venous blood from each sample was transferred with a pipette into a cryovial and frozen at -80 ℃ until qPCR was performed. Each cryovial was labelled with a patient name, sample number, medium type (EDTA), the date of collection, and study supervisor details.

3.9.2 Nucleic acid purification

Nucleic acid was purified on behalf of the primary investigator by the DVTD, as described [11 in preparation]. Whole blood aliquots were thawed on ice prior to extraction and 50 µL used for processing with the automated MagMAX™ Express Magnetic Particle Processor (Applied Biosystems, Foster City, California, USA). The 5x MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems, catalogue no. 4462359) was used according to the manufacturer guidelines. VetMAX™Xeno™ Internal Positive Control - VIC™ Assay (Applied Biosystems, catalogue no. A29767), 2 µL per sample, was added to the lysis buffer during extraction. Extracted DNA was

kept at -20 ℃ until RLB and qPCR were conducted. DNA concentration was not measured, but the Xeno internal control served as a proxy for the efficiency of the extraction.

3.9.3 qPCR assay

The qPCR assay consisted of a single group-specific *Babesia* primer set (Integrated DNA Technologies, distributed by Whitehead Scientific) targeting the 18S gene and two hydrolysis (TaqMan) MGB (non-fluorescent quencher) probes (Applied Biosystems, catalogue no. 4316034) that were specific for *B. rossi* and *B. vogeli*. The probes were labelled with FAM (*B. rossi*) and NED (*B. vogeli*) [11 in preparation]. Primer and probe sequences as in [11 in preparation].

The qPCR assay was performed on behalf of the primary investigator by the DVTD according to standard operating procedures (QA/BS/SOP PRO 027 Version1) [11 in preparation]. The reaction consisted of *Babesia* spp. forward and reverse primers (final concentration of 200 nM for each primer), *B. rossi* and *B. vogeli* probes (final concentration of 250 nM for both probes), 1 µL of VetMAX^{TM}Xeno^{TM} DNA primer probe mix_VIC (10 uM), 12.5 µL VetMAX^{TM}-Plus qPCR Master Mix (Applied Biosystems, catalogue no. 4415327), 2 µL sample nucleic acid extract and nuclease-free water *quantum satis* (qs) to make up a total reaction volume of 25 µL/reaction. Each sample was run in duplicate and for each reaction plate one positive and one negative control was added. The positive control consisted of a plasmid construct. The negative control was nucleic acid extracted from a sample confirmed to be healthy and uninfected. The reaction plate was loaded into a StepOnePlus real-time thermo-cycler (Applied Biosystems, Foster City, California, USA) and run using a pre-programmed thermo-cycling protocol, as described by the manufacturer: polymerase enzyme activation took place at 95 ℃ for 10 min (1 cycle), followed by 40 amplification cycles of denaturation at 95 ℃ for 15 s, and annealing/extension at 60 ℃ for 45 s respectively. A cooling stage followed at 40 ℃ for 30 s.

3.9.4 Correlations between qPCR and manual counts

The absolute parasite count per sample for capillary and venous manual counts were determined by the following formula:

% parasitaemia x total red cell count/L = absolute parasite count in the sample.

The absolute parasite counts were log transformed by a base 2 log in order to draw statistical correlations between qPCR C_q and the manual counts on a logarithmic scale.

3.9.5 qPCR dilution series

The methodology for preparation and conduction of the dilution series is described above. One hundred and fifty µL was retained from each sample in the dilution series run in triplicate and analysed by the DVTD as previously described [11 in preparation] in order to evaluate the diagnostic ability of qPCR to detect a positive sample at different dilutions. A total of 24 samples were analysed.

3.10 Repeatability of methodology

Fifteen dogs from the infected study population were sampled in replicate. Fifteen repeat capillary blood smears were sampled from patients. Fifteen repeat venous blood smears were made from the EDTA samples, and fifteen infected venous samples were analysed in repeat by both flow cytometry and qPCR. Analysis of all replicate blood smears were subjected to random, blinded examination.

3.11 Limitations

Limitations across the study design and data analysis included:

- Multiple institutions and clinics were utilized during sampling to ensure that the sample population was collected within the prescribed time. Consequently, sampling was not done solely by the primary investigator.
- Capillary smears sampled ranged in technique and quality due to various individuals involved in the sampling process. Analysis of smears that were of sub-standard quality made parasite quantification more tedious in the sense of having to search for areas on the smear of sufficient quality to calculate parasite density.
- Data capture sheets were at times completed by individuals other than the primary investigator and subsequently data capture forms were not always completed to satisfaction.

- All light microscopic analysis of blood smears were done by the primary investigator, which limited analysis of intra-operator variability during assessment.
- Only one successful dilution series was recorded for the assays. Ideally three dilution series done on separate occasions should have been conducted.
- Only the single SYBR Green I dye was used for staining of nucleic acid during flow cytometric analysis. Duel-staining might have facilitated easier differentiation of cells such as mature red blood cells, reticulocytes and white blood cells.
- The correction factor utilized in this study is novel in nature and has much scope for improvement and refinement. In particular, the correction factor does not account for normoblasts or infected reticulocytes. It's inclusion as part of the analysis though proved essential due to the use of a single colour dye for flow cytometric analysis.
- Due to the nature of the study certain statistical variables could not be analysed to satisfaction, including ROC curve analysis for adjusted flow cytometry and kappa for unadjusted flow cytometry.
- RLB and qPCR analysis was only run on cryopreserved samples after all study sampling was concluded. Ideally the samples should have been run as soon as possible after sampling, but for practical reasons all analyses were conducted together in an attempt to standardize the assessment.

3.12 Ethics

The study included client-owned dogs, naturally infected with babesiosis that presented for care at the OVAH, DAC and MAHC. Informed owner consent for sampling was obtained by providing an information pamphlet on canine babesiosis (appendix E) and owner consent form (appendix F). Ethical clearance was granted (V060-16; 7 June 2016) by the University of Pretoria's Animal Ethics Committee and the research protocol was registered (V060-16; 5 September 2016).

3.13 Statistical analysis

Quantitative data analysis was performed using IBM SPSS Statistics version 24, (International Business Machines Corp., Armonk, NY, USA) and MedCalc for Windows, version 18 (MedCalc Software, Ostend, Belgium). The normality of quantitative data was assessed by performing the

Anderson-Darling test. Normally distributed data was descriptively presented as the mean and non-normal quantitative data were assessed using median and range due to the violation of the normality assumption for these data. Variability in quantitative data was assessed by describing standard deviations (SD) and interquartile ranges (IQR). Agreement between assays for diagnosis of *Babesia* was assessed by calculating absolute agreement, kappa (κ), and prevalence-adjusted bias-adjusted kappa (PABAK). Spearman's rho (r_s) was used to describe the correlations among the parasite quantification methods and *P*-value calculated to determine significance. Scatter plots were used to graphically present correlations between the assays and a linear regression line fitted to indicate the direction of the relationship. The coefficient of determination (R^2) was indicated on each scatter plot to demonstrate how close the data are fitted to the regression line. Statistical results were interpreted at the 5 % level of significance. Repeatability of assays was assessed by calculating the mean coefficient of variation (COV) at a 95 % confidence interval (CI) and COV range. Wilcoxon signed-rank tests were used to compare samples run in replicate to assess repeatability. A receiver-operating characteristics (ROC) curve analysis was employed to measure diagnostic test performance and the Youden J index was used to identify the most efficient cut-off value for classification of case and control dogs.

Chapter 4: Results

4.1 Study population

A total of sixty animals were sampled during the course of the study. Data from a total of ten animals were excluded from the data set, because they did not conform to the study inclusion criteria. Data from nine of the ten animals were excluded subsequent to RLB analysis as follows: Of the infected dogs three were positive for *B. vogeli*; one dog was positive for an undetermined *Ehrlichia/Anaplasma* sp., one animal was positive for both *B. vogeli* and *E. canis*, one dog was very faintly positive for *A. platys*, and one animal was faintly positive for an undetermined *Theileria*/*Babesia* sp. Two dogs from the apparent healthy control population were faintly positive for undetermined *Babesia* spp. Data from only one animal was excluded based on a capillary blood smear that was of insufficient quality due to a poorly defined red cell area to allow parasite quantification.

4.2 Descriptive statistics

4.2.1 Population and sampling variables

Basic epidemiological and sampling data were collected and are presented in Table 1. Each attribute is presented as a proportion of the population size sampled for its parent variable. Location was recorded to evaluate were the majority of samples were obtained. Variables such as sex, breed and age were noted to enable commentary on any signalment-related predispositions for *Babesia* infection. The bleeding sites for both capillary and venous blood samples were recorded to evaluate sampling preferences during the study. Location, sex and breed results were recorded for the entire study population. Age and bleeding site results were only noted for the infected population, because these variables were not recorded for the control population during sampling.

Table 1 Basic epidemiological, signalment and bleeding site data for the study cohort

Variable	Location			Sex		Breed		Age			Ear sampled			Vein sampled	
	OVAH	MAHC	DAC	Male	Female	Purebred	Mixed	< 2 years	> 2 years	Unknown	Left	Right	Both	Jugular	Cephalic
Proportion (n)	0.44 (22)	0.40 (20)	0.16 (8)	0.62 (31)	0.38 (19)	0.54 (27)	0.46 (23)	0.45 (18)	0.48 (19)	0.07 (3)	0.45 (18)	0.10 (4)	0.45 (18)	0.85 (34)	0.15 (6)

DAC: Doornpoort Animal Clinic
MAHC: Mamelodi Animal Health Clinic
n: Population size
OVAH: Onderstepoort Veterinary Academic Hospital

Table 2 presents the results of variables of dog owner knowledge of *Babesia* and important variables related to any history of *Babesia* in the dogs sampled. The results were recorded in a questionnaire presented to each owner during the sampling process. Significant questions in the questionnaire explored the relation between the owner's awareness of what *Babesia* is and whether the dog received regular tick prophylactic treatment. Investigation into the dog's history of *Babesia* infection and treatment prior to sampling formed part of the sample exclusion criteria.

Table 2 Level of dog owner knowledge and previous Babesia history for the study cohort

Variable	Owner knows what *Babesia* is		Owner gives regular tick prophylaxis to patient		Dog has had *Babesia* before		Dog has been treated for *Babesia* before	
	Yes	No	Yes	No	Yes	No	Yes	No
Proportion (n)	0.75 (30)	0.25 (10)	0.20 (8)	0.80 (32)	0.10 (4)	0.90 (36)	0.10 (4)	0.90 (36)

n: Population size

4.2.2 Clinical and haematological variables

Both clinical and haematological variables of interest were recorded for each dog in the infected population. Table 3 shows the basic descriptive statistical results for the variables temperature, reticulocyte % and haematocrit. Temperature was not measured in the control population and is therefore absent from Table 3, but remains an important variable in the assessment of the infected dogs at presentation. The haematological variables of interest included reticulocyte % and haematocrit and were recorded for both the infected and control populations.

Table 3 Descriptive statistics for temperature, reticulocyte % and haematocrit

Variable	Population size	Mean ± SD	Median	IQR	Q1; Q3	Min; Max	Pr > A-Sq
Temperature (℃)	*40	39.68 ± 0.63	39.70	0.90	(39.2; 40.1)	(38.3; 41)	> 0.250
Reticulocyte %	*40	4.18 ± 3.95	2.50	6.30	(0.95; 7.25)	(0.39; 14.3)	< 0.005
	**10	0.88 ± 0.66	0.70	0.70	(0.4; 1.1)	(0.3; 2.2)	0.053
Haematocrit	*40	0.26 ± 0.13	0.23	0.18	(0.16; 0.34)	(0.11; 0.59)	< 0.005
	**10	0.51 ± 0.07	0.51	0.09	(0.47; 0.56)	(0.37; 0.62)	> 0.250

%: Percentage
**: control population
*: infected population
℃: degrees Celsius
IQR: Inter-quartile range
Min; Max: Minimum and Maximum
Pr > A-Sq: Anderson-Darling test significance, where $P < 0.05$ indicates non-normal distribution
Q1; Q3: Quartile 1 and Quartile 3
SD: Standard deviation

Descriptive statistical results for clinical variables including hydration status, collapse, mucous membrane colour and capillary refill time are presented in Table 4. These clinical variables provided additional objective measures of disease.

Table 4 Descriptive statistics for hydration, collapse, mucous membrane colour and capillary refill time

Variable	Hydration		Collapse		Mucous membrane colour				Capillary refill time	
	Yes	No	Yes	No	Pink	Pale-pink	Pale	Cyanotic	Less than 2 s	2 s or longer
Proportion (n)	0.28 (11)	0.72 (29)	0.10 (4)	0.90 (36)	0.40 (16)	0.08 (3)	0.50 (20)	0.02 (1)	0.65 (26)	0.35 (14)

n: Population size
s: seconds

4.2.3 Descriptive statistics for manual count, flow cytometry and qPCR

Table 5 contains the descriptive statistical results for the three different methods used to evaluate parasite density. Results for both the infected and control populations are presented respectively. Assessing the normality distribution for each variable enables the choice of either parametric or non-parametric statistical tests.

Table 5 Descriptive statistics for capillary and venous manual count, unadjusted and adjusted flow cytometry, and qPCR

Variable	Population size	Mean ± SD	Median	IQR	Q1; Q3	Min; Max	Pr > A-Sq
Capillary manual count (%)	*40	3.92 ± 8.83	1.00	1.64	(0.38; 2.03)	(0.1; 43.08)	< 0.005
	**10	0.00 ± 0.00	0.00	0.00	(0; 0)	(0; 0)	NA
Venous manual count (%)	*40	0.59 ± 1.19	0.21	0.41	(0.08; 0.49)	(0; 6.87)	< 0.005
	**10	0.00 ± 0.00	0.00	0.00	(0; 0)	(0; 0)	NA
Unadjusted flow cytometry (%)	*40	2.33 ± 2.46	1.52	2.75	(0.51; 3.26)	(0.13; 9.97)	< 0.005
	**10	0.37 ± 0.20	0.35	0.33	(0.17; 0.51)	(0.12; 0.71)	> 0.250
Adjusted flow cytometry (%)	*40	1.80 ± 2.17	1.10	2.61	(0.14; 2.75)	(0; 8.45)	< 0.005
	**10	0.08 ± 0.12	0.005	0.13	(0.005; 0.13)	(0; 0.33)	< 0.005
qPCR (C_q)	*40	27.58 ± 3.23	27.68	4.53	(25.6; 30.13)	(20.85; 33.61)	> 0.250

%: Percentage
**: control population
*: infected population
C_q: Quantification cycle
IQR: Inter-quartile range
Min; Max: Minimum and Maximum
NA: Not applicable
Pr > A-Sq: Anderson-Darling test significance, where $P < 0.05$ indicates non-normal distribution
Q1; Q3: Quartile 1 and Quartile 3
SD: Standard deviation

4.3 Statistical agreement of manual counts, flow cytometry and qPCR for the diagnosis of *Babesia*

Agreement between the three methods employed in this study and RLB as a means of diagnosing *Babesia* infection were evaluated and presented in Table 6. Additionally, agreements between capillary and venous manual parasite counts are also recorded in Table 6.

The scale for interpretation of the kappa statistic is presented in Table 7. RLB was chosen as the reference diagnostic test (test 1) to enable evaluation of the gold standard capillary manual count along with the other diagnostic modalities as a means of diagnosis. The absolute agreement and kappa allows evaluation of how each diagnostic modality (manual, flow cytometry and qPCR) compared to the RLB reference test. A 95 % CI for capillary manual counts and qPCR are absent from Table 6, because the agreement was perfect with RLB. Kappa agreement could not be calculated for the unadjusted flow cytometry, because the entire negative control population gave false-positive results.

Table 6 Agreements between RLB, capillary and venous manual count, unadjusted and adjusted flow cytometry, and qPCR for the diagnosis of *Babesia*

Test 1	Test 2	Population size	Agreement (%)	Kappa (κ)	Kappa interpretation	95 % CI	PABAK
RLB	**Capillary manual count**	50	100	1.000	Very good	-	1.000
RLB	**Venous manual count**	50	88	0.694	Good	(0.475; 0.914)	0.760
RLB	**Unadjusted flow cytometry**	50	80	-	-	-	-
RLB	**Adjusted flow cytometry**	50	80	0.325	Fair	(0.061; 0.689)	0.600
RLB	**qPCR**	50	100	1.000	Very good	-	1.000
Capillary manual count	**Venous manual count**	50	88	0.694	Good	(0.475; 0.914)	0.760

CI: Confidence interval
κ: Kappa
PABAK: Prevalence-adjusted bias-adjusted kappa

Table 7 Scale of interpretation for kappa statistic

Kappa	**< 0.20**	**0.21-0.40**	**0.41-0.60**	**0.61-0.80**	**0.81-1.00**
Agreement	Poor	Fair	Moderate	Good	Very good

[190]

4.4 Correlations between manual counts, flow cytometry and qPCR for the determination of parasite density

Table 8 presents the Spearman's rho correlations and the significance of the correlations for the three diagnostics tools used to evaluate parasite density in this study. Assessing the significance of the correlations between manual counts, flow cytometry and qPCR speaks to the core questions and hypotheses of the study.

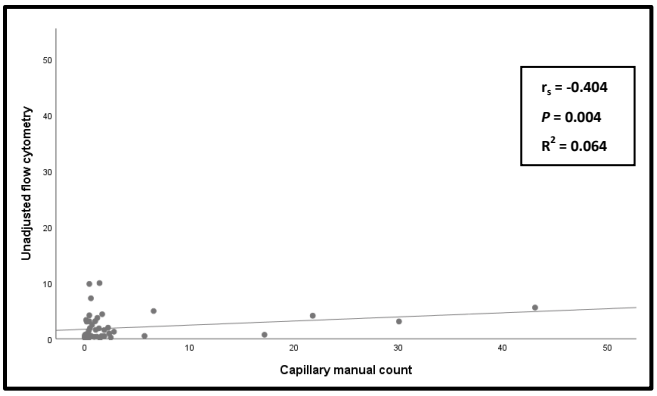
Table 8 Spearman's rho correlations between capillary and venous manual counts, unadjusted and adjusted flow cytometry, and qPCR

Test 1	Test 2	Population size	Spearman's rho (r_s)	*P* value
Capillary manual count	**Venous manual count**	50	0.793	< 0.001
Unadjusted flow cytometry	**Adjusted flow cytometry**	50	0.996	< 0.001
Unadjusted flow cytometry	**Capillary manual count**	50	0.404	0.004
Unadjusted flow cytometry	**Venous manual count**	50	0.467	< 0.001
Adjusted flow cytometry	**Capillary manual count**	50	0.399	0.004
Adjusted flow cytometry	**Venous manual count**	50	0.465	< 0.001
qPCR	**Capillary manual count**	50	-0.526	< 0.001
qPCR	**Venous manual count**	50	-0.500	< 0.001

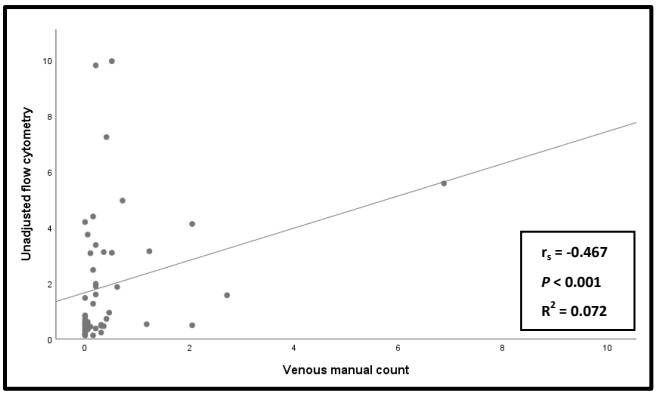
P value: Probability value
r_s: Spearman rank-order correlation coefficient

Figure 1 demonstrates the correlations that unadjusted flow cytometry has with a) capillary manual count; b) venous manual count; and c) adjusted flow cytometry. Figure 2 shows the correlations that adjusted flow cytometry has with a) capillary manual count; and b) venous manual count. Figure 3 presents the correlation results that qPCR has with a) capillary manual count; and b) venous manual count. Figure 4 demonstrates the correlation between capillary manual count and venous manual count. A linear regression line fitted to each scatter plot aids in visualizing whether the correlation is positive or negative.

a)



b)



c)

