

**Prevalence of co-infection with *Ehrlichia* spp. or
Theileria spp. in dogs naturally infected with
babesiosis in the Eastern Cape**

by

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The author, Henry Cloete, has obtained applicable research ethics approvals for the work described in this dissertation. Ethical approval for the study was granted by the Research Ethics Committee and the Animal Ethics Committee of the University of Pretoria, Faculty of Veterinary Science (REC061-20; Appendix I).

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.

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List of Abbreviations

Alb – Albumin
ARC – Absolute Reticulocyte Count
CBC – Complete Blood Count
CD – Cluster of Differentiation
CI – Confidence Interval
DNA – Deoxyribonucleic acid
EDTA – Ethylenediaminetetraacetic Acid
Glob – Globulin
HCT – Haematocrit
HGB – Haemoglobin
IFAT – Immunofluorescence Antibody Test
IFN- γ – Interferon-gamma
IL – interleukin
IQR – Interquartile Range
Kg – Kilogram
MCH – Mean Corpuscular Haemoglobin
MCHC – Mean Corpuscular Haemoglobin Concentration
MCP – Monocyte chemotactic protein
MCV – Mean Corpuscular Volume
MSVC – Mdantsane State Veterinary Clinic
NPV – Negative Predictive Value
NRBC/ 100 WBC – Nucleated Red Blood Cells / 100 White Blood Cells
NRR – No Reference Range
PCR – Polymerase Chain Reaction
PCV – Packed Cell Volume
PPV – Positive Predictive Value
qPCR – Quantitative Polymerase Chain Reaction
RBC – Red Cell Count
RDW – Red cell Distribution Width
RLB – Reverse Line Blot
TNF- α – Tumour Necrosis Factor-alpha
TSP – Total Serum Protein

USA – United States of America

WCC – White Cell Count

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Summary

This study describes the prevalence of *Ehrlichia canis* or *Theileria equi* co-infections in dogs that are naturally infected and clinically sick due to babesiosis. Canine babesiosis and ehrlichiosis are tick-borne diseases of great significance in South Africa. Theileriosis in dogs in South Africa is still poorly understood. Co-infection with multiple tick-borne pathogens has been documented and is perceived as a common occurrence in South Africa. The Mdantsane State Veterinary Clinic in Mdantsane, Eastern Cape Province, South Africa was used as the centre where sample collection took place. Limited data on the prevalence and distribution of tick-borne diseases in the Eastern Cape Province are available and thus this population of dogs can be viewed as a novel study population.

The main objective of this study was to determine the prevalence of co-infections with *E. canis* or *T. equi* in dogs with babesiosis in the Eastern Cape province. Possible associations of population characteristics and haematological and biochemistry measures with a co-infection in these dogs were also investigated.

The study population included 150 dogs naturally infected with babesiosis that presented to the Mdantsane State Veterinary Clinic between January 2021 and November 2021. Quantitative polymerase chain reaction was used to confirm the *Babesia* spp. that the dogs were infected with and to identify co-infections. Association with co-infection for the following parameters were evaluated: sex, breed, age, duration of sickness, leukocyte count, band neutrophil count, monocyte count, platelet count, absolute reticulocyte count, and serum globulin concentration. Positive and negative predictive values of monocytosis, leukopenia, band neutrophilia, thrombocytopenia, and non-regenerative absolute reticulocyte count for co-infection were also calculated.

Babesia rossi was identified in 149/150 samples and *B. vogeli* in only 1/150 samples. A co-infection prevalence of 2.0% (3/149; 95% confidence interval: 0.4–5.7) with *B. rossi* and *E. canis* was found. No other co-infections were reported. No investigated variables showed significant associations with co-infections. Monocytosis, in particular, was not associated with co-infection.

Co-infection with other tick-borne pathogens in dogs with babesiosis is uncommon in the Eastern Cape province. These findings raise the possibility that *B. rossi* may have a protective effect against other tick-borne pathogens. It is advised that further testing, ideally with the use of polymerase chain reaction, be done when a co-infection is suspected to practice responsible antibiotic stewardship.

Chapter 1: Introduction

1.1 Background

Canine babesiosis and ehrlichiosis are tick-borne infections of great significance in South Africa and both may result in severe clinical disease (Van Heerden, 1982; Rautenbach, Boomker, and De Villiers, 1991; Collett, 2000). The clinical importance of theileriosis, a tick-borne disease caused by a piroplasm of the genus *Theileria*, in dogs in South Africa is still poorly understood (Rosa *et al.*, 2014). A co-infection with multiple tick-borne pathogens is possible and has been documented in individual animals. This occurrence has been attributed to single tick species having the ability to act as a vector for multiple pathogens and secondly due to heavy tick infestations (Kordick *et al.*, 1999; Shaw *et al.*, 2001). Additionally, the infection rates of important tick vectors may play a critical role in determining the likelihood of co-infections in affected populations (Rocha *et al.*, 2022).

At least seven different *Babesia* species have been identified in domestic dogs across the globe and are grouped into two groups based on their morphology. The large *Babesia* species include *B. canis*, *B. vogeli* and *B. rossi*. The small *Babesia* species include *B. gibsoni*, *B. conradae*, and *B. negevi*. In addition to these, several unnamed genetically unique *Babesia* species have been identified (Matjila *et al.*, 2008a; Birkenheuer *et al.*, 2020). In South Africa, canine babesiosis is predominantly caused by two of the large *Babesia* species, namely *Babesia rossi* which is considered to be very virulent, and to a lesser extent by *B. vogeli* (Matjila *et al.*, 2008a). *Babesia gibsoni* has been reported only once in South Africa in a three-month-old pit bull terrier pup shortly after being imported from the United States of America (USA) (Matjila *et al.*, 2007).

Various *Ehrlichia* spp. have been found to infect dogs globally, however, not all have been shown to cause clinical disease. These include, but are not limited to, *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. equi*, *E. muris*, *E. risticii*, and *E. ruminantium* (Kelly, 2000; Sainz *et al.*, 2015). Only *E. canis* has been identified to cause clinical disease in South Africa. *Ehrlichia ruminantium*, specifically the Pretoria North genotype has been identified to infect dogs in southern Africa (Allsopp and Allsopp, 2001; Allsopp, 2010). Antibodies against *E. chaffeensis* have been identified in dogs in a study done in Bloemfontein, Free State, South Africa, but this species was not found to infect and cause clinical disease in dogs and is likely due to serological cross-reactivity between *Ehrlichia* infections (Pretorius and Kelly, 1998).