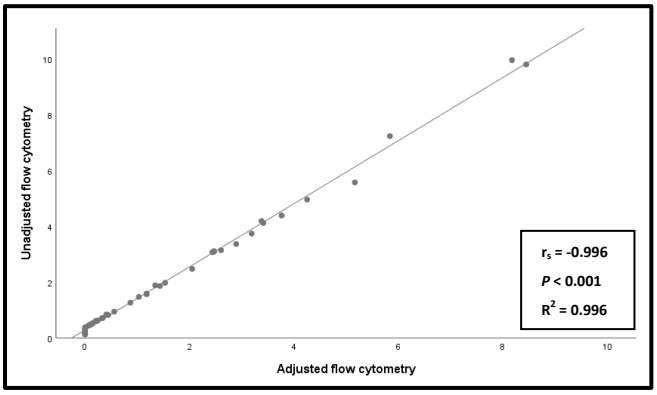
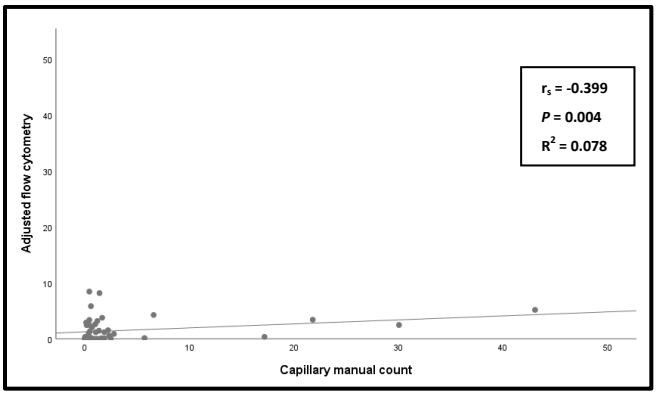


Figure 1 Correlations between unadjusted flow cytometry and capillary manual count (a), venous manual count (b) and adjusted flow cytometry (c)

a)



b)

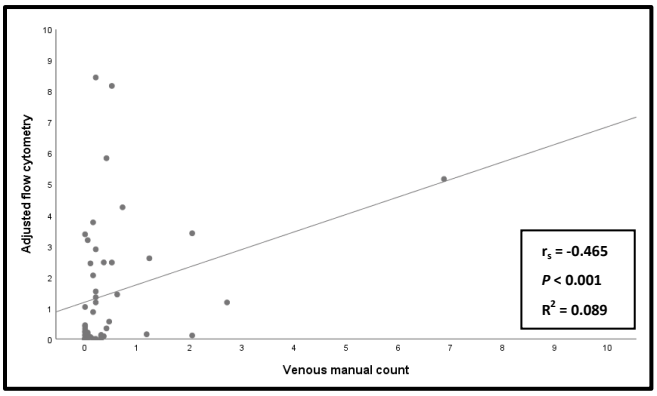
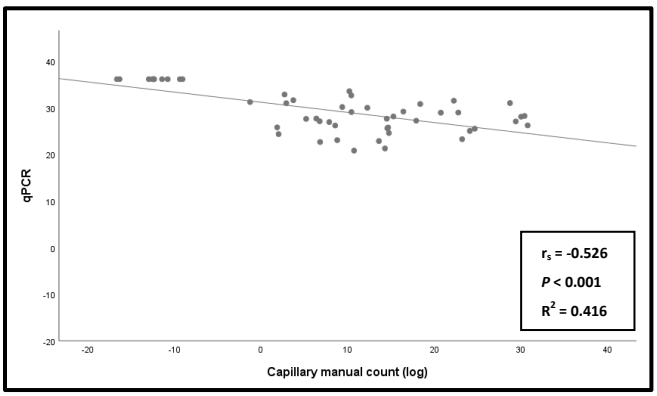


Figure 2 Correlations between adjusted flow cytometry and capillary manual count (a) and venous manual count (b)

a)



b)

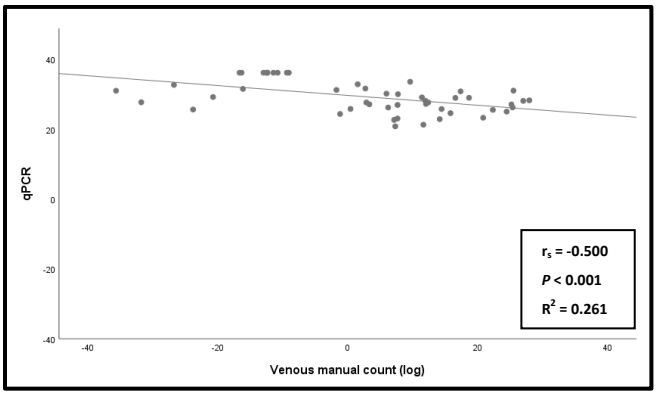


Figure 3 Correlations between qPCR and capillary manual count (a) and venous manual count (b)

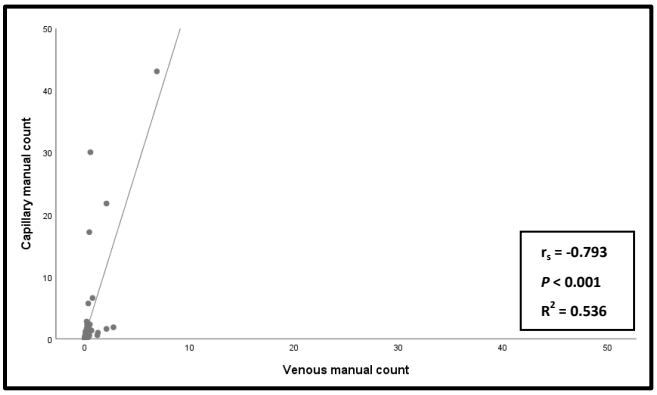


Figure 4 Correlation between capillary manual count and venous manual count

4.5 Repeatability analysis of manual count, flow cytometry and qPCR

4.5.1 Descriptive statistics for replicate analysis

Descriptive statistics for both the original and replicate samples used for replicate analyses by manual count, flow cytometry and qPCR are shown in Table 9. Comparing descriptive statistics between the original and replicate samples allows evaluation of the repeatability of each assay.

Table 9 Descriptive statistics for replicate analysis of capillary and venous manual counts, unadjusted and adjusted flow cytometry, and qPCR

Variables	Population size	Mean ± SD	Median	IQR	Q1; Q3	Min; Max	Pr > A-Sq
Capillary manual count_1 (%)	15	5.62 ± 11.69	1.39	2.36	(0.41; 2.77)	(0.26; 43.08)	< 0.005
Capillary manual count_2 (%)	15	5.68 ± 11.48	1.44	2.56	(0.57; 3.13)	(0.05; 43.74)	< 0.005
Venous manual count_1 (%)	15	1.02 ± 1.80	0.36	1.18	(0.05; 1.23)	(0; 6.87)	< 0.005
Venous manual count_2 (%)	15	1.09 ± 1.95	0.31	1.23	(0.1; 1.33)	(0; 7.74)	< 0.005
Unadjusted flow cytometry_1 (%)	15	2.92 ± 2.90	1.57	3.79	(0.61; 4.39)	(0.17; 9.97)	0.032
Unadjusted flow cytometry_2 (%)	15	2.81 ± 2.70	1.72	3.74	(0.65; 4.39)	(0.14; 9.26)	0.046
Adjusted flow cytometry_1 (%)	15	2.32 ± 2.51	1.18	3.56	(0.2; 3.76)	(0; 8.17)	0.038
Adjusted flow cytometry_2 (%)	15	2.23 ± 2.35	1.33	3.38	(0.27; 3.66)	(0; 7.57)	0.041
qPCR_1 (C_q)	15	26.65 ± 3.44	27.12	6.12	(22.9; 29.02)	(20.85; 31.57)	> 0.250
qPCR_2 (C_q)	15	26.66 ± 3.46	27.13	6.42	(22.8; 29.17)	(20.84; 31.46)	> 0.250

%: Percentage
_1: Original sample data
_2: Replicate sample data
C_q: Quantification cycle
IQR: Inter-quartile range
Min; Max: Minimum and Maximum
Pr > A-Sq: Anderson-Darling test significance, where $P < 0.05$ indicates non-normal distribution
Q1; Q3: Quartile 1 and Quartile 3
SD: Standard deviation

4.5.2 Statistics for repeatability assessment

Statistical results for original and replicate samples of the repeatability analysis of manual count, flow cytometry and qPCR are presented in Table 10. Evaluating the mean COV and Wilcoxon signed-rank test significance enables repeatability analysis of each diagnostic modality.

Table 10 Repeatability analysis for capillary and venous manual counts, unadjusted and adjusted flow cytometry, and qPCR

Variables	Mean COV ± SD	95 % CI	COV range	WSRT Sig.	Decision on H_0
Capillary manual count_1 (%) & Capillary manual count_2 (%)	33.58 ± 21.71	(21.56; 45.6)	(1.09; 94.43)	0.348	Retain the null hypothesis
Venous manual count_1 (%) & Venous manual count_2 (%)	48.97 ± 52.68	(19.79; 78.14)	(0; 141.42)	0.570	Retain the null hypothesis
Unadjusted flow cytometry_1 (%) & Unadjusted flow cytometry_2 (%)	7.44 ± 6.56	(3.81;11.08)	(0.25;25.46)	0.256	Retain the null hypothesis
Adjusted flow cytometry_1 (%) & Adjusted flow cytometry_2 (%)	12.47 ± 13.49	(5.0;19.95)	(3.65; 48.1)	0.249	Retain the null hypothesis
qPCR_1 (C_q) & qPCR_2 (C_q)	0.32 ± 0.27	(0.17; 0.47)	(0.02; 0.74)	0.733	Retain the null hypothesis

%: Percentage
_1: Original sample data
_2: Replicate sample data
CI: Confidence interval
COV: Coefficient of variation (%)
C_q: Quantification cycle
H_0: Null hypothesis, where the median difference between the two variables equals 0
SD: Standard deviation
WSRT Sig.: Wilcoxon signed-rank test significance at significance level .05

4.6 Test performance of manual count, flow cytometry and qPCR for *Babesia* diagnosis

The ROC curve analysis which includes an AUC and *P*-value, as well as the Youden index J for manual counts, flow cytometry and qPCR of the study population are presented in Table 11. RLB was chosen as the reference diagnostic test. ROC curve analysis could not be conducted for the adjusted flow cytometry due to the bias that would be introduced into the analysis by a tie between the positive and negative actual state groups. The AUC and significance of the ROC curves analysis allows making an assessment of the diagnostic test performance of each assay.

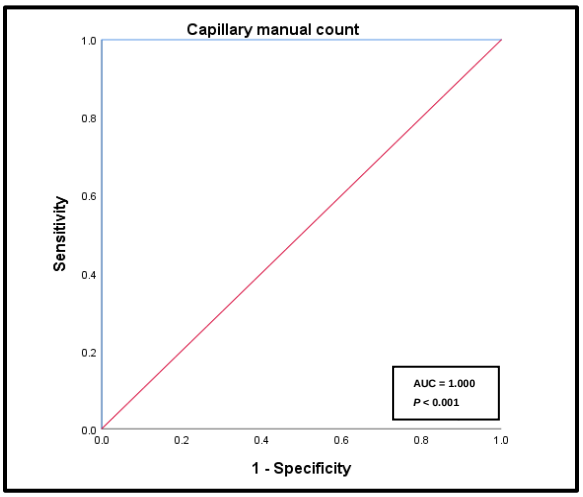
Table 11 ROC curve analysis and Youden J index of capillary and venous manual counts, unadjusted flow cytometry, and qPCR

Variable	Population size	AUC	*P* value	95 % CI	Youden J index
Capillary manual count	50	1.000	<0.001	(1; 1)	1.000
Venous manual count	50	0.925	<0.001	(0.853; 0.997)	0.850
Unadjusted flow cytometry	50	0.850	<0.001	(0.74; 0.96)	0.650
qPCR	50	1.000	<0.001	(1; 1)	1.000

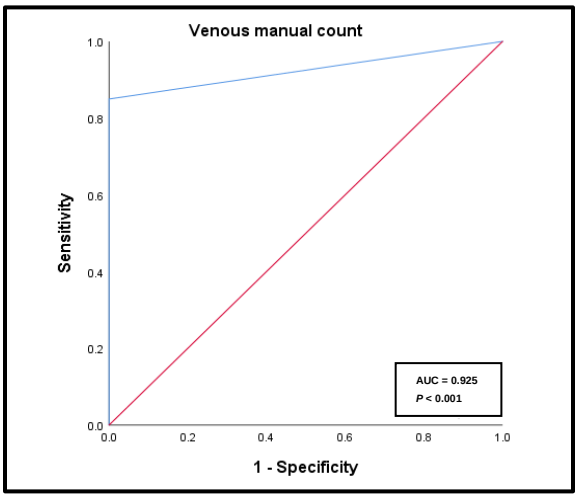
AUC: Area under the ROC curve
CI: Confidence interval
P-value: Probability value, where for the null hypothesis the true area = 0.5

Figure 5 demonstrates the ROC curves for a) capillary manual count; b) venous manual count; c) unadjusted flow cytometry; and d) qPCR. ROC curves for both the capillary manual count and qPCR have a perfect AUC indicating excellent diagnostic test performance compared to the RLB.

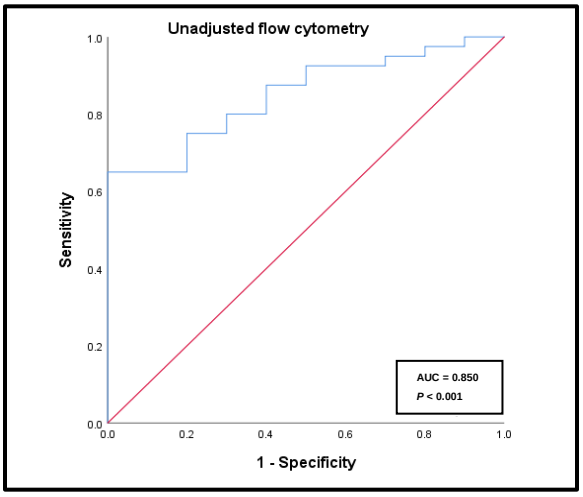
a)



b)



c)



d)

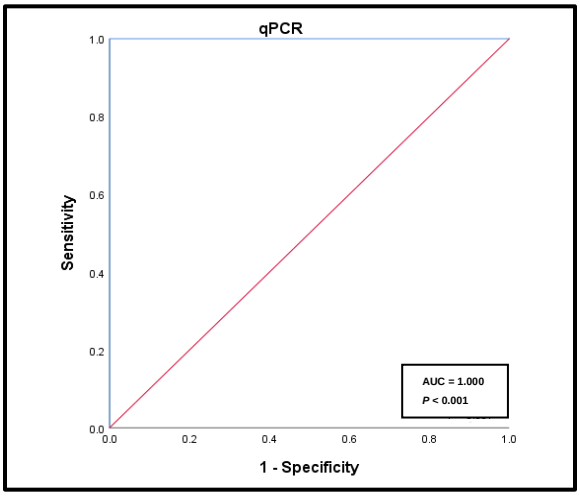


Figure 5 ROC curve analysis of capillary manual counts (a), venous manual counts (b), unadjusted flow cytometry (c) and qPCR (d)

4.7 Limits of detection for manual count, flow cytometry and qPCR

Table 12 demonstrates the dilution series results for a five-fold dilution over six serial dilutions and analysed by venous manual count, unadjusted and adjusted flow cytometry and qPCR. Results for the capillary manual count are absent, because of the practical difficulty in obtaining a sufficient quantity of capillary blood sample needed for a dilution series. Both the initial undiluted and control sample blood results are also included in Table 12. Results for the qPCR controls are absent, because no output was given for negative samples evaluated by qPCR. Evaluation of the mean of each dilution run in triplicate enables evaluation of the diagnostic ability of each modality to detect a positive sample at various dilutions.

Table 12 Dilution series analysis by venous manual count, unadjusted and adjusted flow cytometry, and qPCR

Variable	5^0 Mean	5^{-1} Mean	5^{-2} Mean	5^{-3} Mean	5^{-4} Mean	5^{-5} Mean	5^{-6} Mean	5^C Mean
Venous manual count (%)	0.273	0.103	0.000	0.000	0.000	0.000	0.000	0.000
Unadjusted flow cytometry (%)	2.608	0.651	0.274	0.263	0.244	0.158	0.142	0.367
Adjusted flow cytometry (%)	2.214	0.274	0.000	0.000	0.000	0.000	0.000	0.006
qPCR (C_q)	26.715	26.973	29.306	31.933	36.016	36.582	38.169	-

5^0: Mean of undiluted infected samples run in triplicate
5^C: Mean of healthy control samples run in triplicate
C_q: Quantification cycle
%: Percentage
5^{-1} to 5^{-6}: Mean of series of serial five-fold dilutions run in triplicate

4.8 Flow cytometric analysis using SYBR Green I

Figures 6 and 7 demonstrate the optimized RBC gate placed in the red cell area of both a healthy (Fig. 6a) and *Babesia*-infected (Fig. 7a) sample on a FSC vs. SSC scatter dot plot. The gated red blood cell event population is also plotted on a FL-1 vs. FL-4 scatter dot plot for both the healthy (Fig. 6b) and *Babesia*-infected (Fig. 7b) samples as presented in Figures 6 and 7 to demonstrate the FL-1 positive event distribution. The same gating template was applied to both the healthy and *Babesia*-infected samples. The lack of FL-1 positive events in the upper-left quadrant of the healthy sample (Fig. 6b) demonstrates the absence of SYBR Green I stained parasites. The distinct FL-1 positive events population seen in the upper-left quadrant of the *Babesia*-infected sample (Fig. 7b) clearly demonstrates the SYBR Green I stained pRBCs. The results presented in Figures 6 and 7 are examples from the study population that demonstrate the efficiency of SYBR Green I in staining *Babesia* parasites.

a) b)

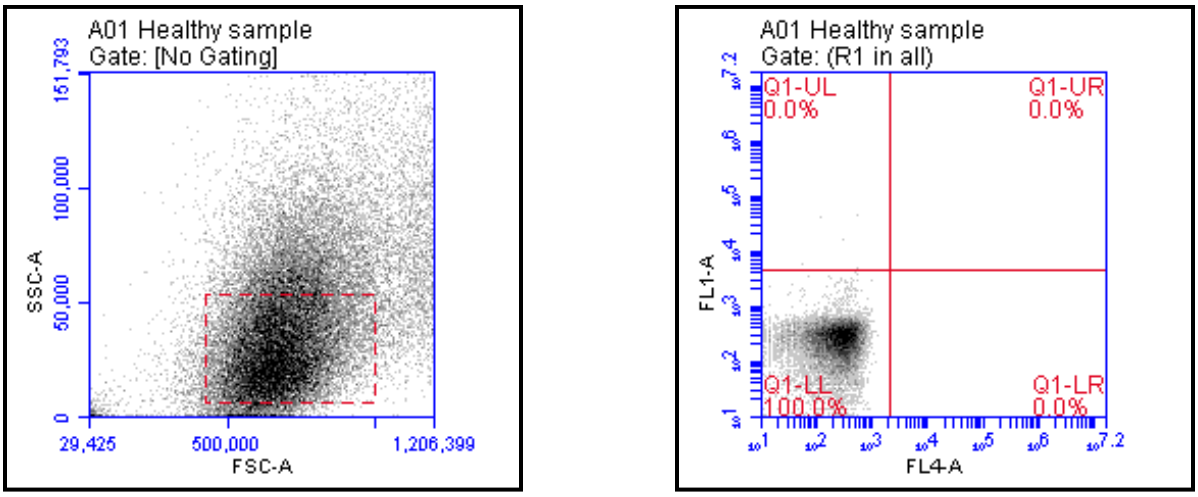


Figure 6 Scatter dot plots of the gated RBC event population in a healthy whole blood sample, unfixed and stained at a 1:000 dilution with 5 µL input SYBR Green I at 37 ℃

a) A final gate was established in the red cell zone of a healthy sample; b) No FL-1 positive events were observed in the healthy sample.

a) b)

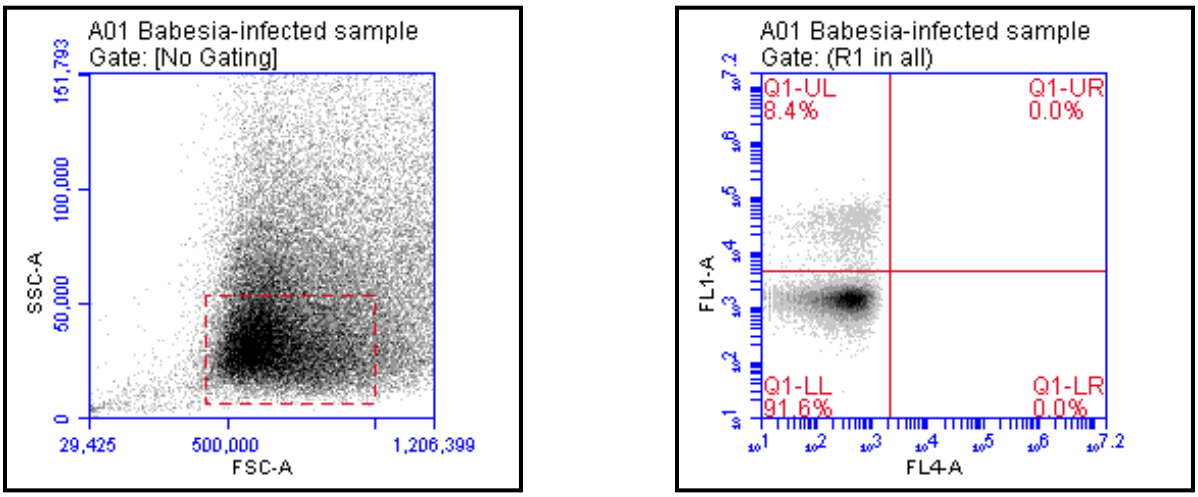


Figure 7 Scatter dot plots of the gated RBC event population in a *Babesia*-infected whole blood sample, unfixed and stained at a 1:000 dilution with 5 µL input SYBR Green I at 37 ℃

a) A final gate was established in the red cell zone of an infected sample; b) FL-1 positive events were observed in the *Babesia*-infected sample.

Chapter 5: Discussion

5.1 Overview

B. rossi is the most prevalent *Babesia* parasite in South Africa [1] and the density of parasitaemia resulting from its infection has previously been investigated by use of light microscopy in both capillary and venous blood [2]. The importance of exploring parasitaemia in both capillary and venous blood has been emphasized in several *Babesia* studies [2, 150, 151]. Manual counts by light microscopy are at present still the gold standard for parasite identification and quantification [130], despite its limitations having been reviewed [3, 4]. Our study sought to investigate alternative diagnostic tools to conventional microscopy to assess the parasitaemia of *B. rossi*. Flow cytometry has been used in a prior study that investigated *B. rossi*-infected whole blood [174], but has never before been used to detect or quantify *B. rossi* parasitaemia. Molecular diagnostics like qPCR have been used with success in investigations of *B. rossi* and other *Babesia* parasites [9, 10, 11 in preparation], but comparing microscopy, flow cytometry and qPCR as a means of diagnosis and as tools for determining parasite density in one study provides the opportunity to correlate their diagnostic and quantitative ability with each other. Studies which compared these three tools as a means of diagnosis and to determine parasite density in investigations of malaria in humans [13] and to diagnose *Babesia* in cattle [28] prompted this study to adapt a similar approach for canine babesiosis. In particular, this study also explored the validity of using a SYBR Green I nucleic acid dye in flow cytometric analysis of *B. rossi*. SYBR Green I has been utilized effectively in an investigation of *B. divergens* [12], but its application in canine *Babesia* has not been explored. Alternative means of diagnosis and determining parasitaemia are required as both a research tool and in the field. More objective diagnostic results facilitated by modalities that have higher throughput and that can detect and quantify parasitaemia with ease will likely aid future studies investigating the relationship between *B. rossi* parasite biomass, disease severity and parasite pathogenesis.

5.2 Study population and data capture

5.2.1 Data excluded from the data set

Sixty animals were sampled in total during the course of the study. Ten samples were excluded from the study based on the exclusion criteria. Different *Babesia* spp. and parasites similar to *Babesia* have been recorded in South Africa [1, 74]. The importance of screening for co-infections by use of RLB has also been reported [145]. RLB is a sensitive diagnostic modality when compared to light microscopy and flow cytometry. Although qPCR [11 in preparation] and PCR [145] is likely more sensitive compared to RLB, the nature of this study's investigation of qPCR dictated the use of RLB as a reference for the inclusion and exclusion criteria and against which to compare the diagnostic tools investigated. The study relates specifically to *B. rossi* and samples confirmed by RLB to have mixed infections or that contained parasites other than *B. rossi* were excluded to avoid discrepancies during quantification. Data from nine cases were excluded because of co-infection with *E. canis*, *B. vogeli*, or unidentified *Babesia* spp. Blood smear quality is important to ensure parasite quantification in each area of interest on the smear. One smear was excluded due to lack of an appropriate RBC area. Attempting parasite quantification in poor quality smears would bias results due to inappropriate representation of the various areas of interest on the smear.

5.2.2 Descriptive data on patient sampling, history and epidemiology

Descriptive data on patient sampling, history and epidemiology is presented in Tables 1 and 2. Three sampling locations were utilized during collection to ensure that sampling was completed within the prescribed timeline. A dry spring and summer led to a decrease in tick vector activity and reduced *Babesia* case numbers in the region sampled. The majority of samples were collected at OVAH (44 %) and MAHC (40 %). Male patients were overrepresented (62 %) during sampling, likely due to an increased risk of contracting babesiosis [64]. When considering the total study population an almost even mixture of purebred (54 %) and mixed breeds (46 %) were sampled. Interestingly, the majority of the infected population were mixed breeds (57.5 %), similar to the sampling results of a previous study [64]. Toy breeds were in the minority (7.5 %) of infected cases and included a Chihuahua and two Pekingese, likely attributed to a lower risk of exposure in toy breeds when compared to larger breed dogs [64]. German shepherd dogs were overrepresented in the control population (60 %), because blood donors were utilized as

controls and German shepherd dogs are commonly used as donors. Approximately half of the infected population was under two years of age (45 %) and the remaining half older than two years (48 %). Almost half of the capillary blood samples were collected from the left ear (45 %), likely as a result of chance and not related to any sampling preferences. The remaining capillary blood samples were taken from both ears (45 %) due to the need for replicate smears and from the right ear (10 %). Almost all of the venous blood samples were collected from the jugular vein (85 %), which was likely related to sampling preference. A good percentage of clients had knowledge of what *Babesia* was (75 %) and yet despite their awareness only a fraction (20 %) regularly gave their dogs some form of tick prophylaxis. A small percentage (10 %) of infected patients had a history of *Babesia* infection, all of which had also received treatment for the illness. No dogs were excluded based on their history subsequent to sampling.

5.2.3 Descriptive clinical and haematological data

Clinical variables of interest in the study included temperature, hydration status, collapse, mucous membrane colour and capillary refill time. The majority of the infected population presented with pyrexia (mean 39.7 ℃, SD ± 0.63) and the data showed a normal distribution (A-Sq $P > 0.250$) as seen in Table 3. Temperature was not captured in the control dog population. The majority of dogs in the infected population presented hydrated (72 %), able to stand (90 %), had pale mucous membranes (50 %) and had a capillary refill time of less than two seconds (65 %), as seen in Table 4. The haematological variables of interest for the infected population provided in Table 3 and include haematocrit and reticulocyte % and had a non-normal distribution (A-Sq $P < 0.005$). Results suggest that most of the infected dogs could be classified as anaemic based on haematocrit (median 0.23, range 0.16-0.34, IQR 0.18) and a degree of reticulocytosis (median 2.5 %, range 0.95-7.25 %, IQR 6.3 %) which are likely associated with the RBC destruction caused by the *B. rossi* infection [82]. In contrast, dogs from the control population all had a healthy haematocrit (mean 0.51, SD ± 0.07) and reticulocyte % (mean 0.88 %, SD ± 0.66 %). Pyrexia and anaemia has a documented clinical association with *Babesia*-infected animals [82] and is usually sufficient to prompt blood smear analysis for *Babesia* parasites in a sick dog. Analysis of the clinical parameters of the infected population in this study indicated that pyrexia and anaemia were the most common clinical abnormalities detected.

5.3 Validation of flow cytometry using SYBR Green I in canine babesiosis

5.3.1 Overview

Flow cytometric analysis has been employed in various studies investigating non-parasite aspects of the disease caused by *Babesia*, including *B. rossi* [174] and *B. vogeli* [137]. Never before has flow cytometry been used in the detection or quantification of *B. rossi*. SYBR Green I dye has been enlisted in several malaria studies as a stain for parasites [164, 169, 170], but its use in *Babesia* parasitaemia research has been limited to *B. divergens* [12]. The SYBR Green I dye used in this study proved effective in both identifying and quantifying *B. rossi* parasitaemia. Fig. 6a shows a gate placed in the RBC zone of whole blood from a healthy dog whole blood. A subsequent FL-1 vs. FL-4 dot plot of the same sample (Fig. 6b) shows no parasite-related events in the upper left quadrant. The same gate placed in the RBC zone of a *B. rossi*-infected event population (Fig. 7a) demonstrates a distinctive positively stained population of parasite-infected red cells in the upper left quadrant of the dot plot (Fig. 7b). Flow cytometry using SYBR Green I does not cause excessive background autofluorescence in a healthy sample and can effectively be utilized to differentiate RBCs from pRBCs in a *B. rossi*-infected sample.

5.3.2 Correction factor methodology for adjusted flow cytometry

Flow cytometry facilitates higher throughput capabilities and less subjectivity [13], but unwanted background fluorescence may be problematic [139]. Excess background fluorescence can cause result discrepancies at low parasitaemias [156]. Reticulocyte interference is arguably the most important confounder in cytometric RBC analysis due to the problems of non-specific staining of reticulocytes [139, 156, 157]. Although other potential confounders such as normoblasts and Howell-Jolly bodies have been recorded [6], their influence was regarded as negligible compared to the issues introduced by reticulocytes in this study. Conventionally, alternative staining methodologies are used to try and combat the issues of unwanted background fluorescence [135, 136] and have been successfully applied. Our study investigated a non-conventional approach to the problem by introducing a correction factor to the equation that mathematically adjusts the raw flow cytometric results to compensate for both autofluorescence and reticulocyte interference. The results still yielded only a rough estimate of parasitaemia and the methodology would only prove valuable for routine use with further refinement. To estimate parasitaemia a gate was set to encompass a sample assumed to be representative of the red cell population in the red cell zone on a FSC and SSC dot plot. For

each sample a small but significant percentage of the total RBC population would consist of reticulocytes. It was assumed that the reticulocyte percentage in the gated population would be representative of the total RBC population.

Several limitations of this study render the adjusted flow cytometry results as estimates of parasitaemia at best. Our methodology did not account for infected reticulocytes and research into the predilection of *B. rossi* for immature RBCs should be explored, as has been done for *B. gibsoni* [162, 163]. A further concern was the sensitivity and specificity of the assay. Depending on an assay's performance and limitations it may fail to detect very low parasitaemias compared to more sensitive methods. Our approach does however suggest that further investigation of such an approach in future studies would be useful, because of the significant improvement seen in the mathematically adjusted results. The need for a correction factor was most evident in review of the healthy control samples and samples that recorded very low parasitaemias. All ten of the control samples returned positive, unadjusted flow cytometry results (providing a false-positive *Babesia* diagnosis), despite testing negative by light microscopy, RLB and qPCR. After mathematical correction, only 5/10 samples still showed a very low false-positive parasitaemia. Although further refinement of the technique is surely needed the correction factor obviously improved the results dramatically.

5.3.3 Overall test performance

The parasitaemia as determined by adjusted flow cytometry (median 1.1, range 0.14-2.75, IQR 2.61) was similar compared to the parasitaemia of capillary manual counts (median 1.0, range 0.38-2.03, IQR 1.64) in the infected population. Adjusted flow cytometry parasitaemia was, as expected, higher compared to the parasitaemia of venous manual counts (median 0.21, range 0.08-0.49, IQR 0.41) in the infected population. The adjusted flow cytometry results yielded a fair agreement ($\kappa = 0.325$) in comparison to the reference RLB for the diagnosis of *Babesia*. Significant correlations for parasite quantification were observed between the adjusted flow cytometry and capillary manual count ($r_s = 0.399$, $P = 0.004$, Fig. 2a), as well as the venous manual count ($r_s = 0.465$, $P < 0.001$, Fig. 2b). Correlations between the adjusted flow cytometry and manual counts were not high and despite proving statistically significant leave much room for improvement. The correlation between unadjusted and adjusted flow cytometry ($r_s = 0.996$, $P < 0.001$, Fig. 1c) was very high and significant due to the obvious relationship between the data results. Interestingly, the adjusted flow cytometric results showed an almost negligible difference to the unadjusted flow cytometry when both the former and latter were compared to the other

diagnostic modalities. This suggests that although background and reticulocyte interference are documented problems, the importance of the impact they have on parasite quantification results need to be investigated further. The mean COV (12.47) and COV range (3.65; 48.1) indicated an acceptable level of repeatability of the adjusted flow cytometry assay methodology with a negligible difference between the original (median 1.18, range 0.2-3.76, IQR 3.56) and repeat (median 1.33, range 0.27-3.66, IQR 3.38) samples of the replicate population. The Wilcoxon signed-rank test significance (0.249) enabled retention of the null hypothesis, meaning no significant differences were recorded between replicate adjusted flow cytometry samples. ROC curve analysis could not be conducted on the adjusted flow cytometry, because an average of the background autofluorescence observed in the control samples was used in the correction factor in both healthy and infected animals. ROC curve results on the adjusted flow cytometry would therefore be biased due to a tie between the actual infected and control groups. The dilution series evaluated the diagnostic ability of the adjusted flow cytometry and demonstrated that after only one five-fold dilution (5^{-1}) of an infected sample the assay could no longer detect parasitaemia, which was similar to what was found with the venous manual count dilution series (5^{-1}). Taken together these data indicate that our hypothesis regarding the potential validity to use SYBR Green I dye as a nuclear marker for flow cytometric analysis to detect and quantify *B. rossi* was correct. However, further investigation and refinement of the methodology is needed before employment in routine diagnosis and parasite quantification can be considered.

5.4 *B. rossi* detection and quantification by light microscopy

Light microscopy is the gold standard for parasite detection and quantification [130] and was chosen as the reference modality against which flow cytometry and qPCR were compared to evaluate parasitaemia. Various methods are used for blood smear evaluation and this study employed a semi-quantitative non-volumetric method [94, 148, 149] as an effective compromise between accuracy and practicality [2]. Parasitaemia was determined by examining a set number of cells, instead of a fixed number of fields. Depending on the degree of anaemia of an infected dog, significantly fewer RBCs would have been assessed in anaemic dogs if the analysis was based on parasites per field and this would have resulted in discrepancies in the repeatability of the analysis. The highest density of RBCs was along the straight margin of the blood smears and lowest in the feather edge, but pRBCs were recorded in highest abundance along the

feather edge. All slides were scored by the same operator and therefore operator-dependent variability was not investigated in this study.

The differences in parasite density found in capillary and venous blood is documented for *B. rossi* [2] and other *Babesia* parasites [150, 151] and prompted a comparison between blood sourced from both sites. The capillary parasitaemia (median 1.0, range 0.38-2.03, IQR 1.64) was significantly higher than the venous parasitaemia (median 0.21, range 0.08-0.49, IQR 0.41) for the majority of the *B. rossi*-infected population, similar to observations made in a previous study of *B. rossi* [2]. Interestingly, 5/40 (12.5 %) infected samples had a higher venous parasitaemia than capillary parasitaemia. This phenomenon was also recorded in a prior study where a subset of venous samples 6/100 (6 %) had a higher venous parasitaemia compared to capillary parasite density [2] and suggest that sequestration, if indeed present in *B. rossi*, only occurs in some individuals or is associated with certain stages of infection. Sequestration may be associated with specific parasite genotypes [31], but further investigations are needed. Very good agreement was recorded for capillary manual smear evaluation ($\kappa = 1.0$) and venous manual smear evaluation ($\kappa = 0.694$) compared to the reference RLB for diagnosis of *Babesia*. Capillary and venous manual parasite count had a fairly high and significant correlation ($r_s = 0.793$, $P < 0.001$, Fig. 4) for determining parasite density. A small difference in capillary manual counts was recorded between the original (median 1.39, range 0.41-2.77, IQR 2.36) and repeat (median 1.44, range 0.57-3.13, IQR 2.56) samples of the replicate population. The mean COV (33.58) and COV range (1.09; 94.43) recorded for capillary manual counts implied poor repeatability, but the Wilcoxon-signed rank test significance (0.348) indicated that differences between replicate smears were not statistically significant. Similarly, the mean COV (48.97) and COV range (0; 141.42) for venous manual counts also reflected poor repeatability, but the Wilcoxon signed-rank test significance (0.570) suggested that the differences were not statistically significant. A small difference in venous manual counts was recorded between the original (median 0.36, range 0.05-1.23, IQR 1.18) and repeat (median 0.31, range 0.1-1.33, IQR 1.23) samples of the replicate population. Repeatability of parasite quantification using light microscopy proved especially difficult at low parasitaemias and related to the uneven distribution of RBCs and pRBCs on a blood smear. The manual scoring technique used in this study is not very sensitive at low parasitaemias. ROC curve analysis revealed excellent test performance (AUC = 1.0, Youden J index = 1.0) for capillary manual count compared to the RLB reference (Fig. 5a) for diagnosis of *Babesia*. Likewise, good diagnostic test performance was observed for venous manual counts (AUC = 0.925, Youden J index = 0.85, Fig. 5b). This was expected, because light microscopy is currently the gold standard for parasite diagnosis

and quantification [130]. A dilution series for capillary manual count could not be performed for practical reasons (the volume capillary blood collected from a needle prick is too small). The dilution series of the venous manual count showed that after only one five-fold dilution (5^{-1}) of an infected sample the assay could no longer detect a positive sample. Results concur with the study hypothesis that there is a statistically significant correlation between the parasitaemias recorded for *B. rossi* in capillary and venous blood determined by manual count in the same dog. Furthermore, results show that capillary parasitaemias are generally higher compared to venous parasitaemias, which indicate that parasite sequestration may be occurring.

5.5 *B. rossi* detection and quantification by flow cytometry

Flow cytometry has never before been used in the detection or quantification of *B. rossi* parasitaemia. Parasite density detected by flow cytometry in this study was estimated by counting the number of SYBR Green I positive RBCs per total number of RBCs [139]. The methodology using SYBR Green I which was described previously in a *Babesia* investigation [12] was adapted in this study for the analysis of *B. rossi*-infected whole blood and the final gate construct utilized for the analysis of the study population was optimized prior to data acquisition (addendum G). A variety of variables were tested during the initial optimization process, but further refinement of the cytometric methodology used in this study is obviously warranted. Sample storage, preparation, processing and data analysis all influence the final results and have a bearing on the accuracy of estimating the sample parasitaemia. Optimization of the method used for sample preparation prior to analysis and creating a standard gating template used during data analysis were the starting points in this portion of the study. It is advisable to use a standard gating template during analysis and to avoid adjusting the gate after events are recorded. Adjustments to the gate subsequent to event acquisition could bias results and lead to discrepancies when comparing samples from different dogs or when evaluating replicate results from the same dog. This study used a standard, optimized method for all sample processing and the same unadjusted, optimized gating template was used throughout sample analysis to standardize results for the study population. Although SYBR Green I is an effective DNA marker for the detection of babesiosis using flow cytometry [12], analysis was limited by use of SYBR Green I as a single dye reagent. Lack of a duel- or tri-staining methodology complicated distinguishing pRBCs from reticulocytes. Unresolved issues of reticulocyte interference remain that relate to a lack of knowledge regarding to what extend *B. rossi* targets immature red cells,

as is seen with other *Babesia* parasites [158]. Cytometric profiling can however be performed relatively quickly and provides the advantages of automation [13], despite the problems of background and reticulocyte interference encountered [139]. The correction factor method introduced in this study is novel and highlights the need for further investigation and refinement. The higher parasite counts measured by unadjusted flow cytometry in comparison to adjusted flow cytometry in this study however attest to the importance of correcting for background fluorescence [156, 157].