Theileria annae has long been identified as Europe's leading cause of canine theileriosis. In recent years, however, there has been much disagreement as to its classification under the genus *Theileria*

and it has been suggested that it be reclassified under the genus *Babesia* and named *Babesia vulpes* (Miró *et al.*, 2015; Baneth *et al.*, 2015, Baneth, 2018). *Theileria equi*, which causes the disease known as equine piroplasmiasis in equids, and *T. annulata*, which causes the disease known as bovine tropical theileriosis in bovines, have also been reported in dogs globally (Ghauri *et al.*, 2019; Ullah *et al.*, 2021). Apart from *Theileria equi*, the *Theileria* spp. known to cause disease in dogs in South Africa has not been positively identified (Rosa *et al.*, 2014). Theileriosis has not been reported in dogs in the Eastern Cape province. One study identified dogs to be infected and clinically sick due to a *Theileria* spp. that was closely related to an unidentified species of *Theileria* known to cause disease in sable antelope (Matjila *et al.*, 2008b). The deoxyribonucleic acid (DNA) sequence of the agent is not available (Matjila *et al.*, 2008b; Rosa *et al.*, 2014)

Reports focused on the prevalence of *Babesia* spp. infection with *Ehrlichia* spp. or *Theileria* spp. co-infections in dogs in South Africa are scarce. Few studies have directly or indirectly reported on this relationship and only one of these included data from the Eastern Cape Province where co-infections were not reported (Du Plessis *et al.*, 1990; Böhm *et al.*, 2006; Matjila *et al.*, 2008a; Rautenbach, Schoeman and Goddard, 2018). A survey conducted in 1993 that focused on canine babesiosis demonstrated that most veterinary practitioners in South Africa reported that they consider ehrlichiosis an important complicating factor in babesiosis infections (Collett, 2000). This belief likely stemmed from reports by several investigators regarding the importance and the difficulties in diagnosing such co-infections predating this survey (Van Heerden, Reyers and Stewart, 1983; Irwin and Hutchinson, 1991).

1.2 Aims and objectives

The objectives of this study were firstly to determine the prevalence of co-infections with *Ehrlichia canis* or *Theileria equi*, known to cause clinical disease, in dogs naturally infected with babesiosis in the Eastern Cape province and secondly to identify host and environmental factors associated with co-infection.

1.3 Benefits

This study provides information regarding the prevalence of co-infections with common and highly virulent canine vector-borne pathogens as well as an emerging pathogen in South Africa. This study was the first of its kind performed in the Eastern Cape Province and provides great insight into the disease management of cases presented to local veterinary and primary animal healthcare clinics. Lastly, this study fulfils the requirements of the principal investigator's MSc degree.

Chapter 2: Literature Review

2.1 Canine babesiosis

Babesia rossi is transmitted by the tick vector *Haemaphysalis elliptica*, which has a wide distribution in South Africa and has been found on large numbers of dogs in the Eastern Cape Province (Horak *et al.*, 2009). *Rhipicephalus sanguineus* has been identified as the vector for *B. vogeli* (Uilenberg *et al.*, 1989) and was found to heavily infest ruminant hosts in the Eastern Cape province (Iweriebor *et al.*, 2017), confirming its establishment in the environment. Both *B. rossi* and *B. vogeli* are large *Babesia* species and can readily be diagnosed by blood film examination and visualisation of parasites in erythrocytes (Schoeman, 2009).

Babesia rossi is known to cause peracute and acute disease with clinical signs that typically include pale mucous membranes, icterus, depression, tachycardia, tachypnoea, anorexia, weakness, splenomegaly, and pyrexia (Uilenberg *et al.*, 1989; Schoeman, 2009; Leisewitz *et al.*, 2019a). These clinical signs are believed to manifest due to tissue hypoxia secondary to anaemia and due to an accompanying systemic inflammatory response syndrome caused by marked cytokine release (Schoeman, 2009; Sudhakara Reddy *et al.*, 2016; Leisewitz *et al.*, 2019b). In severe cases, multiple organ failure with resulting complications such as acute renal failure, hepatopathy with marked icterus, acute respiratory distress syndrome, cerebral pathology, pancreatitis, and immune-mediated haemolytic anaemia are often seen (Schoeman, 2009; Leisewitz *et al.*, 2019a). Haematological abnormalities include a significant reduction in red cell count, haemoglobin concentration, platelet count, and packed cell volume (PCV) percentage, which is often associated with a weak regenerative response (Seejarim *et al.*, 2023). A relatively normal white cell count with elevated band neutrophils (left shift) has been reported (Leisewitz *et al.*, 2019a), indicative of a marked systemic inflammatory response (Schoeman, 2009; Scheepers *et al.*, 2011). Additionally, a monocytosis has been reported as a common haematological finding in dogs with *B. rossi* (Scheepers *et al.*, 2011).

Serum biochemical abnormalities include mild hypoalbuminaemia, hyperbilirubinaemia, and increased concentrations of creatinine and urea in cases of acute renal injury (Schoeman, 2009; Koster, Lobetti and Kelly, 2015; Sudhakara Reddy *et al.*, 2016; Leisewitz *et al.*, 2019a). Increased serum liver enzyme activities are reported in some cases (Schoeman, 2009).

Babesia vogeli is much less virulent compared to *B. rossi* and causes a moderate, often clinically unnoticed infection in mature dogs (Di Cataldo *et al.*, 2020). It has only been positively identified in the Gauteng, Free State, and Mpumalanga provinces of South Africa using polymerase chain reaction

(PCR) (Matjila *et al.*, 2004; Kolo *et al.*, 2014). Puppies may be severely affected and tend to present with marked anaemia (Schoeman, 2009).

2.2 Canine ehrlichiosis

Ehrlichia canis is transmitted by the tick vector *Rhipicephalus sanguineus*, which it shares with *B. vogeli* (Uilenberg *et al.*, 1989; Fourie *et al.*, 2013) and is considered a pathogen of clinical importance in the South African dog population. An overall seroprevalence of 42% has been reported in dogs from the Bloemfontein area, Free State province (Pretorius and Kelly, 1998). In poorer areas, the seroprevalence was slightly higher at 48 % (Pretorius and Kelly, 1998). Moreover, a seroprevalence of 41.7% has been reported in the uMkhanyakude district of the KwaZulu-Natal province (Mofokeng *et al.*, 2020). In addition, *Ehrlichia ruminantium*, specifically the Pretoria North genotype, has been identified to infect dogs in southern Africa and may also cause clinical disease (Allsopp and Allsopp, 2001; Allsopp, 2010). In South Africa, *E. canis* outbreaks are of concern in kennels (police, security, and commercial kennels) as well as in areas where tick control is inadequately implemented (Immelman and Button, 1973). Due to the difficulty in identifying *E. canis* morulae on peripheral stained blood smears and the limited accessibility of PCR testing, veterinary practitioners rely heavily on serological methods for the diagnosis of ehrlichiosis (Kelly, 2000; Waner *et al.*, 2001). In geographical regions where the disease is endemic the reliability of serological testing is however challenged (Waner *et al.*, 1997).

Ehrlichiosis is characterised by various clinical signs which range from asymptomatic to severe acute and chronic disease (Thirumalapura and Walker, 2015; Kelly, 2000). Affected dogs show signs like anorexia, fever, lymphadenomegaly, pale mucous membranes, peripheral oedema, and in severe cases, a haemorrhagic diathesis, renal failure, acute respiratory distress syndrome, nervous and ocular involvement (Thirumalapura and Walker, 2015; Kelly, 2000). The following laboratory abnormalities have been documented in both experimentally and naturally infected dogs during the various phases of the disease: thrombocytopenia, regenerative or non-regenerative anaemia, hypoalbuminaemia, hyperglobulinaemia, hyperproteinaemia, leukopenia, leukocytosis, absolute lymphocytosis, absolute neutropenia, pancytopenia, and elevated liver enzyme activities (Kelly, 2000).

The clinical manifestation of disease in dogs infected with *E. ruminantium* has not been described and it is not definitively known whether dogs can become clinical or remain asymptomatic carriers (Allsopp and Allsopp, 2001; Allsopp, 2010). There is no published data available on the tick vector for *E. ruminantium* infections in canine patients. However, the tick species *Amblyomma hebraeum* and *A.*

variegatum are considered the major vectors of the pathogen to cattle, sheep and goats (Petney, Horak and Rechav, 1987).

2.3 Canine theileriosis

The tick vector for theileriosis in dogs in South Africa has also not been identified. It may be possible that the same vectors that transmit *T. equi* to horses and other equids and *Theileria* sp. (sable) to sable and other antelope, could also act as the vectors for transmitting these pathogens to dogs. Multiple ticks in the genera *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus* have been identified as potential vectors of *T. equi* across the world (Scoles and Ueti, 2015). Theileriosis in roan antelope has been studied and it was found that the *Theileria* sp (sable), to which the previously defined *Theileria* sp. dog (Matjila *et al.*, 2008b) is closely related, induces clinical disease when transmitted by the tick vector *Rhipicephalus evertsi evertsi* (Steyl *et al.*, 2012). Diagnosis of infection with *Theileria* spp. in South African dogs has only been by PCR, and no circulating parasitaemia has been reported (Matjila *et al.*, 2008b; Rosa *et al.*, 2014). Dogs with theileriosis appear to present with similar clinical findings to dogs with babesiosis, which include pale mucous membranes, bleeding tendencies, lethargy, fever, and anorexia (Matjila *et al.*, 2008b; Rosa *et al.*, 2014). Haematology results from dogs infected with these pathogens have shown anaemia and/or thrombocytopenia, which may be secondary to an immune-mediated syndrome (Matjila *et al.*, 2008b; Rosa *et al.*, 2014). No serum protein abnormalities associated with *Theileria* spp. infections have been reported in South African studies.

2.4 Co-infections with multiple tick-borne pathogens

The first South African publication to publish data on the incidence of *Babesia* and *Ehrlichia* co-infections found a co-infection prevalence of 32% based on immunofluorescence antibody testing (IFAT) from a cohort of dogs from the Onderstepoort region in South Africa (Du Plessis *et al.*, 1990). *Ehrlichia canis* antibody titers have however been reported to rise and peak 2 – 5 months post-infection and may persist in blood and tissue samples for extended periods (Kelly, 2000). This 32% seroprevalence may thus reflect exposure rather than active infection. Two studies using PCR and reverse line blot (RLB) assays have been done. The first study reported 560 dogs from a study population of 1138 sampled across all, bar the Limpopo and Northern Cape, provinces to be infected with at least one tick-borne pathogen. Of these, 12/560 had a co-infection with *B. rossi* and *E. canis* whilst 7/560 were co-infected with *B. vogeli* and *E. canis*. One dog was co-infected with both *B. rossi* and *B. vogeli*, whilst one other was co-infected with *B. rossi*, *B. vogeli* and *E. canis*. A large group of dogs (82/560) were infected with a novel species of *Theileria* not previously defined. Three dogs were co-infected with this species of *Theileria* and *E. canis*. (Matjila *et al.*, 2008a). The most recent study

published in 2019 focused on animals from the Gauteng region and reported an *E. canis* or *E. ruminantium* co-infection prevalence of 2% in dogs with *B. rossi*. No co-infections with *Ehrlichia* spp. were found in dogs with *B. vogeli* (Rautenbach, Schoeman and Goddard, 2018). Little is known regarding the vector-borne pathogens of companion animals elsewhere in sub-Saharan Africa, while global reports of co-infections are often limited to smaller case series or focus on pathogens such as *Borrelia burgdorferi*, *Leishmania infantum*, and *Anaplasma* spp., which are not of significant clinical concern in South Africa (Suksawat *et al.*, 2001; De Tommasi *et al.*, 2013; Noden and Soni, 2015; Pantchev *et al.*, 2015; Attipa *et al.*, 2018).

2.5 Associations with co-infections

It has been reported that 72% of veterinary practitioners used monocytosis or cytological evidence of monocyte activation as an indicator of co-infection with ehrlichiosis, whilst 42% used thrombocytopenia and 23% a normal or low white cell count (Collett, 2000). The only study to have addressed possible associations of haematological variables with *Ehrlichia* and *Babesia* co-infections reported very low positive predictive values (PPVs) and high negative predictive values (NPVs) for thrombocytopenia (PPV – 2.1%; NPV – 100%) or leukopenia (PPV – 1.3%; NPV – 97.4%) from a cohort of 198 dogs with a co-infection prevalence of 2% (Rautenbach, Schoeman and Goddard, 2018).