A higher unadjusted flow cytometry parasitaemia (median 1.52, range 0.51-3.26, IQR 2.75) compared to both capillary manual count and venous manual count was observed for the infected population. Unadjusted flow cytometry recorded excessively high parasite density, because of the lack of correction for background fluorescence. Diagnostic agreement between unadjusted flow cytometry and RLB could not be determined for the study population, because of the false-positive results seen amongst the negative controls (attributed to background fluorescence). Significant correlations for parasite quantification were recorded between the unadjusted flow cytometry and capillary manual counts (r_s = 0.404, *P* = 0.004, Fig. 1a), as well as the venous manual counts (r_s = 0.467, *P* < 0.001, Fig. 1b), despite the absence of correction for background fluorescence. Correlations between the unadjusted flow cytometry and manual counts were not high and despite proving statistically significant leave much room for improvement. The mean COV (7.44) and COV range (0.25-25.46) for unadjusted flow cytometry implied good repeatability of the assay and the Wilcoxon signed-rank test significance (0.256) confirmed that no significant differences were measured between repeat samples. Only a small difference was seen between the original (median 1.57, range 0.61-4.39, IQR 3.79) and repeat (median 1.72, range 0.65-4.39, IQR 3.74) unadjusted flow cytometry samples of the replicate population. ROC curve analysis provided fair diagnostic test performance for unadjusted flow cytometry (AUC = 0.85, Youden J index = 0.65, Fig. 5c). The dilution series for unadjusted flow cytometry yielded positive values throughout the dilution (5^0 to 5^{-6}), but biased the evaluation of the diagnostic ability of the modality due to lack of correction for background fluorescence. Indeed, the true dilution series results for flow cytometry is likely closer to 5^{-1} as seen with the adjusted flow cytometry results and similar to the venous manual count. Overall, the results from the flow cytometric analysis concur with the study hypotheses that there are statistically significant correlations between the parasitaemia of *B. rossi* in both capillary and venous blood determined by manual count when compared to the estimated parasite density of *B. rossi* in venous blood determined by flow cytometry in the same dog. The potential of using flow

cytometry as an alternative diagnostic tool to detect and quantify *B. rossi* parasitaemia has been demonstrated, but the methodology presented here requires further refinement.

5.6 *B. rossi* detection and quantification by qPCR

Despite the high costs attributed to qPCR for routine *Babesia* testing it remains a very sensitive diagnostic modality [144] and has been used successfully in the study of *B. rossi* and other *Babesia* parasites [9, 10, 11 in preparation]. Although conventional PCR has been employed for the detection of *B. rossi* to good effect [178, 179], the quantitative ability of qPCR made it attractive for use in this study. The output for qPCR is C_q, which is not an absolute measure of parasite density. A higher parasite burden in a sample will result in a higher parasite DNA signal or fluorescence detected during analysis, an earlier rise in the amplification curve, and subsequently a lower C_q recorded. The relationship between C_q and parasitaemia is therefore inversely proportional. This study sought to investigate the correlations between qPCR C_q and % parasitaemia as determined by capillary and venous manual count.

Correlations between C_q and % parasitaemia become problematic, because C_q is a log number and the percentage parasitaemia is a ratio. The C_q of qPCR is a log value, because of the indirect proportional relationship between C_q and the target copy number of parasite DNA detected during each cycle of the amplification process. Theoretically, the C_q multiplies by a base 2 log function during each subsequent cycle. A ratio (% parasitaemia) and absolute value (C_q) are hence not mathematically comparable. This is further compounded by the fact that anaemia reduces the number of red blood cells per unit blood volume. Percentage parasitaemia, being a ratio, requires conversion to an absolute number of parasitized red cells per unit volume and then log transformation to make comparisons between it and C_q mathematically possible. Absolute parasite counts per sample were thus determined for both capillary and venous manual count samples and log transformed in order to produce variables that could be correlated to C_q.

The use of C_q to estimate *B. rossi* parasitaemia have been reported and merit further investigation [11 in preparation]. The qPCR C_q of the infected population yielded values suggesting good assay performance (geometric mean C_q 27.58, SD ± 3.23). Very good agreement was found between the qPCR (κ = 1.0) and reference RLB for the diagnosis of *Babesia*. Significant correlations for parasite quantification were recorded between qPCR C_q

and both capillary manual count parasitaemia (r_s = -0.526, P < 0.001, Fig. 3a) and venous manual count parasitaemia (r_s = -0.500, P < 0.001, Fig. 3b). The negative correlation to qPCR is due to the inverse relationship of C_q and parasitaemia i.e. the higher the parasitaemia, the lower the C_q. Correlations between the qPCR and manual counts were not poor, but despite proving statistically significant leave scope for improvement. The mean COV (0.32) and COV range (0.02-0.74) of qPCR indicated excellent repeatability and no significant differences were found between repeat samples based on the Wilcoxon signed-rank test (0.733). A negligible difference was seen between the original (mean 26.65, SD ± 3.44) and repeat (mean 26.66, SD ± 3.46) qPCR samples of the replicate population. The qPCR demonstrated the highest repeatability compared to manual count and flow cytometry. ROC curve analysis revealed excellent diagnostic test performance for qPCR (AUC = 1.0, Youden J index = 1.0, Fig. 5d), similar to the gold standard capillary manual count. The dilution series for qPCR was able to detect reliable C_q up to a dilution of 5^{-4} based on the assay C_q cut-off of 36.12 [11 in preparation]. The evaluation of the diagnostic ability of the qPCR assay shows that it is able to detect a positive sample at a much lower dilution compared to the other modalities investigated. The qPCR findings in this study concur with previous investigations where light microscopy and PCR [28] or qPCR [13] were compared by demonstrating that qPCR is far more sensitive and reliable in parasite detection. Although high diagnostic costs limit its use as a routine screening test for *B. rossi*, its value in a laboratory or research setting requiring quantitative parasite analysis or detection of low density parasitaemias is promising. Results from the qPCR analysis agree with the study hypotheses that there are statistically significant correlations between the parasitaemia of *B. rossi* in both capillary and venous blood determined by manual count when compared to the estimated parasite density of *B. rossi* in venous blood determined by qPCR in the same dog. The value of qPCR as an alternative molecular diagnostic technique to detect *B. rossi* parasitaemia in whole blood has been demonstrated and further investigations into using C_q to quantify and estimate actual % *B. rossi* parasitaemia in whole blood should be explored.

Chapter 6: Conclusion

6.1 Problem statement

Light microscopy is the current gold standard modality for *B. rossi* parasite detection and quantification. The process of quantifying parasites by manual count using microscopy is very time-consuming and labour intensive. A clear need exists to find alternative diagnostic tools as well as alternative means to quantify parasite density. An automated method of *B. rossi* parasite detection and quantification would provide more objective results and facilitate higher throughput when investigating *B. rossi* parasitaemia in whole blood or tissue.

6.2 Experimental overview and findings

The similarities between babesiosis and malaria have resulted in collaborative research across both fields that have enabled mutual benefit. Understanding the pathogenesis of *B. rossi* and defining the link between parasitaemia and disease severity are crucial to understanding the parasite-host interaction. *B. rossi* parasite quantification plays an essential role in investigations of its pathogenesis, but the current gold standard manual count by light microscopy is labour intensive. Flow cytometric detection and quantification of *Babesia* have been well documented in species other than *B. rossi.* This study demonstrated the validity of using SYBR Green I as a DNA marker for detecting and quantifying *B. rossi* parasitaemia in fresh whole blood. Comparative analysis in this study demonstrated significant correlations between *B. rossi* diagnostic detection and parasite quantification in venous blood measured by manual count, flow cytometry and qPCR. The well-documented differences between peripheral capillary and central venous parasitaemia may be related to disease pathogenesis. A significant correlation was found between *B. rossi* parasite densities determined by manual count in both capillary and venous whole blood. Furthermore, *B. rossi* parasitaemia in capillary blood detected by manual count showed significant correlations to parasite densities in venous blood measured by flow cytometry and qPCR, which suggested that automated methods of *B. rossi* parasite quantification may be a suitable alternative to the time consuming gold standard light microscopy. The use of qPCR for *B. rossi* parasite detection and quantification proved efficient

in this study and flow cytometry showed promise if problems presented by background fluorescence and reticulocyte detection can be refined.

6.3 Implications, limitations and future perspectives

By having demonstrated the potential of flow cytometry and qPCR as alternative diagnostic tools for *B. rossi* and as a means of parasite quantification, future studies might now rely more heavily on these quicker and more objective automated methods. The feasibility of using flow cytometry or qPCR in different settings merits further investigation as they may have limited usefulness compared to manual counting in the field. The use of flow cytometry and qPCR for diagnosis of *B. rossi* (positive or negative) is impractical in the field. Parasite quantification is however different in that manual methods are labour intensive and open to significant inter-observer variation. For the diagnosis of *B. rossi* light microscopy may still be considered the most efficient tool, because blood smear analysis is fairly sensitive for *B. rossi* detection and not labour intensive. However, in a setting where *B. rossi* quantification is needed then automated methods such as flow cytometry or qPCR may present alternatives that are much more suited to the task and less labour intensive compared to manual counting. The species of *Babesia* investigated may also factor into the choice of modality, where light microscopy used for the detection of a species like *B. vogeli* (which typically presents with a very low parasitaemia) may lack sensitivity to diagnose infection compared to flow cytometry or qPCR which may tilt the scale in favour of using more sensitive, automated diagnostic tools. Evaluation of RBCs using flow cytometry is complex and issues of background fluorescence and reticulocyte detection need to be addressed. SYBR Green I as a single nucleic acid dye proved efficient as a DNA marker for *B. rossi*, but the use of dual- or tri-staining methodologies might facilitate easier differentiation between parasitized and non-parasitized cells and hence provide more reliable results without the need for adjustment. The mathematical correction factor used in this study to address the problem of background showed potential, but is unrefined and influenced by unknowns such as the extent to which *B. rossi* shows a predilection for reticulocyte infection. This study is the first to explore the correlations between manual count, flow cytometry and qPCR for *B. rossi* diagnosis and quantification and more work is needed to investigate the full potential of automated diagnostic tools. The novel use of flow cytometry to investigate *B. rossi* parasite density in this study sets precedence for future studies to exploit the use of this modality in their investigations of *B. rossi*.

Bibliography

1. Matjila PT, Leisewitz AL, Jongejan F, Penzhorn BL. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. Vet Parasitol. 2008;155 1-2:152-7.
2. Bohm M, Leisewitz AL, Thompson PN, Schoeman JP. Capillary and venous *Babesia canis rossi* parasitaemias and their association with outcome of infection and circulatory compromise. Vet Parasitol. 2006;141 1-2:18-29.
3. Payne D. Use and limitations of light microscopy for diagnosing malaria at the primary health care level. Bulletin of the World Health Organization. 1988;66 5:621.
4. O'meara WP, McKenzie FE, Magill AJ, Forney JR, Permpanich B, Lucas C, *et al*. Sources of variability in determining malaria parasite density by microscopy. The American journal of tropical medicine and hygiene. 2005;73 3:593-8.
5. Mosqueda J, Olvera-Ramirez A, Aguilar-Tipacamu G, Canto GJ. Current advances in detection and treatment of babesiosis. Curr Med Chem. 2012;19 10:1504-18.
6. Shapiro HM, Apte SH, Chojnowski GM, Hänscheid T, Rebelo M, Grimberg BT. Cytometry in malaria: a practical replacement for microscopy? Current protocols in cytometry. 2013:11.20. 1-11.20. 3.
7. Yamasaki M, Hwang S-J, Ohta H, Yamato O, Maede Y, Takiguchi M. Flow cytometry to evaluate the level of *Babesia gibsoni* parasitemia *in vivo* and *in vitro* by using the fluorescent nucleic acid stain SYTO16. Japanese Journal of Veterinary Research. 2008;55 4:129-36.
8. Bicalho KA, Ribeiro MF, Martins-Filho OA. Molecular fluorescent approach to assessing intraerythrocytic hemoprotozoan *Babesia canis* infection in dogs. Vet Parasitol. 2004;125 3-4:221-35.
9. Wang C, Ahluwalia SK, Li Y, Gao D, Poudel A, Chowdhury E, *et al*. Frequency and therapy monitoring of canine *Babesia* spp. infection by high-resolution melting curve quantitative FRET-PCR. Vet Parasitol. 2010;168 1-2:11-8.
10. Costa LM, Jr., Zahler-Rinder M, Ribeiro MF, Rembeck K, Rabelo EM, Pfister K, *et al*. Use of a real-time PCR for detecting subspecies of *Babesia canis*. Vet Parasitol. 2012;188 1-2:160-3.

11. Troskie M, De Villiers L, Leisewitz AL, Quan M. Development of a real-time PCR assay for detection and differentiation of *B. rossi* and *B. vogeli* DNA in canine blood. 2018. In preparation.
12. Rossouw I, Maritz-Olivier C, Niemand J, van Biljon R, Smit A, Olivier NA, *et al*. Morphological and molecular descriptors of the developmental cycle of *Babesia divergens* parasites in human erythrocytes. PLoS Negl Trop Dis. 2015;9 5:e0003711.
13. Campo JJ, Aponte JJ, Nhabomba AJ, Sacarlal J, Angulo-Barturen I, Jimenez-Diaz MB, *et al*. Feasibility of flow cytometry for measurements of *Plasmodium falciparum* parasite burden in studies in areas of malaria endemicity by use of bidimensional assessment of YOYO-1 and autofluorescence. J Clin Microbiol. 2011;49 3:968-74.
14. Clark I, Jacobson L. Do babesiosis and malaria share a common disease process? Annals of tropical medicine and parasitology. 1998;92 4:483-8.
15. Reyers F, Leisewitz A, Lobetti R, Milner R, Jacobson L. Canine babesiosis in South Africa: more than one disease? Does this serve as a model for *falciparum* malaria? Annals of Tropical Medicine and Parasitology. 1998;92 4:503-11.
16. Krause PJ, Daily J, Telford SR, Vannier E, Lantos P, Spielman A. Shared features in the pathobiology of babesiosis and malaria. Trends Parasitol. 2007;23 12:605-10.
17. Lyke KE, Diallo DA, Dicko A, Kone A, Coulibaly D, Guindo A, *et al*. Association of intraleukocytic *Plasmodium falciparum* malaria pigment with disease severity, clinical manifestations, and prognosis in severe malaria. The American journal of tropical medicine and hygiene. 2003;69 3:253-9.
18. Cunnington AJ, Walther M, Riley EM. Piecing together the puzzle of severe malaria. Sci Transl Med. 2013;5 211:211ps18.
19. Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature. 2002;415 6872:673-9.
20. Sherman IW, Eda S, Winograd E. Cytoadherence and sequestration in *Plasmodium falciparum*: defining the ties that bind. Microbes and Infection. 2003;5 10:897-909.
21. O'Connor RM, Long JA, Allred DR. Cytoadherence of *Babesia bovis*-infected erythrocytes to bovine brain capillary endothelial cells provides an *in vitro* model for sequestration. Infect Immun. 1999;67 8:3921-8.
22. Gohil S, Kats LM, Sturm A, Cooke BM. Recent insights into alteration of red blood cells by *Babesia bovis*: moovin' forward. Trends Parasitol. 2010;26 12:591-9.

23. Allred DR, Al-Khedery B. Antigenic variation and cytoadhesion in *Babesia bovis* and *Plasmodium falciparum*: different logics achieve the same goal. Molecular and biochemical parasitology. 2004;134 1:27-35.
24. Hutchings CL, Li A, Fernandez KM, Fletcher T, Jackson LA, Molloy JB, *et al*. New insights into the altered adhesive and mechanical properties of red blood cells parasitized by *Babesia bovis*. Mol Microbiol. 2007;65 4:1092-105.
25. Collett MG. Survey of canine babesiosis in South Africa. J S Afr Vet Assoc. 2000;71 3:180-6.
26. Schoeman JP. Canine babesiosis. Onderstepoort J Vet Res. 2009;76 1:59-66.
27. Irwin PJ. Canine babesiosis: from molecular taxonomy to control. Parasite Vectors. 2009;2 Suppl 1:S4.
28. Costa-Junior LM, Rabelo EM, Martins Filho OA, Ribeiro MF. Comparison of different direct diagnostic methods to identify *Babesia bovis* and *Babesia bigemina* in animals vaccinated with live attenuated parasites. Vet Parasitol. 2006;139 1-3:231-6.
29. Irwin PJ. Canine babesiosis. Vet Clin North Am Small Anim Pract. 2010;40 6:1141-56.
30. Penzhorn BL. Why is Southern African canine babesiosis so virulent? An evolutionary perspective. Parasites & vectors. 2011;4 1:1.
31. Matjila PT, Carcy B, Leisewitz AL, Schetters T, Jongejan F, Gorenflot A, *et al*. Preliminary evaluation of the BrEMA1 gene as a tool for associating *Babesia rossi* genotypes and clinical manifestation of canine babesiosis. J Clin Microbiol. 2009;47 11:3586-92.
32. Cunnington AJ, Riley EM, Walther M. Stuck in a rut? Reconsidering the role of parasite sequestration in severe malaria syndromes. Trends Parasitol. 2013;29 12:585-92.
33. Cunnington AJ, Bretscher MT, Nogaro SI, Riley EM, Walther M. Comparison of parasite sequestration in uncomplicated and severe childhood *Plasmodium falciparum* malaria. J Infect. 2013;67 3:220-30.
34. Schnittger L, Rodriguez AE, Florin-Christensen M, Morrison DA. *Babesia*: a world emerging. Infect Genet Evol. 2012;12 8:1788-809.
35. Solano-Gallego L, Baneth G. Babesiosis in dogs and cats: expanding parasitological and clinical spectra. Vet Parasitol. 2011;181 1:48-60.
36. Uilenberg G, Franssen FF, Perie NM, Spanjer AA. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Vet Q. 1989;11 1:33-40.

37. Hauschild S, Shayan P, Schein E. Characterization and comparison of merozoite antigens of different *Babesia canis* isolates by serological and immunological investigations. Parasitology research. 1995;81 8:638-42.
38. Hauschild S, Schein E. The subspecies specificity of *Babesia canis*. Berl Munch Tierarztl Wochenschr. 1996;109 6-7:216-9.
39. Lewis BD, Penzhorn BL, Lopez-Rebollar LM, De Waal DT. Isolation of a South African vector-specific strain of *Babesia canis*. Vet Parasitol. 1996;63 1-2:9-16.
40. Schetters TP, Moubri K, Precigout E, Kleuskens J, Scholtes NC, Gorenflot A. Different *Babesia canis* isolates, different diseases. Parasitology. 1997;115 (Pt 5) 05:485-93.
41. Zahler M, Schein E, Rinder H, Gothe R. Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. Parasitology Research. 1998;84 7:544-8.
42. Uilenberg G. *Babesia*: a historical overview. Vet Parasitol. 2006;138 1-2:3-10.
43. Lack JB, Reichard MV, Van Den Bussche RA. Phylogeny and evolution of the Piroplasmida as inferred from 18S rRNA sequences. International journal for parasitology. 2012;42 4:353-63.
44. Birkenheuer AJ, Neel J, Ruslander D, Levy MG, Breitschwerdt EB. Detection and molecular characterization of a novel large *Babesia* species in a dog. Vet Parasitol. 2004;124 3-4:151-60.
45. Patton WS. Preliminary report on a new piroplasm (*Piroplasma gibsoni* sp. nov.) found in the blood of the hounds of the Madras Hunt and subsequently discovered in the blood of the jackal Canis aureus. Bull Soc Pathol Exot. 1910;3:274-80.
46. Kjemtrup AM, Wainwright K, Miller M, Penzhorn BL, Carreno RA. *Babesia conradae*, sp. Nov., a small canine *Babesia* identified in California. Vet Parasitol. 2006;138 1-2:103-11.
47. Zahler M, Rinder H, Schein E, Gothe R. Detection of a new pathogenic *Babesia microti*-like species in dogs. Vet Parasitol. 2000;89 3:241-8.
48. García ATC. Piroplasma infection in dogs in northern Spain. Veterinary parasitology. 2006;138 1:97-102.
49. Yeagley TJ, Reichard MV, Hempstead JE, Allen KE, Parsons LM, White MA, *et al*. Detection of *Babesia gibsoni* and the canine small *Babesia* 'Spanish isolate' in blood samples obtained from dogs confiscated from dog fighting operations. Journal of the American Veterinary Medical Association. 2009;235 5:535-9.
50. Baneth G, Florin-Christensen M, Cardoso L, Schnittger L. Reclassification of *Theileria annae* as *Babesia vulpes* sp. nov. Parasites & vectors. 2015;8 1:207.