Chapter 3: Materials and Methods

3.1 Animals and Study design

This project was a cross-sectional, observational study of dogs naturally infected with *Babesia* spp. Sampling was done between January and November 2021. A minimum sample size of 140 dogs was calculated to estimate a co-infection prevalence of 10% with 5% precision and 95% confidence (Thrusfield, 2007). One hundred and fifty samples were collected by the primary investigator from privately owned dogs, positively diagnosed with babesiosis that were clinically sick and presented to the Mdantsane State Veterinary Clinic (MSVC) for treatment. The MSVC is in Mdantsane, which is the second largest urban township in South Africa and forms part of the Buffalo City Metropolitan Municipality (-32.944, 27.778). The diagnosis of babesiosis was made by visualisation of piroplasms on a peripheral Diff-Quik stained blood smear using light microscopy and then confirmed by quantitative real-time PCR (qPCR) (Bhoora *et al.*, 2010; Troskie *et al.*, 2018; Nkosi *et al.*, 2022). Inclusion criteria were dogs that were clinically sick, privately owned, weighed more than 3 kilograms (kg), and were over the age of 12 weeks. Dogs of any breed and either sex were accepted. All animals were subjected to a full physical examination. Observations including the signalment, a brief history, and physical examination findings for each animal were recorded on a data capture sheet (Appendix I). Dogs were excluded if there was a clinical suspicion based on history and clinical examination of comorbidity with other non-tick-borne infectious diseases (such as canine parvoviral enteritis and canine distemper) which may affect the haematology and biochemistry results. Dogs were also excluded if they had received treatment for a suspected tick-borne disease in the four weeks before presentation to the MSVC with any of the following drugs: imidocarb dipropionate, diminazene aceturate, tetracycline antibiotics, clindamycin or corticosteroids. Before sampling commenced, owner consent was obtained from the owner or the person responsible for the animal (Appendix II). Ethical approval for the study was granted by the Research Ethics Committee and the Animal Ethics Committee of the University of Pretoria, Faculty of Veterinary Science (REC061-20). A permit granting permission to do the research in terms of Section 20 of the Animal Diseases Act, 1984 (Act no. 35 of 1984) was obtained from the Department of Agriculture, Land Reform and Rural Development (12/11/1/1/6 (1625 AC)).

3.2 Sample Collection

Peripheral venous blood was collected at presentation and before any treatment. The collected blood was divided into one ethylenediaminetetraacetic acid (EDTA) vacutainer tube (3 millilitres (mL)) and one serum vacutainer tube (3mL) (BD Biosciences, Becton Dickinson Pty. Ltd.). Two blood smears were

prepared from the EDTA sample, fixed in alcohol, stored at room temperature, and submitted to the Clinical Pathology Laboratory at the Faculty of Veterinary Science, University of Pretoria. The serum tubes were centrifuged after clot formation, whereafter the serum was harvested using a pipette and stored in separate sterile sample tubes. The non-coagulated blood and serum samples were kept at 4°C from the time of sampling and during overnight delivery to the laboratory for assay the following day. Analysis was performed within 4 hours of arrival at the laboratory and within 24 hours following sampling. Laboratory analysis included a complete blood count (CBC) with an absolute reticulocyte count (ARC), and serum protein concentration measurement. After analysis, the blood cells were harvested from the EDTA samples and together with the remaining serum samples were stored in cryovials at -80°C. The whole blood EDTA pellets were submitted in two batches to the Veterinary Tropical Diseases Laboratory, Faculty of Veterinary Science, University of Pretoria for qPCR to be performed.

3.3 Methodologies

3.3.1 Quantitative real-time polymerase chain reaction (qPCR)

Nucleic acid was purified and extracted from the stored whole blood EDTA pellet (200 µL) using the MagMAX™ Total Nucleic Acid Isolation kit (Thermo Fisher Scientific, USA) and MagMAX™ Express Particle Processor following manufacturer instructions. The qPCR assays were performed using specific probes for *B. rossi* (TGGCTTTTGCCTATTA), *B. vogeli* (AGTTTGCCATTCGTTTGG), *E. canis* (AGCCTCTGGCTATAGGA) and *T. equi* (AAATTAGCGAATCGCATGGCTT) on the StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA). This was done according to standard operating procedures described for each of these pathogens in validation studies performed at the Veterinary Tropical Diseases Laboratory, Faculty of Veterinary Science, University of Pretoria (Bhoora *et al.*, 2010; Troskie *et al.*, 2018; Nkosi *et al.*, 2022). These validation studies reported acceptable efficiency for each assay. The qPCR cycling conditions consisted of one cycle of polymerase activation at 95 °C for 10 seconds and 40 cycles each of denaturing at 95 °C for one second and extension at 60 °C for 20 seconds. A no-template control assay is routinely run by the laboratory and because the tests were previously validated, a negative control group was not included in the study.

3.3.2 Haematology

The samples were analysed with the ADVIA 2101 (Siemens) automated haematology analyser. The following variables were produced and evaluated for possible trends that may indicate a co-infection:

1. Haemoglobin (HGB)
2. Red cell count (RCC)
3. Haematocrit (HCT)
4. Mean corpuscular volume (MCV)
5. Mean corpuscular haemoglobin (MCH)
6. Mean corpuscular haemoglobin concentration (MCHC)
7. Red cell distribution width (RDW)
8. White cell count (WCC)
9. Segmented neutrophils
10. Band neutrophils
11. Lymphocytes
12. Monocytes
13. Eosinophils
14. Basophils
15. Other white blood cells
16. Platelet count
17. New red blood cells per 100 white blood cells (NRB/100 WBC)
18. Reticulocyte percentage
19. ARC

Differential leukocyte counts and blood cell morphological evaluations were done by veterinary laboratory technicians per a standard protocol published in the Atlas of Veterinary Hematology, 1st Ed, 2001 (Blue, 2002). The presence of blood-borne parasites was visually noted, and the sample condition was assessed.

The haematocrit and regenerative response of the bone marrow, in terms of the ARC, was further investigated. The classification used for both the severity of anaemia and the reticulocyte response has previously been applied in a study investigating dogs naturally infected with *B. rossi* (Scheepers et al., 2011). Anaemia was classified as very severe if the HCT was <0.13 L/L, severe if HCT = $0.13-0.19$ L/L, moderate if HCT = $0.20-0.29$ L/L and mild if HCT = $0.30-0.36$ L/L (Scheepers et al., 2011). The reticulocyte response was classified as marked if the ARC was $>300 \times 10^9/L$, moderate if ARC = $150-300 \times 10^9/L$ and mild if ARC = $80-150 \times 10^9/L$. An ARC below $80 \times 10^9/L$ was classified as non-regeneration (Scheepers et al., 2011).