51. Macintire DK, Boudreaux MK, West GD, Bourne C, Wright JC, Conrad PA. *Babesia gibsoni* infection among dogs in the southeastern United States. Journal of the American Veterinary Medical Association. 2002;220 3:325-9.
52. Birkenheuer AJ, Correa MT, Levy MG, Breitschwerdt EB. Geographic distribution of babesiosis among dogs in the United States and association with dog bites: 150 cases (2000-2003). Journal of the American Veterinary Medical Association. 2005;227 6:942-7.
53. Apanaskevich DA, Horak I, Camicas J-L. Redescription of *Haemaphysalis* (Rhipistoma) *elliptica* (Koch, 1844), an old taxon of the *Haemaphysalis* (Rhipistoma) *leachi* group from East and southern Africa, and of *Haemaphysalis* (Rhipistoma) *leachi* (Audouin, 1826) (Ixodida, Ixodidae). Onderstepoort Journal of Veterinary Research. 2007;74 3:181-208.
54. Nuttall GHF. On haematozoa occurring in wild animals in Africa. Parasitology. 1910;3 01:108-16.
55. Horak IG. Ixodid ticks collected at the Faculty of Veterinary Science, Onderstepoort, from dogs diagnosed with *Babesia canis* infection. Journal of the South African Veterinary Association. 1995;66 3:170-1.
56. Oyamada M, Davoust B, Boni M, Dereure J, Bucheton B, Hammad A, *et al*. Detection of *Babesia canis rossi*, *B. canis vogeli*, and *Hepatozoon canis* in dogs in a village of eastern Sudan by using a screening PCR and sequencing methodologies. Clinical and diagnostic laboratory immunology. 2005;12 11:1343-6.
57. Sasaki M, Omobowale O, Tozuka M, Ohta K, Matsuu A, Nottidge HO, *et al*. Molecular survey of *Babesia canis* in dogs in Nigeria. J Vet Med Sci. 2007;69 11:1191-3.
58. Solano-Gallego L, Sainz Á, Roura X, Estrada-Peña A, Miró G. A review of canine babesiosis: the European perspective. Parasites & vectors. 2016;9 1:336.
59. Bourdoiseau G. Canine babesiosis in France. Vet Parasitol. 2006;138 1-2:118-25.
60. Irwin PJ, Jefferies R. Arthropod-transmitted diseases of companion animals in Southeast Asia. Trends Parasitol. 2004;20 1:27-34.
61. Martinod S, Laurent N, Moreau Y. Resistance and immunity of dogs against *Babesia canis* in an endemic area. Vet Parasitol. 1986;19 3-4:245-54.
62. Taboada J, Harvey J, Levy M, Breitschwerdt E. Seroprevalence of babesiosis in Greyhounds in Florida. Journal of the American Veterinary Medical Association. 1992;200 1:47-50.
63. Hornok S, Edelhofer R, Farkas R. Seroprevalence of canine babesiosis in Hungary suggesting breed predisposition. Parasitology research. 2006;99 6:638-42.

64. Mellanby R, Handel I, Clements D, C Bronsvoort B, Lengeling A, Schoeman J. Breed and sex risk factors for canine babesiosis in South Africa. Journal of veterinary internal medicine. 2011;25 5:1186-9.
65. Keller N, Jacobson LS, Nel M, Clerq M, Thompson PN, Schoeman JP. Prevalence and risk factors of hypoglycemia in virulent canine babesiosis. Journal of Veterinary Internal Medicine. 2004;18 3:265-70.
66. Jacobson LS. The South African form of severe and complicated canine babesiosis: clinical advances 1994-2004. Vet Parasitol. 2006;138 1-2:126-39.
67. Solano-Gallego L, Trotta M, Carli E, Carcy B, Caldin M, Furlanello T. *Babesia canis canis* and *Babesia canis vogeli* clinicopathological findings and DNA detection by means of PCR-RFLP in blood from Italian dogs suspected of tick-borne disease. Vet Parasitol. 2008;157 3-4:211-21.
68. Schetters TP, Kleuskens J, Scholtes N, Pasman J, Bos H. Vaccination of dogs against *Babesia canis* infection using antigens from culture supernatants with emphasis on clinical babesiosis. Veterinary parasitology. 1994;52 3:219-33.
69. Schetters T. Vaccination against canine babesiosis. Trends in Parasitology. 2005;21 4:179-84.
70. Schetters TP, Strydom T, Crafford D, Kleuskens JA, van de Crommert J, Vermeulen AN. Immunity against *Babesia rossi* infection in dogs vaccinated with antigens from culture supernatants. Vet Parasitol. 2007;144 1-2:10-9.
71. Schetters T, Moubri K, Cooke B. Comparison of *Babesia rossi* and *Babesia canis* isolates with emphasis on effects of vaccination with soluble parasite antigens: a review. Journal of the South African Veterinary Association. 2009;80 2:75-8.
72. Moubri K, Kleuskens J, Van de Crommert J, Scholtes N, Van Kasteren T, Delbecq S, *et al*. Discovery of a recombinant *Babesia canis* supernatant antigen that protects dogs against virulent challenge infection. Veterinary Parasitology. 2018;249:21-9.
73. Shakespeare AS. The incidence of canine babesiosis amongst sick dogs presented to the Onderstepoort Veterinary Academic Hospital. J S Afr Vet Assoc. 1995;66 4:247-50.
74. Matjila PT, Penzhorn BL, Bekker CP, Nijhof AM, Jongejan F. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Vet Parasitol. 2004;122 2:119-25.
75. Lounsbury CP. Transmission of malignant jaundice of the dog by a species of tick. Agric J Cape Good Hope. 1901;19:714-24.

76. Lounsbury CP. Ticks and malignant jaundice of the dog. Journal of Comparative Pathology and Therapeutics. 1904;17:113-29.
77. Penzhorn BL, Vorster I, Harrison-White RF, Oosthuizen MC. Black-backed jackals (*Canis mesomelas*) are natural hosts of *Babesia rossi*, the virulent causative agent of canine babesiosis in sub-Saharan Africa. Parasites & vectors. 2017;10 1:124.
78. Matjila PT, Leisewitz AL, Jongejan F, Bertschinger HJ, Penzhorn BL. Molecular detection of *Babesia rossi* and *Hepatozoon* sp. in African wild dogs (*Lycaon pictus*) in South Africa. Vet Parasitol. 2008;157 1-2:123-7.
79. Köster LS, Lobetti R, Kelly P. Canine babesiosis: a perspective on clinical complications, biomarkers, and treatment. Journal of Veterinary Medicine: Research and Reports. 2015;6:119-28.
80. Scheepers E, Leisewitz AL, Thompson PN, Christopher M. Serial haematology results in transfused and non-transfused dogs naturally infected with *Babesia rossi*. Journal of the South African Veterinary Association. 2011;82 3:136-43.
81. Jacobson L. Cerebellar ataxia as a possible complication of babesiosis in two dogs. Journal of the South African Veterinary Association. 1994;65 3:130.
82. Jacobson LS, Clark IA. The pathophysiology of canine babesiosis: new approaches to an old puzzle. J S Afr Vet Assoc. 1994;65 3:134-45.
83. Malherbe W. Clinico-pathological studies of *Babesia canis* infection in dogs. V. The influence of the infection on kidney function. Journal of the South African Veterinary Association. 1966;37 3:261-4.
84. Lobetti R, Jacobson L. Renal involvement in dogs with babesiosis. Journal of the South African Veterinary Association. 2001;72 1:23-8.
85. Defauw P, Schoeman J, Smets P, Goddard A, Meyer E, Liebenberg C, *et al*. Assessment of renal dysfunction using urinary markers in canine babesiosis caused by *Babesia rossi*. Veterinary parasitology. 2012;190 3:326-32.
86. 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 4:471-6.
87. Welzl C, Leisewitz A, Jacobson L, 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 3:158-62.

88. Mohr A, Lobetti R, Van der Lugt J. Acute pancreatitis: a newly recognised potential complication of canine babesiosis. Journal of the South African Veterinary Association. 2000;71 4:232-9.
89. Dvir E, Lobetti RG, Jacobson LS, Pearson J, Becker PJ. Electrocardiographic changes and cardiac pathology in canine babesiosis. Journal of Veterinary Cardiology. 2004;6 1:15-23.
90. Lobetti R. Cardiac involvement in canine babesiosis. Journal of the South African Veterinary Association. 2005;76 1:4-8.
91. Jacobson L, Lobetti R. Rhabdomyolysis as a complication of canine babesiosis. Journal of small animal practice. 1996;37 6:286-91.
92. Lobetti RG. Canine babesiosis. The Compendium on continuing education for the practicing veterinarian (USA). 1998.
93. 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;196 2:213-7.
94. Vaughan-Scott T: Serum concentrations of tumour necrosis factor in dogs naturally infected with Babesia canis and its relation to severity of disease. 2001.
95. Köster LS, Van Schoor M, Goddard A, Thompson P, Matjila P, Kjelgaard-Hansen M. C-reactive protein in canine babesiosis caused by *Babesia rossi* and its association with outcome. Journal of the South African Veterinary Association. 2009;80 2:87-91.
96. Leisewitz AL, Jacobson LS, Morais HS, Reyers F. The mixed acid-base disturbances of severe canine babesiosis. Journal of Veterinary Internal Medicine. 2001;15 5:445-52.
97. Schoeman JP, Rees P, Herrtage ME. Endocrine predictors of mortality in canine babesiosis caused by *Babesia canis rossi*. Veterinary parasitology. 2007;148 2:75-82.
98. Lobetti R, Dvir E, Pearson J. Cardiac troponins in canine babesiosis. Journal of Veterinary Internal Medicine. 2002;16 1:63-8.
99. Lobetti R, Kirberger R, Keller N, Kettner F, Dvir E. NT-ProBNP and cardiac troponin I in virulent canine babesiosis. Veterinary parasitology. 2012;190 3:333-9.
100. Malherbe WD. The manifestations and diagnosis of *Babesia* infections. Annals of the New York Academy of Sciences. 1956;64 2:128-46.
101. Maegraith B, Gilles HM, Devakul K. Pathological processes in *Babesia cam's* infections. Zeitschrift fur Tropenmedizin und Parasitologie. 1957;8 4:485-514.
102. Schetters TP, Eling W. Can *Babesia* infections be used as a model for cerebral malaria? Parasitology today. 1999;15 12:492-7.

103. Cooke BM, Mohandas N, Cowman AF, Coppel RL. Cellular adhesive phenomena in apicomplexan parasites of red blood cells. Vet Parasitol. 2005;132 3-4:273-95.
104. Berendt AR, Ferguson DJ, Newbold CI. Sequestration in *Plasmodium falciparum* malaria: sticky cells and sticky problems. Parasitol Today. 1990;6 8:247-54.
105. Aikawa M, Miller L: Structural alteration of the erythrocyte membrane during malarial parasite invasion and intraerythrocytic development. In: *Ciba Foundation symposium 1983*: 45.
106. Gruenberg J, Allred DR, Sherman IW. Scanning electron microscope-analysis of the protrusions (knobs) present on the surface of *Plasmodium falciparum*-infected erythrocytes. J Cell Biol. 1983;97 3:795-802.
107. Raventos-Suarez C, Kaul DK, Macaluso F, Nagel RL. Membrane knobs are required for the microcirculatory obstruction induced by *Plasmodium falciparum*-infected erythrocytes. Proc Natl Acad Sci U S A. 1985;82 11:3829-33.
108. Crabb BS, Cooke BM, Reeder JC, Waller RF, Caruana SR, Davern KM, *et al*. Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. Cell. 1997;89 2:287-96.
109. Aikawa M, Rabbege J, Uni S, Ristic M, Miller LH. Structural alteration of the membrane of erythrocytes infected with *Babesia bovis*. The American journal of tropical medicine and hygiene. 1985;34 1:45-9.
110. Wright IG. An electron microscopic study of intravascular agglutination in the cerebral cortex due to *Babesia argentina* infection. International journal for parasitology. 1972;2 2:209-10.
111. Wright IG, Goodger BV. Acute *Babesia bovis* infections: renal involvement in the hypotensive syndrome. Zeitschrift für Parasitenkunde. 1979;59 2:115-9.
112. Aikawa M, Pongponratn E, Tegoshi T, Nakamura K, Nagatake T, Cochrane A, *et al*. A study on the pathogenesis of human cerebral malaria and cerebral babesiosis. Mem Inst Oswaldo Cruz. 1992;87 Suppl 3:297-301.
113. Genton B, D'Acremont V, Rare L, Baea K, Reeder JC, Alpers MP, *et al*. *Plasmodium vivax* and mixed infections are associated with severe malaria in children: a prospective cohort study from Papua New Guinea. PLoS medicine. 2008;5 6:e127.
114. Beeson JG, Brown GV. Pathogenesis of *Plasmodium falciparum* malaria: the roles of parasite adhesion and antigenic variation. Cell Mol Life Sci. 2002;59 2:258-71.
115. Smith JD, Gamain B, Baruch DI, Kyes S. Decoding the language of *var* genes and *Plasmodium falciparum* sequestration. Trends in parasitology. 2001;17 11:538-45.

116. Allred DR. Immune evasion by *Babesia bovis* and *Plasmodium falciparum*: cliff-dwellers of the parasite world. Parasitol Today. 1995;11 3:100-5.
117. Newbold C, Craig A, Kyes S, Rowe A, Fernandez-Reyes D, Fagan T. Cytoadherence, pathogenesis and the infected red cell surface in *Plasmodium falciparum*. Int J Parasitol. 1999;29 6:927-37.
118. Leech J, Barnwell J, Miller L, Howard R. Identification of a strain-specific malarial antigen exposed on the surface of *Plasmodium falciparum*-infected erythrocytes. Journal of experimental medicine. 1984.
119. Su X-z, Heatwole VM, Wertheimer SP, Guinet F, Herrfeldt JA, Peterson DS, *et al*. The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. Cell. 1995;82 1:89-100.
120. del Portillo HA, Lanzer M, Rodriguez-Malaga S, Zavala F, Fernandez-Becerra C. Variant genes and the spleen in *Plasmodium vivax* malaria. International journal for parasitology. 2004;34 13:1547-54.
121. Anstey NM, Handojo T, Pain MC, Kenangalem E, Tjitra E, Price RN, *et al*. Lung injury in *vivax* malaria: pathophysiological evidence for pulmonary vascular sequestration and post-treatment alveolar-capillary inflammation. The Journal of infectious diseases. 2007;195 4:589-96.
122. Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, Alonso PL, *et al*. Key gaps in the knowledge of *Plasmodium vivax*, a neglected human malaria parasite. The Lancet infectious diseases. 2009;9 9:555-66.
123. Wright I, Goodger B, Clark I. Immunopathophysiology of *Babesia bovis* and *Plasmodium falciparum* infections. Parasitology Today. 1988;4 8:214-8.
124. Parrodi F, Wright I, Kerr J, Dobson C. *In vitro* adherence of erythrocytes infected with *Babesia bigemina* and *Babesia rodhainito* thrombospondin. International journal for parasitology. 1990;20 7:899-903.
125. Al-Khedery B, Allred DR. Antigenic variation in *Babesia bovis* occurs through segmental gene conversion of the *ves* multigene family, within a bidirectional locus of active transcription. Molecular microbiology. 2006;59 2:402-14.
126. O'Connor RM, Allred DR. Selection of *Babesia bovis*-infected erythrocytes for adhesion to endothelial cells coselects for altered variant erythrocyte surface antigen isoforms. The Journal of Immunology. 2000;164 4:2037-45.

127. Sondgeroth KS, McElwain TF, Allen AJ, Chen AV, Lau AO. Loss of neurovirulence is associated with reduction of cerebral capillary sequestration during acute *Babesia bovis* infection. Parasites & vectors. 2013;6 1:181.
128. Purchase H. Cerebral babesiosis in dogs. The Veterinary Record. 1947;59 20:269.
129. Botha H. The cerebral form of babesiosis in dogs. Journal of the South African Veterinary Association. 1964;35 1:27-8.
130. Tavares R, Staggemeier R, Borges A, Rodrigues M, Castelan L, Vasconcelos J, *et al*. Molecular techniques for the study and diagnosis of parasite infection. Journal of Venomous Animals and Toxins including Tropical Diseases. 2011;17 3:239-48.
131. Jacobson LS, Reyers F, Berry W, Viljoen E. Changes in haematocrit after treatment of uncomplicated canine babesiosis: a comparison between diminazene and trypan blue, and an evaluation of the influence of parasitaemia. Journal of the South African Veterinary Association. 1996;67 2:77-82.
132. Moody A. Rapid diagnostic tests for malaria parasites. Clin Microbiol Rev. 2002;15 1:66-78.
133. Bose R, Jorgensen WK, Dalgliesh RJ, Friedhoff KT, de Vos AJ. Current state and future trends in the diagnosis of babesiosis. Vet Parasitol. 1995;57 1-3:61-74.
134. Izumiyama S, Omura M, Takasaki T, Ohmae H, Asahi H. *Plasmodium falciparum*: development and validation of a measure of intraerythrocytic growth using SYBR Green I in a flow cytometer. Experimental parasitology. 2009;121 2:144-50.
135. Malleret B, Claser C, Ong AS, Suwanarusk R, Sriprawat K, Howland SW, *et al*. A rapid and robust tri-color flow cytometry assay for monitoring malaria parasite development. Sci Rep. 2011;1:118.
136. Roobsoong W, Maher SP, Rachaphaew N, Barnes SJ, Williamson KC, Sattabongkot J, *et al*. A rapid sensitive, flow cytometry-based method for the detection of *Plasmodium vivax*-infected blood cells. Malar J. 2014;13 1:55.
137. Carli E, Tasca S, Trotta M, Furlanello T, Caldin M, Solano-Gallego L. Detection of erythrocyte binding IgM and IgG by flow cytometry in sick dogs with *Babesia canis canis* or *Babesia canis vogeli* infection. Vet Parasitol. 2009;162 1-2:51-7.
138. Radcliff G, Jaroszeski MJ. Basics of flow cytometry. Flow Cytometry Protocols. 1998:1-24.
139. Grimberg BT. Methodology and application of flow cytometry for investigation of human malaria parasites. J Immunol Methods. 2011;367 1-2:1-16.

140. Qurollo BA, Archer NR, Schreeg ME, Marr HS, Birkenheuer AJ, Haney KN, *et al*. Improved molecular detection of *Babesia* infections in animals using a novel quantitative real-time PCR diagnostic assay targeting mitochondrial DNA. Parasites & vectors. 2017;10 1:128.
141. Schneider P, Wolters L, Schoone G, Schallig H, Sillekens P, Hermsen R, *et al*. Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium falciparum*. J Clin Microbiol. 2005;43 1:402-5.
142. Hwang SY, Kim SH, Lee GY, Hang VT, Moon CS, Shin JH, *et al*. A novel real-time PCR assay for the detection of *Plasmodium falciparum* and *Plasmodium vivax* malaria in low parasitized individuals. Acta Trop. 2011;120 1-2:40-5.
143. Osoga J, Waitumbi J, Guyah B, Sande J, Arima C, Ayaya M, *et al*. Comparative evaluation of fluorescent *in situ* hybridization and Giemsa microscopy with quantitative real-time PCR technique in detecting malaria parasites in a holoendemic region of Kenya. Malaria journal. 2017;16 1:297.
144. Gama BE, Silva-Pires Fdo E, Lopes MN, Cardoso MA, Britto C, Torres KL, *et al*. Real-time PCR versus conventional PCR for malaria parasite detection in low-grade parasitemia. Exp Parasitol. 2007;116 4:427-32.
145. Yisaschar-Mekuzas Y, Jaffe CL, Pastor J, Cardoso L, Baneth G. Identification of *Babesia* species infecting dogs using reverse line blot hybridization for six canine piroplasms, and evaluation of co-infection by other vector-borne pathogens. Veterinary parasitology. 2013;191 3:367-73.
146. Van Heerden J, Reyers F, Stewart CG. Treatment and thrombocyte levels in experimentally induced canine ehrlichiosis and canine babesiosis. Onderstepoort J Vet Res. 1983;50 4:267-70.
147. Lewis BD, Penzhorn BL, Lopez Rebollar LM. Immune responses to South African *Babesia canis* and the development of a preliminary vaccine. J S Afr Vet Assoc. 1995;66 2:61-5.
148. Jacobson LS, Reyers F, Berry W. Comparison of the effects of diminazene and trypan blue in canine babesiosis. Article abstract. 1994.
149. Pardini AD: The pathology and pathogenesis of canine cerebral babesiosis. 2000.
150. Mendis K, Carter R. Clinical disease and pathogenesis in malaria. Parasitology Today. 1995;11 5:PTI1-PTI16.

151. Callow LL, Pepper PM. Measurement of and correlations between fever, changes in the packed cell volume and parasitaemia in the evaluation of the susceptibility of cattle to infection with *Babesia argentina*. Australian Veterinary Journal. 1974;50 1:1-5.
152. Njunda A, Assob N, Nsagha S, Kamga F, Mokenyu M, Kwenti E. Comparison of capillary and venous blood using blood film microscopy in the detection of malaria parasites: A hospital based study. Scientific Journal of Microbiology. 2013;2 5:89-94.
153. Ouedraogo JB, Guiguemde TR, Gbary AR. Etude comparative de la densite parasitairede *Plasmodium falciparum* dans le sang capillaire et dans le sang veineux chez des porteurs asymptomatiques (Région de Bobo-Dioulasso, Burkina Faso). Médecine d'Afrique Noire. 1991;38 8/9.
154. Ewing SA. Evaluation of methods used to detect *Babesia canis* infections in dogs. The Cornell veterinarian. 1966;56 2:211-20.
155. Sandeu MM, Bayibéki AN, Tchioffo MT, Abate L, Gimonneau G, Awono-Ambéné PH, *et al*. Do the venous blood samples replicate malaria parasite densities found in capillary blood? A field study performed in naturally-infected asymptomatic children in Cameroon. Malaria Journal. 2017;16 1:345.
156. Jiménez-Díaz MB, Rullas J, Mulet T, Fernández L, Bravo C, Gargallo-Viola D, *et al*. Improvement of detection specificity of *Plasmodium*-infected murine erythrocytes by flow cytometry using autofluorescence and YOYO-1. Cytometry Part A. 2005;67 1:27-36.
157. Wongchotigul V, Suwanna N, Krudsood S, Chindanond D, Kano S, Hanaoka N, *et al*. The use of flow cytometry as a diagnostic test for malaria parasites. South-east Asian J Trop Med Public Health. 2004.
158. Borggraefe I, Yuan J, Telford SR, Menon S, Hunter R, Shah S, *et al*. *Babesia microti* primarily invades mature erythrocytes in mice. Infection and immunity. 2006;74 6:3204-12.
159. McQueen PG, McKenzie FE. Age-structured red blood cell susceptibility and the dynamics of malaria infections. Proceedings of the National Academy of Sciences of the United States of America. 2004;101 24:9161-6.
160. Cho JS, Russell B, Kosasaivee V, Zhang R, Colin Y, Bertrand O, *et al*. Unambiguous determination of *Plasmodium vivax* reticulocyte invasion by flow cytometry. Int J Parasitol. 2016;46 1:31-9.
161. Malleret B, Rénia L, Russell B. The unhealthy attraction of *Plasmodium vivax* to reticulocytes expressing transferrin receptor 1 (CD71). International Journal for Parasitology. 2017.

162. Uemura T, Suzuki S, Ohnishi T. Flow cytometric enumeration of reticulocyte in the peripheral blood from canine infected with *Babesia gibsoni*. Zentralbl Veterinarmed B. 1990;37 6:468-72.

163. Yamasaki M, Otsuka Y, Yamato O, Tajima M, Maede Y. The cause of the predilection of *Babesia gibsoni* for reticulocytes. The Journal of veterinary medical science. 2000;62 7:737-41.

164. Theron M, Hesketh RL, Subramanian S, Rayner JC. An adaptable two-color flow cytometric assay to quantitate the invasion of erythrocytes by *Plasmodium falciparum* parasites. Cytometry Part A. 2010;77 11:1067-74.

165. Bennett TN, Paguio M, Gligorijevic B, Seudieu C, Kosar AD, Davidson E, *et al*. Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. Antimicrobial agents and chemotherapy. 2004;48 5:1807-10.

166. Bei AK, DeSimone TM, Badiane AS, Ahouidi AD, Dieye T, Ndiaye D, *et al*. A flow cytometry-based assay for measuring invasion of red blood cells by *Plasmodium falciparum*. American journal of hematology. 2010;85 4:234-7.

167. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrobial agents and chemotherapy. 2004;48 5:1803-6.

168. Briggs C, Jones M. SYBR Green I-induced fluorescence in cultured immune cells: A comparison with Acridine Orange. Acta histochemica. 2005;107 4:301-12.

169. Philipp S, Oberg HH, Janssen O, Leippe M, Gelhaus C. Isolation of erythrocytes infected with viable early stages of *Plasmodium falciparum* by flow cytometry. Cytometry Part A. 2012;81 12:1048-54.

170. Rebelo M, Shapiro HM, Amaral T, Melo-Cristino J, Hänscheid T. Haemozoin detection in infected erythrocytes for *Plasmodium falciparum* malaria diagnosis: Prospects and limitations. Acta tropica. 2012;123 1:58-61.

171. Johnson JD, Dennull RA, Gerena L, Lopez-Sanchez M, Roncal NE, Waters NC. Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. Antimicrobial agents and chemotherapy. 2007;51 6:1926-33.

172. Karl S, Wong RP, St Pierre TG, Davis TM. A comparative study of a flow-cytometry-based assessment of *in vitro Plasmodium falciparum* drug sensitivity. Malaria journal. 2009;8 1:294.

173. Ekland EH, Schneider J, Fidock DA. Identifying apicoplast-targeting antimalarials using high-throughput compatible approaches. The FASEB Journal. 2011;25 10:3583-93.
174. Rautenbach Y, Goddard A, Thompson PN, Mellanby RJ, Leisewitz AL. A flow cytometric assessment of the lymphocyte immunophenotypes in dogs naturally infected with *Babesia rossi*. Veterinary Parasitology. 2017;241:26-34.
175. Goddard A, Leisewitz AL, Kristensen AT, Schoeman JP. Platelet activation and platelet-leukocyte interaction in dogs naturally infected with *Babesia rossi*. The Veterinary Journal. 2015;205 3:387-92.
176. Kaltenboeck B, Wang C. Advances in real-time PCR: application to clinical laboratory diagnostics. Adv Clin Chem. 2005;40 4:219-59.
177. Loftis AD, Reeves WK. Principles of real-time PCR. Veterinary PCR Diagnostics. 2012:3.
178. Duarte SC, Linhares GF, Romanowsky TN, da Silveira Neto OJ, Borges LM. Assessment of primers designed for the subspecies-specific discrimination among *Babesia canis canis*, *Babesia canis vogeli* and *Babesia canis rossi* by PCR assay. Vet Parasitol. 2008;152 1-2:16-20.
179. Birkenheuer AJ, Levy MG, Breitschwerdt EB. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. J Clin Microbiol. 2003;41 9:4172-7.
180. Annoscia G, Latrofa MS, Cantacessi C, Olivieri E, Manfredi MT, Dantas-Torres F, *et al*. A new PCR assay for the detection and differentiation of *Babesia canis* and *Babesia vogeli*. Ticks and Tick-borne Diseases. 2017.
181. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, *et al*. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55 4:611-22.
182. Matsuu A, Ono S, Ikadai H, Uchide T, Imamura S, Onuma M, *et al*. Development of a SYBR green real-time polymerase chain reaction assay for quantitative detection of *Babesia gibsoni* (Asian genotype) DNA. J Vet Diagn Invest. 2005;17 6:569-73.
183. Kim C, Iseki H, Herbas MS, Yokoyama N, Suzuki H, Xuan X, *et al*. Development of TaqMan-based real-time PCR assays for diagnostic detection of *Babesia bovis* and *Babesia bigemina*. The American journal of tropical medicine and hygiene. 2007;77 5:837-41.

184. Peleg O, Baneth G, Eyal O, Inbar J, Harrus S. Use of chimeric DNA-RNA primers in quantitative PCR for detection of *Ehrlichia canis* and *Babesia canis*. Appl Environ Microbiol. 2009;75 19:6393-8.

185. Bhoora R, Quan M, Franssen L, Butler CM, van der Kolk JH, Guthrie AJ, *et al*. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. Vet Parasitol. 2010;168 3-4:201-11.

186. Peleg O, Baneth G, Eyal O, Inbar J, Harrus S. Multiplex real-time qPCR for the detection of *Ehrlichia canis* and *Babesia canis vogeli*. Vet Parasitol. 2010;173 3-4:292-9;.

187. Teal AE, Habura A, Ennis J, Keithly JS, Madison-Antenucci S. A new real-time PCR assay for improved detection of the parasite *Babesia microti*. J Clin Microbiol. 2012;50 3:903-8.

188. Ohmori S, Kawai A, Takada N, Saito-Ito A. Development of real-time PCR assay for differential detection and quantification for multiple *Babesia microti*-genotypes. Parasitol Int. 2011;60 4:403-9.

189. Adaszek L, Winiarczyk S. Application of the SYBR Green real-time HRM PCR technique in the differentiation of the *Babesia canis canis* protozoa isolated in the areas of eastern Poland. Parasitol Res. 2010;106 5:1253-6.

190. Altman DG. Practical statistics for medical research. London: Chapman and Hall; 1991.

Appendix A: Declaration of originality/plagiarism

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

UNIVERSITY OF PRETORIA

FACULTY OF VETERINARY SCIENCE

DECLARATION OF ORIGINALITY

This document must be signed and submitted with every essay, report, project, assignment, mini-dissertation, dissertation and/or thesis

Full names of student: **Dr Lourens de Villiers**

Student number: **29256594**

Declaration:

1. I understand what plagiarism is and am aware of the University's policy in this regard.
2. I declare that this **dissertation** is my own original work. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.
3. I have not used work previously produced by another student or any other person to hand in as my own.
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

Signature of student: _____

Signature of supervisor: _____

Appendix B: Ethical clearance certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**A comparison between manual count, flow cytometry and q PCR as a means of determining *B.rossi* parasitaemia in naturally infected dogs**
PROJECT NUMBER	**V060-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. L de Villiers**

STUDENT NUMBER (where applicable)	**UP_29256594**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Canine**	
NUMBER OF ANIMALS	**50-60**	
Approval period to use animals for research/testing purposes		**June 2016 –June 2017**
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 27 June 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

S*4285-15*

Appendix C: Research committee clearance certificate

Ref: V060-16

5 September 2016

University of Pretoria

Faculty of Veterinary Science
Private Bag X04
Onderstepoort
0110

Tel: +27 12 529 8434
Fax: +27 12 529 8300

Prof. A Leisewitz
Department Companion Animal Studies
(Andrew.leisewitz@up.ac.z)

Dear Prof. Leisewitz

PROTOCOL V060-16 : A comparison between manual count, flow cytometry and q PCR as a means of determining *B.rossi* parasitaemia in naturally infected dogs. (L de Villiers)

I am pleased to inform you that the abovementioned protocol was registered by the Research Committee.

Kind regards

Prof. V Naidoo
RESEARCH COMMITTEE

Copy: Prof J Schoeman, HOD (johan.schoeman@up.ac.za)
Dr L de Villiers, Researcher (DeVilliersL@arc.agric.za)

Appendix D: Data capture sheet

DEPARTMENT OF COMPANION ANIMAL CLINICAL STUDIES
FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Project title: A comparison between manual count, flow cytometry and qPCR as a means of determining *B. rossi* parasitaemia in naturally infected dogs.

(Please encircle where applicable; Grey blocks ☐ are MANDATORY to complete)

PATIENT STICKER / OR COMPLETE DETAILS	**Date:**
Patient name:	**Laboratory number:**
Patient number:	**ClinPath number:**
Species: Canine **Sex: M / F Age:**	**Video/photo collected:**
Breed:	**Name of Student/Vet/Nurse:**
Weight: kg	**Clinician:** Prof A.L. Leisewitz
Owner name:	**Clinician initials (MANDATORY):**
Owner number:	

Provisional Diagnosis: Babesiosis / Healthy (encircle)
Suspected concurrent disease (if any, specify):
History
Chief complaint:
Duration of illness:
Has the dog had *Babesia* before: Y / N (encircle)
Has the dog been treated for *Babesia* before: Y / N (encircle) **If yes, when last:**
Does the client know what *Babesia* is? Y / N (encircle)
Does the client apply tick control to the patient: Y / N (encircle) **If yes, when last?**
Has the patient been splenectomised? Y / N (encircle)
When last did the dog eat:
Clinical examination
Habitus: Collapsed (i.e. unable to stand)**: Y / N** (encircle)
Clinically detectable dehydration (i.e. sunken eyes, decreased skin fold turgidity, dry oral mucosa)**: Y / N** (encircle)
TPR: T: P: R:
Mucous membranes: Pink / Pale / Cyanotic (encircle) **CRT: sec Pulse: Weak / Strong** (encircle)
Appetite:
Peripheral lymph nodes:
Respiratory:
Cardiovascular:
Abdominal/GIT:
Urinary:
Musculo-skeletal:
Mandatory sampling
Blood smear: Left ear Y / N (encircle) **Right ear Y / N** (encircle) ***Babesia* parasites seen Y / N** (encircle)
Venous blood sample (EDTA): Jugular Y / N (encircle) **Cephalic Y / N** (encircle)
Was the bleeding easy? Y / N (encircle) **Was the animal excited before or during sampling? Y / N** (encircle)
Approximate ml collected: ml (minimum 3ml)
Optional in-house tests
Faecal examination: Y / N (encircle) **Colour: Consistency: Ova count:**
Hct and ISA: Hct: ISA:
MAP (Oscillometric, 3 readings): 1. 2. 3.
Initial drugs used to treat the patient:

Researcher: Dr Lourens de Villiers

Appendix E: Project information sheet

DEPARTMENT OF COMPANION ANIMAL CLINICAL STUDIES
FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Project title: A comparison between manual count, flow cytometry and qPCR as a means of determining *B. rossi* parasitaemia in naturally infected dogs.

Project information sheet - Babesiosis

Biliary fever (Babesiosis) / Bosluiskoors (Tick bite fever)

Biliary is a very common disease in the Onderstepoort area accounting for up to 12% of cases in the OVAH each year. Although complications can occur, the disease can easily be treated in the early phases and prevented with proper tick control practices. Biliary fever is caused by the parasite *(Babesia canis)* which is carried by ticks. The parasite infects the dog by means of a tick bite and enters the bloodstream through the tick's saliva. The parasite infects the red blood cells of the dog. Signs of disease will become apparent from 7-21 days after the tick bite. Symptoms of disease are caused by a generalized inflammation and anaemia and can range from mild to fatal. A similar disease in humans is mosquito-borne malaria.

The parasite causes a reaction in the body and the body's' defence mechanisms act to try to eradicate the parasite from the bloodstream. This results in fever and some of the complications. The parasite also directly causes the red blood cells to break up and this could cause the urine to become red when excessive amounts are damaged. The anaemia caused by biliary can be very severe. Complications which can also occur due to the infection include: acute renal failure, acute respiratory distress syndrome (fluid on the lungs), blood clotting abnormalities and liver damage. These patients usually require intensive medical care and carry a poorer prognosis for recovery.

The disease is managed by killing the parasite, giving a blood transfusion if the anaemia is severe and medically managing any complications which may be evident. Early symptoms of infection include lethargy, anorexia, and fever. Diagnosis of infection is easily made on a blood smear and the patient can be treated with a single injection. This injection can harm the patient if the dose is incorrect. It is not advised that you wait for the gums to become pale before considering biliary, as once the patient is anaemic a blood transfusion is often required.

Tick control is essential to prevent the disease. There is no vaccination currently available in SA. Ticks are mainly a problem in the summer months, but if the winter is not very cold they can persist throughout winter. Many tick control products are available. Dips are effective, but require frequent application every 2 weeks and are toxic if overdosed. Gloves must be worn and instructions carefully followed. Most dips, with the exception of Acarins®, are not suitable for puppies under 6 months.
"Spot-on" type products and sprays are also available, and are more efficient, requiring application only every 4-6 weeks. The dog must not be bathed 4 days before or after dipping to allow proper dispersal of the agent. These agents are usually non-toxic and can be used on young dogs.

The Animal Use and Care Committee of the University of Pretoria has approved this study.

Information sheet compiled by:
Prof Andrew Leisewitz
Department of Companion Animal Clinical Studies
Faculty of Veterinary Science, University of Pretoria, Onderstepoort, 0110
Tel: 012 529 8262

Researcher: Dr Lourens de Villiers

Appendix F: Owner consent form

DEPARTMENT OF COMPANION ANIMAL CLINICAL STUDIES
FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Project title: A comparison between manual count, flow cytometry and qPCR as a means of determining *B. rossi* parasitaemia in naturally infected dogs.

Informed Consent

(To be completed by the patient's owner / authorized agent)

Please encircle Yes or No where applicable

1. Have you received the information sheet on canine babesiosis/distemper?
   Yes No
2. Have you had the opportunity to ask questions about the research project?
   Yes No
3. Have you received satisfactory answers to your questions?
   Yes No
4. Have you received enough information about this study?
   Yes No
5. Supply the name of the person to whom you have spoken to:

   .....
6. Do you grant consent that blood samples can be collected from your dog and utilized for research purposes?
   Yes No

I, …….. **(owner name, surname)**, hereby give permission that my dog …….. **(patient name)**, a …….. **(patient breed)**, may participate in this clinical study.

I understand that this study will in no way harm my dog. Furthermore, I understand that the costs of the diagnostic tests conducted for the study will be borne by the trial fund, and that I will only be liable for costs pertaining to the diagnosis and treatment that would in any event be required for my dog, including any complications that may arise as a result of canine babesiosis.

Signed at …….. on the …….. day of …….. 20.....
Signature Owner/Agent ……..
Home Tel: ……..
Work Tel: ……
Cell No: ……

Researcher: Dr Lourens de Villiers

Appendix G: Flow cytometry optimization

G.1 Objective and hypothesis

The objective was to develop and optimize a flow cytometric protocol for analysing *B. rossi*-infected whole blood using a SYBR Green I nucleic acid dye in order to quantify parasitaemia. We hypothesized that if a red cell zone gate could be established that included only RBC events it would enable estimation of parasitaemia through quantification of pRBCs taken as a percentage of the total number of RBC events recorded in the gate.

G.2 Methodology

The flow cytometric quantification of *B. rossi* in this study was based on a method previously described for *B. divergens* [12] which served as a good starting point to develop a novel staining protocol for *B. rossi* using SYBR Green I. The parameters in Rossouw's study for an optimal staining procedure to determine *B. divergens* parasitaemia were reported as follows: 50 µL unfixed *B. divergens* sample preparation was mixed with 20 µL 1:100 SYBR Green I:PBS solution and stained at an incubation temperature of 37 ℃ for 30 min in the dark; two washing steps were included prior to staining; and ten thousand gated events were recorded and plotted on a forward versus side scatter density plot.

The SYBR Green I functioned as a nuclear marker to stain the DNA of *B. rossi* pRBC. Being a dye that stains primarily DNA, but also RNA, all nucleated cells and more than likely, all reticulocytes would also stain. All handling of and staining with SYBR Green I was conducted in a darkened environment due to the light sensitivity of SYBR Green I and the associated risks of photobleaching. Optical filters detected the SYBR Green I dye in the FL-1 channel. Variations of FL-1 against FL-2, FL-3, FL-4, SSC and FSC were plotted in an attempt to determine which filters gave the best event distribution during optimization. Various parameters were investigated and adjusted during the optimization process to determine the optimal staining procedure for *B. rossi*. Parameters that were investigated include: staining at various dilutions of SYBR Green I, namely 1:2000, 1:1500, 1:1000, 1:500, 1:100, 1:50 and 1:10; staining at a variety of input

volumes of SYBR Green I, namely 20 µL, 10 µL and 5 µL; running samples unfixed or fixed with 0.025 % glutaraldehyde working stock (2.5 % glutaraldehyde concentration diluted at a 1:100 ratio with PBS) for 45 min at 4 ℃ prior to staining; staining with SYBR Green I at 37 ℃ and room temperature (23 ℃) respectively; centrifugation of samples at 5000 rcf and 450 rcf respectively; and minimizing the amount of washing steps pre-staining by increasing the amount of PBS used per wash, as well as including a washing step post-staining. Optimization was primarily conducted on healthy whole blood, but also on WBC-enriched canine spleen extract, whole blood enriched with canine spleen extract, and *Babesia*-infected whole blood. Once an optimal staining protocol was determined a standardized gating strategy was formulated.