3.3.3 Serum protein biochemistry

Total protein (TSP) and albumin (Alb) concentrations were measured on the serum samples with the Cobas Integra 400 Plus (Roche) analyser, using the biuret method for total protein concentration measurement and the bromocresol green method for albumin quantification. The globulin (Glob) was also calculated from these measurements using a simple calculation (TSP - Alb = Glob).

3.4 Statistical analysis

Cases were categorised based on the *Babesia* species with which they were infected (i.e., *B. rossi* or *B. vogeli*) and the presence or absence of a co-infection with *E. canis* or *T. equi*. The prevalence of a co-infection was defined as the proportion of *Babesia*-infected dogs that tested positive on PCR for either *E. canis* or *T. equi*. Haematology and biochemistry results for the different groups were tabulated and reported as the median and interquartile range. Box plots were drawn to visually represent the distribution of the data, and the Mann-Whitney U test was used to test for differences between two population medians.

Specific population characteristics, haematology and biochemistry measures of interest were identified for further investigation. These measures of interest included sex, breed, age, duration of sickness, WCC, band neutrophil count, monocyte count, platelet count, ARC and serum Glob concentration. Variables were categorised as in previous studies on co-infections and the clinical description of *Babesia* infections (Reyers *et al.*, 1998; Keller *et al.*, 2004; Schoeman, Rees and Herrtage, 2007; Mellanby *et al.*, 2011; Leisewitz *et al.*, 2019a). Cross-tabulation and Fisher's exact tests were used to assess associations with infection status. Further to this, the PPVs and their 95% confidence intervals (CIs) for leukopenia ($<6 \times 10^9/L$), monocytosis ($>1.35 \times 10^9/L$), thrombocytopenia ($<200 \times 10^9/L$), band neutrophilia ($>0.5 \times 10^9/L$) and non-regenerative ARC ($<80 \times 10^9/L$) as indicators of co-infection with *B. rossi* and *E. canis* or *T. equi* were calculated. The PPVs were calculated as the proportion of dogs presenting with these haematological findings that were co-infected. The NPVs of a normal leukocyte count, monocyte count, platelet count, band neutrophil count, and a mild to marked regenerative ARC for the exclusion of a co-infection with *E. canis* or *T. equi* were also calculated. The NPVs were calculated as the proportion of dogs presenting with these haematological findings that were not co-infected. Statistical tests were performed using Microsoft Excel® (version 16, Microsoft Corporation, Washington, USA) and a commercial software package, (SPSS Statistics version 28® IBM, New York, USA), and a $P < 0.05$ was used to indicate significance.

Chapter 4: Results

4.1 Study sample characteristics

All 150 dogs naturally infected with *Babesia* spp. and sampled were included in the study. The sample included 84/150 (56%) male and 66/150 (44%) female dogs. Mixed-breed dogs (90/150 (60%)) were most prevalent, followed by pit bull terriers 25/150 (17%) and Boerboel dogs 15/150 (10%). The remaining breeds each represented less than 5% of the sample. The median age (interquartile range (IQR)) and weight (IQR) of all the infected dogs were 12 months (8–30) and 18 kg (12–25), respectively. The three *E. canis* – *Babesia* co-infected dogs were all male and consisted of one mixed breed, one Boerboel, and one Rottweiler. The median age (IQR) and weight (IQR) of these dogs were 18 months (9 – 24) and 27.4 kg (15.1 – 36.7), respectively. Evaluation of the sample characteristics is detailed in the supplementary material (Appendix IV).

4.2 Co-infection prevalence

Quantitative PCR results from the 150 samples showed 149/150 (99%) of the dogs to have an infection with *B. rossi*, of which 3/149 (2%) were co-infected with *E. canis*. The remaining dog, 1/150 (1%) had a *B. vogeli* mono-infection. None of the dogs were co-infected with *B. vogeli* and *E. canis*, nor with *B. rossi* and *B. vogeli*. No primary infections or co-infections with *T. equi* were detected. The estimated co-infection prevalence was therefore 2.0% (95%; CI: 0.4 – 5.7).

4.3 Haematology results

Haematology results were available for 148/150 cases, of which 144 were from the *B. rossi* group, three were from the co-infected group and the remaining one was infected with *B. vogeli*. There was no significant difference between the median leukocyte count, monocyte count, platelet count, HCT or ARC of the *B. rossi* and co-infected groups. In the *B. rossi* group, monocytosis was documented in 31/144 (21.4%) dogs, thrombocytopenia in 142/144 (98.6%) and 94/144 (52.8%) had a normal or low WCC. In the co-infected group monocytosis was documented in 1/3 (33.3%) dogs, thrombocytopenia in 3/3 (100%) and a normal or low WCC in 1/3 (33.3%).

The median (IQR) HCT value of the *B. rossi* mono-infected group was 0.18 L/L (0.13 – 0.27) along with a median (IQR) ARC value of 102.1 x10⁹/L (52.9 – 175.1). The median (IQR) HCT and ARC for the co-infected group was 0.18 L/L (0.16 – 0.21); *P* = 0.927 and 38.5 x 10⁹/L (31.3 – 163.5); *P* = 0.279 respectively. No association could be made concerning the degree of anaemia or the reticulocyte response and the co-infection status of the dogs.

An interesting finding was that pit bull terriers, which represented the largest group of purebred dogs, had significantly lower median (IQR) haematocrit and ARC values compared to mixed-breed dogs and other purebred dogs. Pit bull terriers had a median (IQR) HCT of 0.14 L/L (0.11 – 0.2) compared to mixed-breed dogs 0.2 L/L (0.13 – 0.28); $P = <0.006$ and other purebred dogs 0.18 L/L (0.14 – 0.25); $P = <0.042$. This breed had a median (IQR) ARC of $59.9 \times 10^9/L$ (29.2 – 107.6) compared to mixed-breed dogs $107.85 \times 10^9/L$ (55.55 – 205.35); $P = <0.004$ and other purebred dogs $140.2 \times 10^9/L$ (52.9 – 175.1); $P = <0.009$. The findings are reported in Table 1 as median (IQR).