Unstained whole blood was used as a control for each sample. A histogram was used to plot the SYBR Green I stained cells in FL-1 against the total event count. FL-1 was plotted against FL-4 on a dot plot. The unstained control was used to determine accurate placement of the histogram marker over the negative peak of the unstained control and to ensure accurate placement of the quadrant marker to cordon off the FL-1 FL-4 negative event population in the lower-left quadrant of the dot plot. Whole blood from the same dog was then stained according to the optimized staining protocol and a red cell zone (a zone free of FL-1 positive events in healthy whole blood) was determined. The red cell gate was adjusted accordingly to ensure no WBC events were recorded on FL-1 in the gated region and any remaining fluorescence in the delineated red cell zone was regarded as background and reticulocyte interference. The ten healthy control dogs were run as a first batch to ensure a predictable and repeatable gating strategy that allowed an optimized gating template to be generated before the infected dogs were processed as part of the study population. Parasitized whole blood from an infected dog was used to test the optimized red cell gate template by confirming that pRBC events could be recorded on the FL-1 channel in the delineated gate for infected samples. The final gating template consisted of an initial FSC and SSC dot plot and a subsequent dot plot of FL-1 against FL-4 for the optimized red cell gate.

Following the optimization process a final staining protocol was chosen for use in the study. SYBR Green I working stock was prepared at a 1:1000 concentration by diluting 1 µL SYBR Green I stock with 999 µL PBS. The working stock was stored in Eppendorf tubes wrapped in aluminium foil at -20 ℃ in the dark as 20 µL aliquots to minimize freeze-thawing. Prior to flow cytometric analysis samples were prepared as follows:

1. Two falcon tubes were marked ‘unstained’ and ‘stained’ respectively.

2. 50 µL whole blood sample was pipetted into each of the two falcon tubes and each tube was washed with 1.5 mL PBS, vortexed for 5 s, centrifuged at 450 rcf (1725 rpm, where R = 135 mm) for 5 min at 23 ℃ and the supernatant pipetted off.
3. The cell pellet in the tube marked ‘unstained’ was re-suspended in 20 µL PBS, and the pellet in the tube marked ‘stained’ in 15 µL PBS.
4. 5 µL SYBR Green I dye working stock (1:1000 dilution) was added.
5. Both tubes were vortexed for 5 s and incubated in the dark for 30 min at 37 ℃.
6. Following incubation, the contents of each tube were washed with 1.5 mL PBS, vortexed for 5 s, centrifuged at 450 rcf (1725 rpm, where R = 135 mm) for 5 min at 23 ℃ and the supernatant pipetted off.
7. Each tube’s cell pellet was finally re-suspended in 500 µL PBS, vortexed for 5 s and analysed by use of an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA).

G.3 Results

As SYBR Green I is an FL-1 dye the FL-1 channel yielded the best event distribution on a histogram and dot plot (Fig. 8). Higher dilutions of SYBR Green I (1:1500 and 1:2000) caused only a slight decrease in the amount of FL-1 positive events compared to the 1:1000 dilutions (Fig. 9). More concentrated dilutions of SYBR Green I (1:500, 1:100, 1:50, and 1:10) had a more significant effect on the amount of FL-1 positive events measured by pushing the uninfected RBC population into the FL-1 positive quadrant. This observation was made for both healthy (Fig. 10) and *Babesia*-infected (Fig. 11) whole blood samples. The input volume of SYBR Green I had a significant influence on the detection of FL-1 positive events (Fig. 12). 5 µL was selected as the optimal input volume because higher input volumes appeared to push uninfected RBC events into the upper-left FL-1 positive quadrant. The addition of a 0.025 % glutaraldehyde working stock for fixation of samples prior to staining resulted in a marked right shift of the event population on the x-axis of the dot plot into the FL-4 positive quadrant (Fig. 13). Staining at room temperature (23 ℃) did not appear to have any significant influence on the event population distribution compared to staining at 37 ℃ in an incubator (Fig. 14). Centrifugation speed had a negligible influence on results when decreased approximately ten-fold from 5000 rcf to 450 rcf. A washing step before and after staining appeared to decrease the amount of FL-1 events

pushed into the upper-left quadrant. WBC-enriched spleen extract, as well as whole blood enriched with spleen extract at a 1:1 ratio, yielded more FL-1 positive events as opposed to whole blood alone. The WBC-enriched spleen extract alone yielded more FL-1 positive events as opposed to the whole blood and spleen extract mixture (Fig. 15). A final gate was established in the red cell zone of both a healthy (Fig. 16) and *Babesia*-infected (Fig. 17) sample to demonstrate that only pRBCs and no WBCs were detected in the optimized gated region.

a) b)

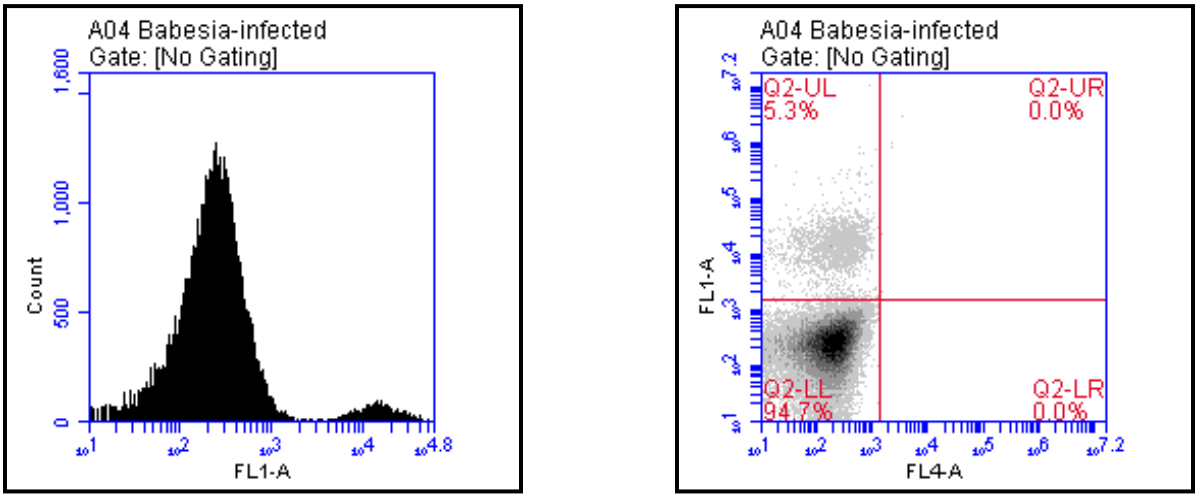
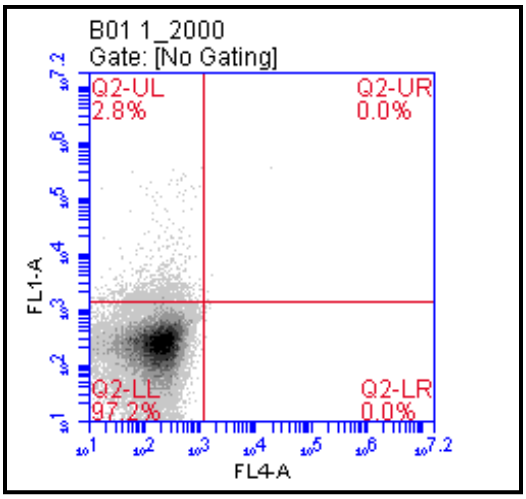


Figure 8 Histogram and scatter dot plot of ungated *Babesia*-infected whole blood, unfixed and stained at a 1:1000 dilution with 5 µL input SYBR Green I at 37 ℃

A distinct FL-1 positive cell population was observed as a right peak on the histogram (a), and an event population in the upper-left quadrant of the dot plot (b).

a)



b)

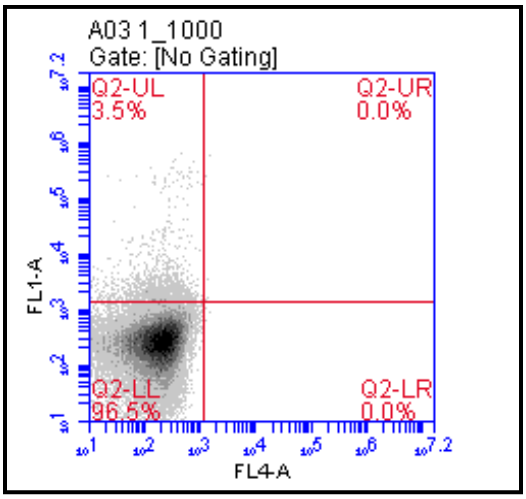
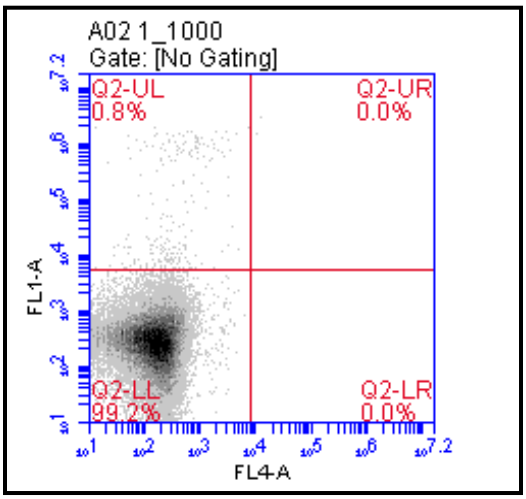


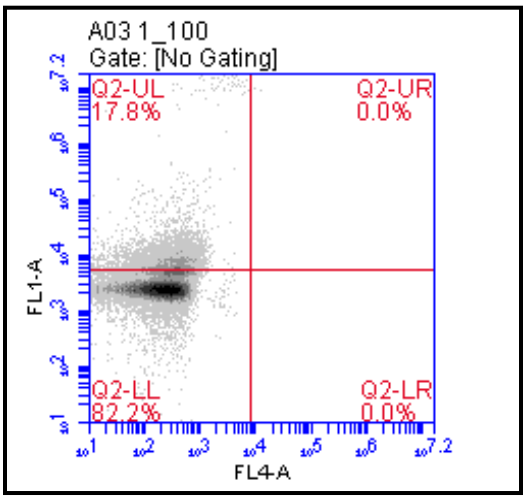
Figure 9 Scatter dot plots of healthy whole blood, unfixed and stained at a 1:2000 and 1:1000 dilution with 10 µL input SYBR Green I at 37 ℃

A slight decrease in FL-1 positive events was observed with a 1:2000 dilution (a) compared to a 1:1000 dilution (b).

a)



b)



c)

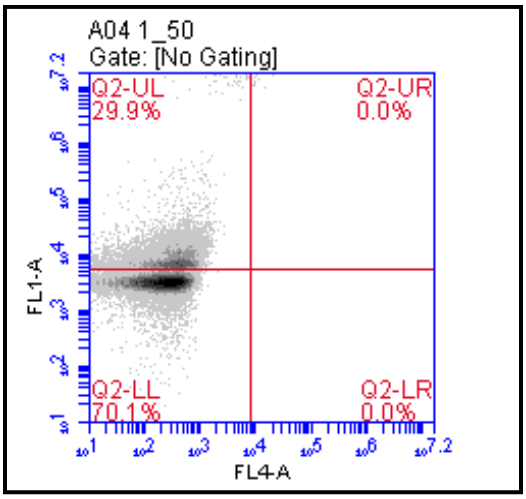
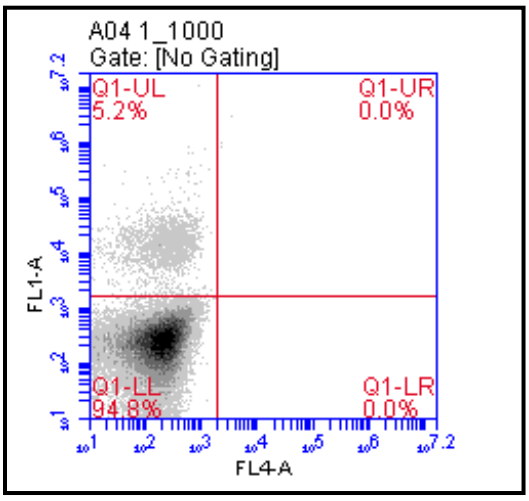


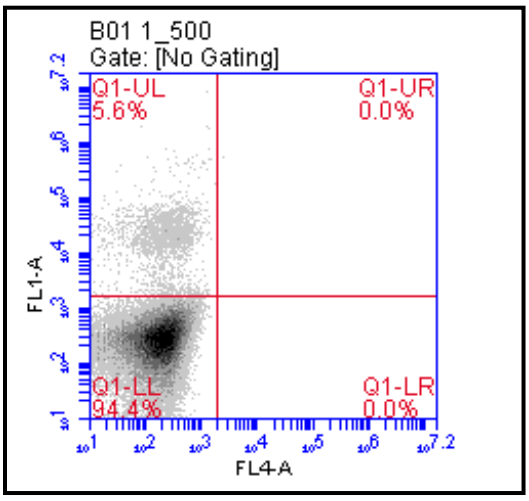
Figure 10 Scatter dot plots of healthy whole blood, unfixed and stained at a 1:1000, 1:100 and 1:50 dilution with 5 µL input SYBR Green I at 37 ℃

An increase in FL-1 positive events was observed with a decrease in dilution from 1:1000 (a), to 1:100 (b), and 1:50 (c).

a)



b)



c)

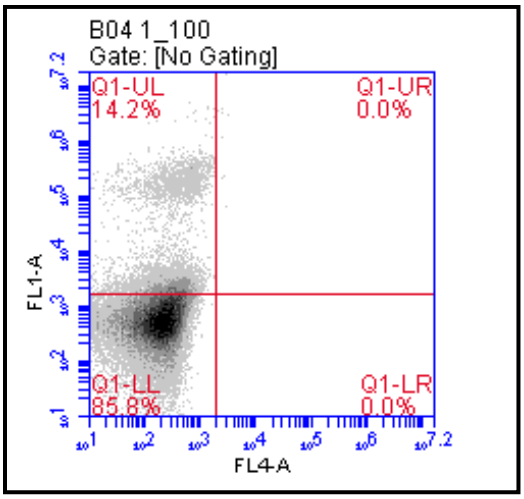
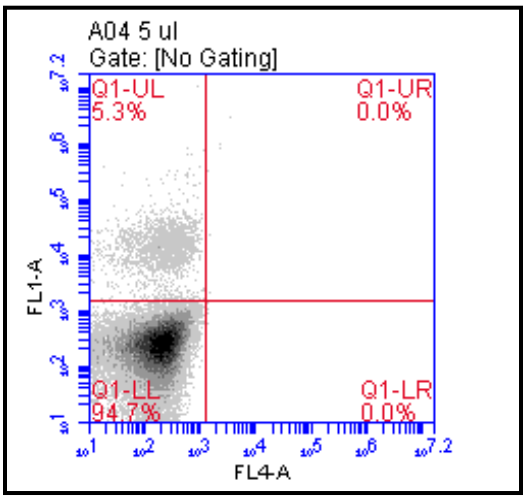


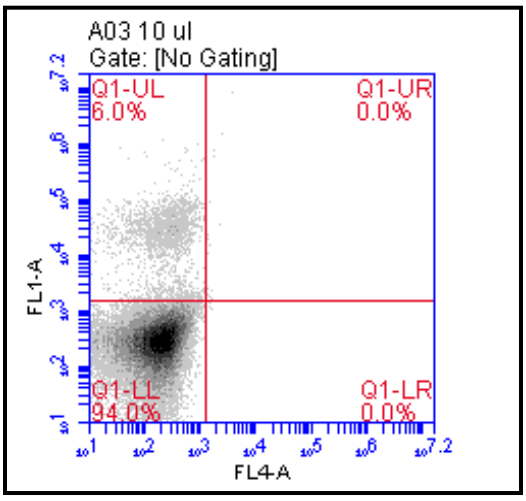
Figure 11 Scatter dot plots of *Babesia*-infected whole blood, unfixed and stained at a 1:1000, 1:500 and 1:100 dilution with 5 µL input SYBR Green I at 37 ℃

An increase in FL-1 positive events was observed with a decrease in dilution from 1:1000 (a), to 1:500 (b), and 1:100 (c).

a)



b)



c)

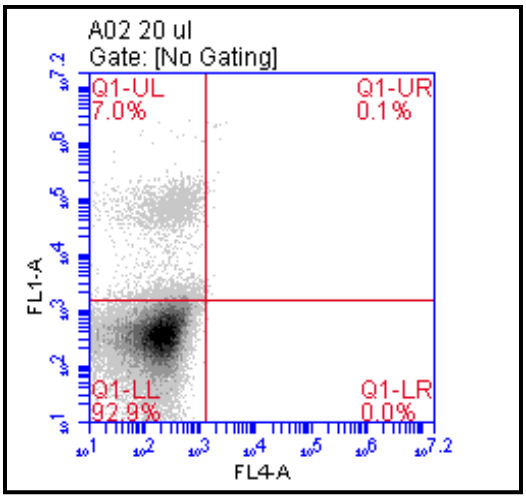
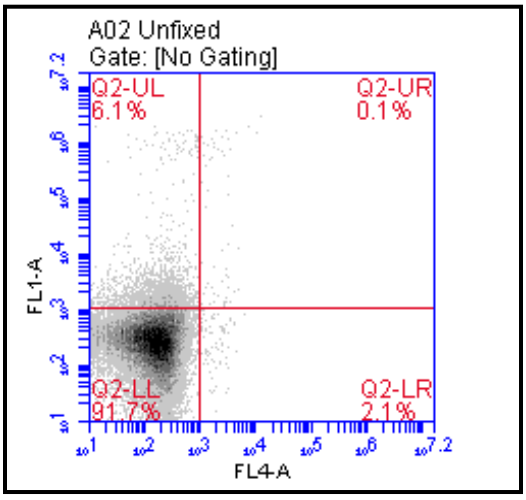


Figure 12 Scatter dot plots of *Babesia*-infected whole blood, unfixed and stained at a 1:1000 dilution with 5 µL, 10 µL and 20 µL input SYBR Green I at 37 ℃

An undesired upwards shift of uninfected RBCs into the FL-1 positive quadrant was observed as the input volume of SYBR Green I was increased from 5 µL (a), to 10 µL (b), and 20 µL (c).

a)



b)

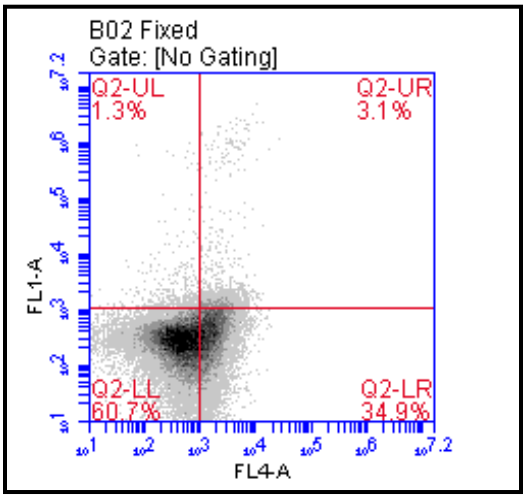
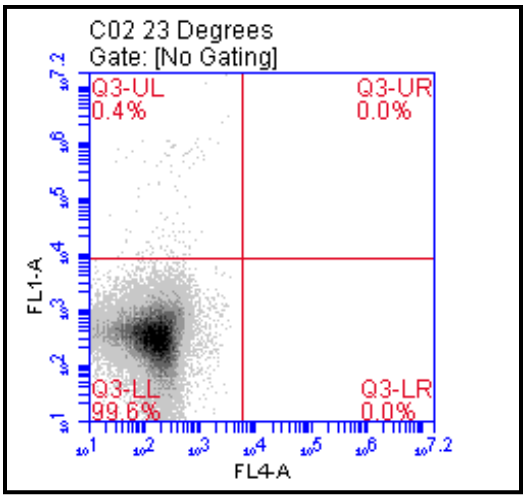


Figure 13 Scatter dot plots of healthy whole blood, fixed and unfixed and stained at a 1:1000 dilution with 20 µL input SYBR Green I at 37 ℃

Unfixed samples (a) yielded a more desirable event population distribution compared to fixed samples (b).

a)



b)