Table 1: Haematocrit and bone marrow response comparison among mixed-breed and purebred dogs

Parameter	Pit bull terriers (n=25)	Mixed-breed dogs (n=90)	P-value ¹	Purebred dogs excluding pit bull terriers (n=35)	P-value ²
Haematocrit (L/L)	0.14 (0.11 – 0.2)	0.2 (0.13 – 0.28)	0.006	0.18 (0.14 – 0.25)	0.042
Absolute Reticulocyte Count ($\times 10^9/L$)	59.9 (29.2 – 107.6)	107.85 (55.55 – 205.35)	0.004	140.2 (52.9 – 175.1)	0.009

1. P -values comparing the HCT and ARC of pit bull terriers and mixed-breed dogs
2. P -values comparing the HCT and ARC of pit bull terriers and other purebred dogs

4.4 Serum protein biochemistry results

Serum biochemistry results were available for 140 dogs in the *B. rossi* group, all three dogs in the co-infected group, and the one infected with *B. vogeli*. The median total serum protein, albumin and globulin concentrations for the *B. rossi* group were not significantly different from the co-infected group. Complete haematology and serum protein biochemistry results are available in Table 2 on the following page, with results reported as median (IQR).

Table 2: Complete haematology and serum protein biochemistry results for the dogs infected with *Babesia rossi*, *Babesia vogeli* and those co-infected with *B. rossi* and *Ehrlichia canis* at the time of presentation

Variable	Laboratory reference interval	<i>Babesia rossi</i> group (n = 146)	<i>Babesia vogeli</i> group (n=1)	Co-infection group (n=3)	P-value ¹
HGB	120 – 180 g/L	53 (39 – 80.75)	120	49 (48 – 67)	0.962
RCC	5.5 – 8.5 x 10 ¹² /L	2.31 (1.67 – 3.66)	5.87	2.23 (2.1 – 2.99)	0.895
HCT	0.37 – 0.55 L/L	0.18 (0.13 – 0.27)	0.42	0.18 (0.16 – 0.21)	0.927
MCV	60 – 77 fL	75.7 (71.23 – 80.58)	72	74 (71.6 – 84.1)	0.870
MCH	Pg (NRR ²)	23 (21.9 – 23.98)	21.4	23.1 (22.4 – 28.1)	0.523
MCHC	32 – 36 g/dL	30 (28.5 – 31.4)	29.7	29.3 (27.5 – 31.2)	0.619
RDW	% (NRR)	16.7 (14.7 – 20.2)	13.8	14.6 (14.4 – 17.8)	0.390
WCC	6 – 15 x 10 ⁹ /L	7.88 (5.54 – 13.19)	1.17	11.42 (2.92 – 12.88)	0.932
Segmented neutrophils	3 – 11.5 x 10 ⁹ /L	5.23 (3.51 – 8.6)	0.44	7.31 (2.13 – 8.63)	0.729
Band neutrophils	0 – 0.5 x 10 ⁹ /L	0.3 (0.13 – 0.82)	0	0.91 (0.03 – 1.42)	0.993
Lymphocytes	1 – 4.8 x 10 ⁹ /L	1.52 (1.03 – 2.31)	0.47	1.42 (0.26 – 2.63)	0.619
Monocytes	0.15 – 1.35 x 10 ⁹ /L	0.75 (0.44 – 1.3)	0.15	0.5 (0.46 – 1.42)	0.839
Eosinophils	0.1 – 1.25 x 10 ⁹ /L	0 (0 – 0.12)	0.01	0 (0 – 0.11)	0.717
Basophils	0 – 0.1 x 10 ⁹ /L	0	0.06	0	1.000
Other white blood cells	% (NRR)	0	0.1	0	1.000
Platelet count	200 – 500 x 10 ⁹ /L	61 (39 -86.75)	70	61 (43 – 75)	0.925
NRB/ 100 WBC	0 - 9	4 (1 – 9)	0	2 (0 – 6)	0.477
Reticulocytes	% (NRR)	3.9 (1.8 – 9.2)	1.2	1.7 (1.1 – 7.8)	0.353
ARC	x 10 ⁹ /L (NRR)	102.1 (52.9 – 175.1)	68	38.5 (31.3 – 163.5)	0.279
Total serum protein	56 – 73 g/L	57.5 (51.35 – 63.65)	42.2	60.6 (55.1 – 74.2)	0.295
Albumin	28 – 41 g/L	23.5 (19.65 – 27.18)	23.9	26 (25.6 – 29)	0.165
Globulin	20 – 41 g/L	32.65 (27.58 – 38.85)	18.3	31.6 (29.5 – 48.2)	0.682

1. P-values comparing haematology and serum protein biochemistry results of the *B. rossi* infected group and the co-infected group

4.5 Associations with co-infection and predictive values

Specific population characteristics and haematological and biochemistry measures of interest were identified to investigate potential association with a co-infection. Cross-tabulations and Fisher’s exact tests showed no significant associations between investigated variables and co-infection (Table 3). For all haematological measurands investigated the PPV (<4%) was very low and the NPV very high (>98%), as can be seen in Table 4.

Table 3: Variables of interest selected for further investigation of possible association with co-infections

Variable	n	<i>B. rossi</i> only (%)	Co-infection (%)	<i>P</i> -value
Sex				0.255
Male	83	80 (96%)	3 (4%)	
Female	66	66 (100%)	0 (0%)	
Breed				0.565
Purebred	60	58 (97%)	2 (3%)	
Mixed-breed	89	88 (99%)	1 (1%)	
Age				0.633
<12 months	55	54 (98%)	1 (2%)	
12-24 months	53	51 (96%)	2 (4%)	
>24 months	41	41 (100%)	0	
Duration of sickness				0.583
1-3 days	77	75 (97%)	2 (3%)	
4-6 days	45	45 (100%)	0	
>7 days	27	26 (96%)	1 (4%)	
Leukocyte count				1.000
Leukopenia (<6 x10 ⁹ /L)	46	45 (98%)	1 (2%)	
Normal (6-15 x10 ⁹ /L)	74	72 (97%)	2 (3%)	
Leukocytosis (>15 x10 ⁹ /L)	27	27 (100%)	0	
Band neutrophil count				0.295
Normal (0 – 0.5 x10 ⁹ /L)	94	93 (99%)	1 (1%)	
High (> 0.5 x10 ⁹ /L)	53	51 (96%)	2 (4%)	
Monocyte count				0.524
Monocytosis (> 1.35 x 10 ⁹ /L)	32	31 (97%)	1 (3%)	
Normal or low (< 1.35 x 10 ⁹ /L)	115	113 (98%)	2 (2%)	
Platelet count				0.959
Normal or high platelet count (> 200 x10 ⁹ /L)	2	2 (100%)	0	
Thrombocytopenia (< 200 x10 ⁹ /L)	145	142 (98%)	3 (2%)	
Absolute reticulocyte count				0.506
Mild to marked ARC (>80 x10 ⁹ /L)	63	62 (98%)	1 (2%)	
Inappropriate ARC (<80 x10 ⁹ /L)	64	62 (97%)	2 (3%)	
Globulin concentration				0.596
Low (<20g/L)	7	7 (100%)	0	
Normal (20 – 41 g/L)	106	104 (98%)	2 (2%)	
High (>41 g/L)	30	29 (97%)	1 (3%)	

Table 4: Positive and negative predictive values of haematological findings for co-infection in *B. rossi* infected dogs

Variable	Cut-off	PPV (%) (95% CI)	NPV (%) (95% CI)
Leukopenia	<6×10 ⁹ /L	2.2% (0.1 – 11.5)	98.0% (93.0 – 99.8)
Monocytosis	>1.35×10 ⁹ /L	3.1% (0.1 – 16.2)	98.3% (93.9 – 99.8)
Thrombocytopenia	<200×10 ⁹ /L	2.1% (0.4-5.9)	100% (15.8-100)
Non-regenerative ARC	<80×10 ⁹ /L	2.9% (0.3-9.9)	98.7% (93.0 - 99.97)
Band neutrophilia	>0.5 x 10 ⁹ /L	3.5% (0.5-13.0)	98.9% (94.2-99.97)

Chapter 5: Discussion

The study results demonstrated a low prevalence of co-infections with multiple tick-borne pathogens in the Eastern Cape Province, which has not previously been identified in this region. A co-infection prevalence of 2% with *B. rossi* and *E. canis* was found. No co-infections with *B. rossi* and *B. vogeli* were reported and neither with *B. vogeli* and *E. canis*, which share the same tick vector. *Theileria equi* was not identified in any of the samples.

From our binomial exact confidence interval calculation, there may still be some uncertainty regarding the true estimate of prevalence likely related to our sample size. Sample size affects the precision of estimating prevalence; thus, a smaller sample size decreases the confidence in the accuracy of the estimated prevalence (Laurin *et al.*, 2021). Previous studies that investigated tick-borne disease prevalence and the prevalence of co-infections with multiple tick-borne pathogens in the South African context, however, have yielded similarly low prevalence results to what has been found in this study (Matjila *et al.*, 2008a; Rautenbach, Schoeman and Goddard, 2018).

The PCR method has been applied for the detection of various organisms of veterinary and medical importance. It is routinely used for the detection of different species of *Babesia*, *Theileria* and *Ehrlichia* and have been reported to have higher sensitivity and specificity when compared to serological assays (Birkenheuer, Levy and Breitschwerdt, 2003; Geysen, Delespoux and Geerts, 2003; Buling *et al.*, 2007). Conventional PCR operates by identifying whether a specific DNA sequence is present or absent qualitatively (Loftis and Reeves, 2012). It has been well described as an effective method used to distinguish *Babesia* species from one another and specifically its use to distinguish *B. rossi* from *B. vogeli* (Birkenheuer, Levy and Breitschwerdt, 2003). The RLB assay, a PCR combined with a blotting procedure, can simultaneously detect and identify various species of tick-borne pathogens in one sample. The limitations of the RLB assay are that it requires a large number of samples to run cost-effectively, and it is a labour-intensive practice with a slow turnaround time (Allsopp *et al.*, 1993; Troskie *et al.*, 2018). It is however still a suitable and effective screening tool. Quantitative real-time PCR is a more recently developed mechanism that has greatly improved the sensitivity and specificity of molecular detection and diagnosis of various organisms of veterinary and medical importance (Lindh *et al.*, 2007; Wengi *et al.*, 2008, Bhoora *et al.*, 2010). It works on detecting and quantifying specific DNA sequences which all take place in a single tube during the cycling process. This makes it superior to the RLB and conventional PCR methods as it eliminates the need for post-PCR manipulation and reduces the risk of sample contamination. Quantitative PCR tests have been developed for

several haemoparasitic pathogens including *B. rossi* and *B. vogeli*, *E. canis* and *T. equi* (Bhoora *et al.*, 2010, Troskie *et al.*, 2018, Nkosi *et al.*, 2022). A qPCR assay is available for *E. ruminantium* (Steyn *et al.*, 2008) but it was not included in our study due to funding limitations.

Fluorescent antibody testing has been used widely for the diagnosis of *E. canis* since its development in 1972 (Kelly, 2000). It is a serologic testing method in which an antigen is detected through fluorochrome tagging of agent-specific antibodies. A study conducted on a cohort of dogs from the Onderstepoort region to investigate *Cowdria ruminantium* as an antigen for the detection of *Ehrlichia canis* antibodies argued for a much higher incidence of co-infection with babesiosis and ehrlichiosis based on serological diagnosis. It has however been reported that animals can test positive using IFAT to detect *Ehrlichia* antibodies for an extended period following infection without being clinically sick (Kelly, 2000). Furthermore, IFAT has the potential to cross-react with other *Ehrlichia* species or other closely related organisms from different genera which may further mislead the practitioner in making a definitive diagnosis (Bunroddith *et al.*, 2018). Detection of *E. canis* antibodies in PCR-negative dogs in endemic areas can reflect prior exposure to the organism without active infection, whereas the detection of *E. canis* DNA in seronegative dogs could be explained by problems related to the technique or by the absence of antibodies during acute phases of infection by *E. canis* (Rajagopal *et al.*, 2009). Positive results using the PCR technique would support the diagnosis of an active infection when antibodies are detected in healthy/ subclinical infected or clinically sick dogs (Rajagopal *et al.*, 2009).