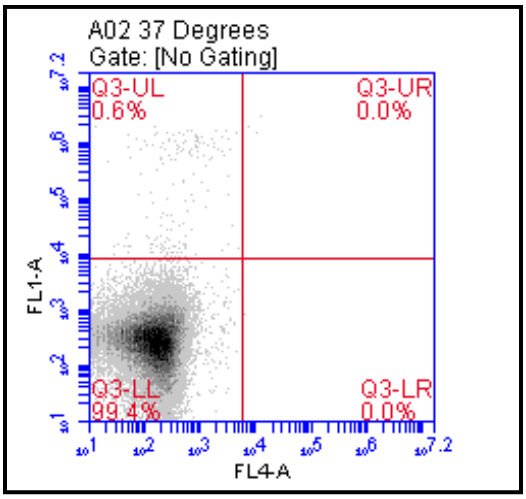
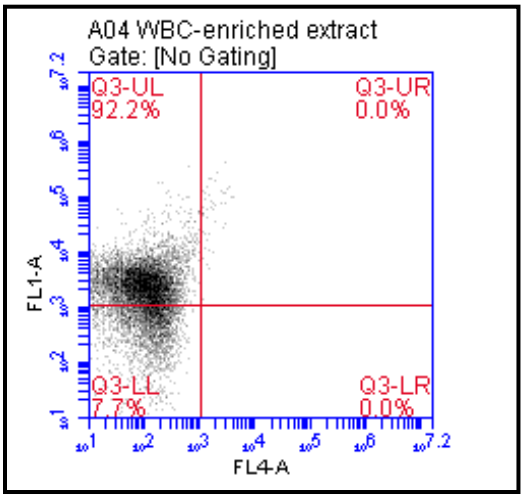


Figure 14 Scatter dot plots of healthy whole blood, unfixed and stained at a 1:1000 dilution with 20 µL input SYBR Green I at 23 ℃ and 37 ℃

Results from staining at room temperature (a) did not differ significantly from staining done at 37 ℃ in an incubator (b).

a)



b)

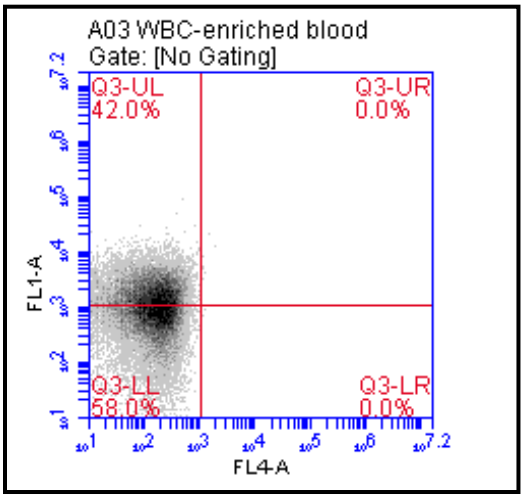
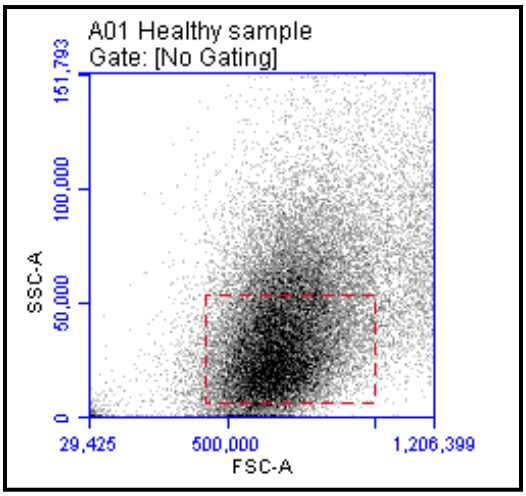


Figure 15 Scatter dot plots of healthy enriched spleen extract and healthy whole blood mixed with spleen extract, unfixed and stained at a 1:100 dilution with 20 µL SYBR Green I input at 23 ℃

Both WBC-enriched spleen extract (a) and whole blood mixed with spleen extract at a 1:1 ratio (b) yielded an increase in FL-1 positive events.

a)



b)

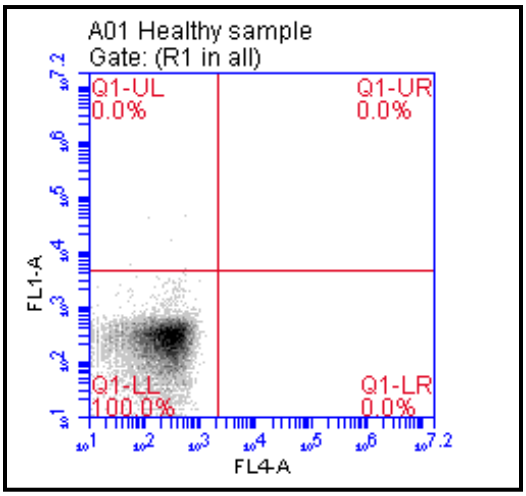


Figure 16 Scatter dot plots of the gated RBC event population in a healthy whole blood sample, unfixed and stained at a 1:000 dilution with 5 µL input SYBR Green I at 37 ℃

A final gate in the red cell zone was established (a) and observed to contain no WBC FL-1 positive events in a healthy whole blood sample (b).

a) b)

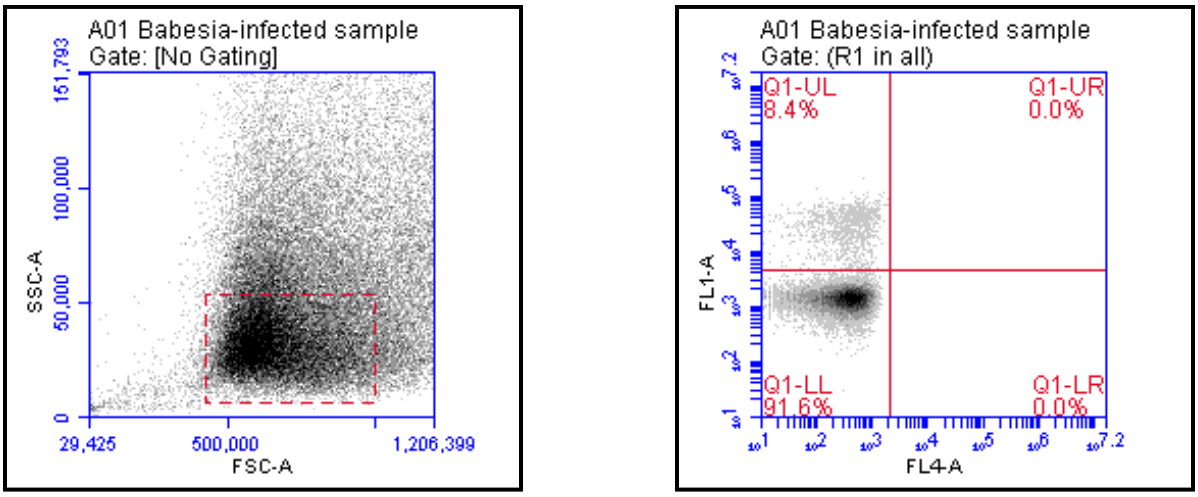


Figure 17 Scatter dot plots of the gated RBC event population in a *Babesia*-infected whole blood sample, unfixed and stained at a 1:000 dilution with 5 µL input SYBR Green I at 37 ℃

A final gate in the red cell zone was established (a) and observed to contain pRBC positive events in a *Babesia*-infected whole blood sample (b).

G.4 Discussion

The optimized flow cytometric protocol was able to discriminate pRBCs from uninfected erythrocytes with the highest resolution and best event distribution by staining 50 µL unfixed whole blood preparation with a 5 µL input of 1:1000 SYBR Green I dilution at 37 ℃ for 30 min in the dark (Fig. 16 & 17). Optimal results for analysing SYBR Green I stained events on a FCS and SSC dot plot were recorded in FL-1 against FL-4 (Fig. 8). SYBR Green I has an excitation maximum of 488 nm and emission maximum of 522 nm, which coincides with the Accuri C6 FL-1 detector that detects emission wavelengths of 530/30 nm (515-545 nm). A 1:1000 SYBR Green I dilution gave the best resolution and event distribution (Fig. 11a). The use of a higher or lower dilution than 1:1000 resulted in sub-optimal results in comparison. Higher dilutions did not appear to have a marked influence on results for healthy blood (Fig. 9), although our study lacked data results for *Babesia*-infected samples at higher dilutions. Lower dilutions (especially dilutions lower than 1:100) had a significant effect on both healthy and infected blood resulting in an upwards shift of the event population into the FL-1 positive quadrant (Fig. 10 & 11). Our observation differed from Rossouw's study on *B. divergens* where a 1:100 dilution was most

informative with regards to population resolution; although it was noted in her study that parasitaemia could be detected accurately with a 1:1000 dilution [12]. A decrease in input volume appeared to have a significant impact on the FL-1 positive event population. An increase in input volume caused an upwards shift of the event population into the FL-1 positive quadrant (Fig. 12). It is likely that an excess of input volume results in an excess of SYBR Green I in the sample. The undesirable event population distribution we observed for unfixed samples coincided with Rossouw's observation that unfixed samples were preferable for recording *B. divergens* events [12] (Fig. 13). Our study lacked result data to investigate the influence of fixation on *Babesia*-infected blood. One of the primarily functions of fixation is to preserve cell integrity over long periods of sample storage. All samples were analysed on the same day of sample collection and as a result fixation was deemed unnecessary in our study. The temperature at which staining was done did not appear to have a significant impact on population resolution or distribution (Fig. 14). Our study lacked data from *Babesia*-infected samples stained at room temperature and 37 ℃ to investigate the influence staining temperature would have on pRBC events. Staining was conducted at 37 ℃ in an incubator. Centrifugation speed had a negligible influence on staining, but was decreased from the recommended 5000 rcf to 450 rcf due to concerns of cell rupture at high speeds. Cell washing was minimized in an attempt to mitigate the risks of disrupting cell integrity by limiting the amount washing prior to staining to a single wash of the whole blood with double the recommended amount of PBS. A post-staining wash step was included due to concerns of an excess of SYBR Green I remaining in the sample post-staining. Although the impact of a post-staining wash step was not investigated in detail during optimization, we suspect it improves results by washing away excess dye not taken up by the cells after staining. Canine spleen extract was added to healthy whole blood to increase the number of nucleated cells in a preparation in an attempt to differentiate the RBC and WBC populations during the optimization process and to rule out the possibility of nucleated cells contaminating the red cell zone. Pure enriched spleen suspension yielded a higher percentage of FL-1 positive events as opposed to a mixture of spleen extract with whole blood (Fig. 15). One of the reasons for this observation would be that WBCs (nucleated cells) are more concentrated in pure spleen suspension and therefore yields more SYBR Green I positive events. Despite not being utilized as part of the final flow cytometric protocol the addition of WBC-enriched medium did assist in event population differentiation and setting up of the final gating template.

Although various parameters were investigated during protocol optimization, both the dilution concentration and input volume of the SYBR Green I dye appeared to play a fundamental role in

ensuring that false-positive events were minimized in the FL-1 positive quadrant. A plausible explanation for why the dilution concentration and input volume parameters are important might be that insufficient dilution and excess input volume both result in an excess of SYBR Green I dye in the sample, thereby causing the dye to stick to or infiltrate uninfected RBCs. The effects of staining duration, the medium used for preparation of SYBR Green I dilutions and washing steps (PBS compared to more ideally recommended buffers such as TAE, TE or TBE buffers to accommodate the optimal pH range of SYBR Green I), the limitations of long-term storage of SYBR Green I working stock aliquots, and the effects of freeze-thawing on dye integrity and event resolution should be considered.

G.5 Conclusion

An optimal sample preparation and staining protocol was developed and a red cell zone gate was successfully established during the optimization process. Taking into account the limitations such as time restrictions and budget the optimization process adequately maximized the chances of detecting *B. rossi* pRBCs in infected samples and estimating parasitaemia. Further investigation is warranted to explore and evaluate the full potential of this novel flow cytometric protocol for the quantification of *B. rossi* parasitaemia. In future the effect of reticulocyte contamination of a gated red cell zone should be investigated by means of a duel or tri-staining technique to detect and quantify *B. rossi*.

Appendix H: Study data set

Table 13 Control population data set

Sample	RBC count (x10^{12}/L)	Ht	Retic %	Gated events	SYBR Green I positive events	Capillary manual count	Venous manual count	Unadjusted flow cytometry	Adjusted flow cytometry	qPCR	RLB
1	6,94	0,47	0,40	15014	76	0,000	0,000	0,506	0,134	*Negative*	*Negative*
2	7,19	0,49	0,40	14929	57	0,000	0,000	0,382	0,010	*Negative*	*Negative*
3	5,61	0,37	0,50	17360	47	0,000	0,000	0,271	0,000	*Negative*	*Negative*
4	8,54	0,58	0,30	17777	22	0,000	0,000	0,124	0,000	*Negative*	*Negative*
5	7,58	0,54	0,30	17301	30	0,000	0,000	0,173	0,000	*Negative*	*Negative*
6	7,87	0,56	0,90	16238	25	0,000	0,000	0,154	0,000	*Negative*	*Negative*
7	5,71	0,42	1,10	21055	100	0,000	0,000	0,475	0,099	*Negative*	*Negative*
8	7,11	0,51	0,90	19769	64	0,000	0,000	0,324	0,000	*Negative*	*Negative*
9	8,69	0,62	1,80	22667	142	0,000	0,000	0,626	0,245	*Negative*	*Negative*
10	7,17	0,51	2,20	21635	154	0,000	0,000	0,712	0,326	*Negative*	*Negative*

Ht: Hematocrit
Retic %: Reticulocyte %

Table 14 Infected population data set

Sample	RBC count (x10^{12} /L)	Ht	Retic %	Gated events	SYBR Green I positive events	Manual count (Capillary)	Manual count (Venous)	Flow cytometry (Unadjusted)	Flow cytometry (Adjusted)	qPCR	RLB
26	6,85	0,46	1,00	11714	84	17,179	0,410	0,717	0,339	26,267	*B. rossi*
27	4,43	0,30	1,50	27060	161	0,154	0,000	0,595	0,216	29,233	*B. rossi*
28	3,75	0,27	0,70	19700	102	0,513	0,051	0,518	0,144	28,187	*B. rossi*
29	5,33	0,34	0,39	16598	76	1,846	0,359	0,458	0,086	23,304	*B. rossi*
30	2,40	0,16	2,80	29447	469	1,026	0,205	1,593	1,178	26,238	*B. rossi*
31	3,61	0,29	1,40	20700	109	0,564	1,179	0,527	0,149	24,624	*B. rossi*
32	3,71	0,24	4,70	22523	331	0,359	0,000	1,470	1,030	25,661	*B. rossi*
33	5,68	0,39	1,50	18439	90	1,590	2,051	0,488	0,110	25,076	*B. rossi*
34	3,11	0,20	8,20	21734	672	30,051	0,513	3,092	2,468	27,276	*B. rossi*
35	1,41	0,11	6,90	21836	1084	6,564	0,718	4,964	4,251	24,388	*B. rossi*
36	1,87	0,13	10,60	20698	868	0,410	0,000	4,194	3,379	31,039	*B. rossi*
37	3,24	0,23	3,10	19561	659	0,103	0,205	3,369	2,894	29,132	*B. rossi*
38	3,51	0,24	2,20	17287	428	0,667	0,154	2,476	2,051	27,702	*B. rossi*
39	1,96	0,13	10,20	21197	2081	0,410	0,205	9,817	8,446	31,678	*B. rossi*
40	2,38	0,17	3,10	17606	328	1,333	0,615	1,863	1,435	23,097	*B. rossi*
41	2,94	0,22	4,90	18750	702	1,179	0,051	3,744	3,190	30,051	*B. rossi*
42	3,09	0,24	8,70	19111	588	0,154	0,103	3,077	2,439	33,612	*B. rossi*
43	3,73	0,26	0,90	18013	85	0,359	0,308	0,472	0,097	25,805	*B. rossi*
44	8,42	0,59	0,90	13145	49	1,128	0,205	0,373	0,000	28,298	*B. rossi*
45	7,71	0,50	0,50	17113	39	2,462	0,308	0,228	0,000	28,133	*B. rossi*
46	1,83	0,14	9,10	22959	433	0,462	0,205	1,886	1,344	32,901	*B. rossi*
47	2,03	0,15	3,70	21053	416	2,205	0,205	1,976	1,532	27,161	*B. rossi*
48	3,13	0,22	7,60	25275	213	0,154	0,000	0,843	0,408	32,709	*B. rossi*
49	4,84	0,32	1,20	19214	84	0,205	0,103	0,437	0,061	30,861	*B. rossi*
50	7,49	0,48	0,40	19149	24	1,436	0,154	0,125	0,000	31,068	*B. rossi*

Ht: Hematocrit
Retic %: Reticulocyte %

Table 15 Replicate population data set

Sample	RBC count (x10^{12} /L)	Ht	Retic %	Gated events	SYBR Green I positive events	Capillary manual count	Venous manual count	Unadjusted flow cytometry	Adjusted flow cytometry	qPCR	RLB
***11**	2,42	0,16	5,90	12108	532	1,641	0,154	4,394	3,764	30,214	*B. rossi*
****11**	2,42	0,16	5,90	11765	492	2,462	0,154	4,182	3,565	30,475	*B. rossi*
***12**	3,06	0,22	1,20	24445	383	1,846	2,718	1,567	1,178	22,902	*B. rossi*
****12**	3,06	0,22	1,20	23751	409	1,436	1,846	1,722	1,331	22,744	*B. rossi*
***13**	2,11	0,15	8,30	25357	1046	21,795	2,051	4,125	3,412	20,845	*B. rossi*
****13**	2,11	0,15	8,30	25395	1116	18,564	1,897	4,395	3,659	20,839	*B. rossi*
***14**	5,69	0,41	1,40	15503	146	2,308	0,462	0,942	0,558	25,546	*B. rossi*
****14**	5,69	0,41	1,40	16197	106	3,077	1,128	0,654	0,275	25,776	*B. rossi*
***15**	5,53	0,36	0,70	18105	61	0,872	0,051	0,337	0,000	29,022	*B. rossi*
****15**	5,53	0,36	0,70	18169	61	0,462	0,205	0,336	0,000	29,171	*B. rossi*
***16**	6,94	0,51	1,30	16073	81	5,692	0,308	0,504	0,127	27,115	*B. rossi*
****16**	6,94	0,51	1,30	16629	80	3,128	0,359	0,481	0,104	27,132	*B. rossi*
***17**	2,49	0,18	8,80	19391	604	0,410	0,359	3,115	2,470	26,981	*B. rossi*
****17**	2,49	0,18	8,80	19454	637	0,615	0,256	3,274	2,616	26,971	*B. rossi*
***18**	4,47	0,33	1,70	19925	251	2,769	0,154	1,260	0,868	28,990	*B. rossi*
****18**	4,47	0,33	1,70	18973	199	5,538	0,103	1,049	0,661	28,729	*B. rossi*
***19**	1,43	0,11	14,30	22792	1651	0,564	0,410	7,244	5,837	31,276	*B. rossi*
****19**	1,43	0,11	14,30	22659	1466	1,128	0,308	6,470	5,174	31,393	*B. rossi*
***20**	2,45	0,16	0,80	20034	1118	43,077	6,872	5,581	5,165	21,316	*B. rossi*
****20**	2,45	0,16	0,80	20589	1095	43,744	7,744	5,318	4,905	21,322	*B. rossi*
***21**	2,18	0,14	5,50	22881	720	0,974	1,231	3,147	2,603	22,699	*B. rossi*
****21**	2,18	0,14	5,50	23022	780	1,282	1,333	3,388	2,831	22,754	*B. rossi*
***22**	1,59	0,14	14,30	21736	2167	1,385	0,513	9,970	8,174	25,837	*B. rossi*
****22**	1,59	0,14	14,30	22441	2079	2,615	0,872	9,264	7,569	25,822	*B. rossi*
***23**	2,34	0,17	0,90	25716	211	0,308	0,000	0,821	0,443	27,737	*B. rossi*
****23**	2,34	0,17	0,90	26596	212	0,462	0,051	0,797	0,419	27,449	*B. rossi*
***24**	2,22	0,16	5,20	22457	136	0,256	0,051	0,606	0,204	27,665	*B. rossi*
****24**	2,22	0,16	5,20	23070	169	0,051	0,000	0,733	0,324	27,873	*B. rossi*
***25**	5,78	0,40	0,70	25116	42	0,359	0,000	0,167	0,000	31,566	*B. rossi*
****25**	5,78	0,40	0,70	24910	34	0,573	0,051	0,136	0,000	31,461	*B. rossi*

*: Original sample data
**: Replicate sample data