Co-infections have been studied widely and it is suggested that the effects of the initial infecting organism or the co-infecting organism can be either enhanced, suppressed, or unaffected based on factors associated with changes in the microenvironment or immunological factors (Cox, 2001). It is known that *B. rossi* infection is associated with marked cytokine derangements, often leading to what has been termed a “cytokine storm” reflecting the hosts’ response to infection (Leisewitz *et al.*, 2019b). These cytokines are intended to stimulate immunity but also play a pivotal role in the pathogenesis of the disease (Day, 2011; Leisewitz *et al.*, 2019b). Significant alterations in key cytokines such as interleukin (IL)-6, IL-8, IL-10 and monocyte chemotactic protein-1 (MCP-1) in dogs infected with *B. rossi* compared to a healthy control group, have been reported (Goddard *et al.*, 2016). Notably, increased serum concentrations of IL-6 and MCP-1 were associated with poorer outcomes, highlighting the potential role of an excessive pro-inflammatory response in disease severity (Goddard *et al.*, 2016). In a previous study, lymphocyte immunophenotyping in dogs infected with *B. rossi* showed a marked reduction in T lymphocytes, particularly cluster of differentiation (CD)4⁺ and CD8⁺

subsets, with more significant reductions in dogs with complicated compared to uncomplicated infections (Rautenbach *et al.*, 2017). This study theorised that the reduction in T lymphocytes, particularly that of CD4⁺ T lymphocytes, may indicate immunosuppression, potentially exacerbated by the reported immunomodulatory effects of diminazene aceturate treatment (Rautenbach *et al.*, 2017). The reduction reported in CD8⁺ T lymphocytes is interesting, as the production of interferon-gamma (IFN- γ) from these, has been shown to inhibit the development of exoerythrocytic forms and ultimately circulating parasitaemia in mice experimentally infected with human malaria (Schofield *et al.*, 1987). In contrast, however, a cytokine profile indicative of a T-helper 1 (Th1)-like response, with high levels of IFN- γ and tumour necrosis factor-alpha (TNF- α) has been observed in *Babesia* spp. infection in mouse models (Hemmer *et al.*, 2000). In mice experimentally infected with *Ehrlichia* spp., IFN- γ plays a significant role in the cell-mediated immune response by activating macrophages and inhibiting replication of *Ehrlichia* spp. (Bitsaktsis *et al.*, 2004). Additionally, in cytokine gene knockout experiments, TNF- α was found to aid in controlling *Ehrlichia* spp. infection (Bitsaktsis *et al.*, 2004). It may thus be possible that the pro-inflammatory immune response elicited in the face of an active *B. rossi* infection could inhibit other pathogens from establishing themselves in the host environment.

The immune-modulatory effect of certain pathogens is highlighted by evidence of vaccinations resulting in a change in the host's susceptibility to unrelated pathogens (Knobel *et al.*, 2022). This was shown to be the case in a study by Gessner *et al.* (2017), who suggested that rabies vaccination may enhance multiple immune responses among children, as is seen by the decreased risk of central nervous system diseases such as cerebral malaria. This theorised protective effect of *B. rossi* infection may explain the low prevalence of co-infections, however, the lack of testing for other *Ehrlichia* and *Theileria* species (or other tick-borne pathogens) in this study could also be part of the reason for the low co-infection prevalence. Interactions with unrelated pathogens have not been described in dogs vaccinated against babesiosis. It may be possible that the use of vaccine adjuvants known to elicit a Th-1 response, as seen in some vaccine antigens (e.g. from influenza virus and *Mycobacterium tuberculosis*) incorporated into cage-like nanoparticle structures or ISCOMs, can enhance the host's response to other pathogens (Rathinasamy *et al.*, 2019). The use of ISCOMs has been suggested as a befitting adjuvant for babesiosis vaccines (Rathinasamy *et al.*, 2019), however further research is necessary to explore this as an option.

Another suggestion may be that attachment of the host vector, *Haemaphysalis elliptica*, results in the development of cutaneous hypersensitivity inhibiting attachment of other ticks and infection with *Ehrlichia* or *Theileria* species. The immune reaction induced by *Rhipicephalus sanguineus* has been

studied and indicated a lack of acquired resistance to this vector in dogs which was suggested to be due to a possible tick saliva-modulating effect (Szabo *et al.*, 1995). No data is available on the host immune response to *H. elliptica*.

Babesia rossi mono-infections accounted for almost all dogs infected and clinically sick in our study, with 149/150 (99.33%) testing positive using qPCR. *Babesia vogeli* infection accounted for only 1/150 (<1%) and was diagnosed in a clinically unwell mixed-breed puppy of 12 weeks of age. This is the first known study to definitively confirm the presence of *B. vogeli* with the use of PCR in the Eastern Cape province. Before this, *B. vogeli* has only been reported in the Gauteng, Freestate, and Mpumalanga provinces of South Africa (Matjila *et al.*, 2004; Kolo *et al.*, 2014). *Babesia vogeli* has been reported to be of greater significance in puppies and typically causes anaemia (Schoeman, 2009). The overall disease presentation of dogs infected with *B. rossi* in our study mirrored previous descriptions of the disease when comparing haematological and serum protein biochemistry investigations as well as the clinical signs of the disease (Schoeman, 2009; Rautenbach, Schoeman and Goddard, 2018; Leisewitz *et al.*, 2019a). It was confirmed that monocytosis, thrombocytopenia and a normal or low leukocyte count, are all common findings associated with a *B. rossi* mono-infection.

It has been reported that dogs infected with *B. rossi* may present with an inappropriate regenerative response in the face of severe anaemia (Reyers *et al.*, 1998; Scheepers *et al.*, 2011) which can be attributed to multiple factors. The destruction of both infected and uninfected red blood cells resulting in iron-restrictive erythropoiesis and acquired iron deficiency due to a direct suppressive effect of the *Babesia* piroplasm has been suggested (Seejarim *et al.*, 2023). A recent study investigating histopathological bone marrow findings in dogs with natural *B. rossi* infection however found sufficient iron stores within the bone marrow, which does not support these theories (Bumby *et al.*, 2022). Canine babesiosis and falciparum malaria have much in common regarding their pathophysiology (Reyers *et al.*, 1998) and thus the potential exists that the same processes in play during falciparum malaria might also inhibit regeneration in canine babesiosis infections (Scheepers *et al.*, 2011; Seejarim *et al.*, 2023). An example is the host immune response which triggers the release of proinflammatory cytokines, which is known to suppress erythrocyte lifespan, hinder iron mobilisation or utilisation, and impair red blood cell generation due to a lack of response to erythropoietin (Scheepers *et al.*, 2011; Bumby *et al.*, 2022). Initial reports on “bilious fever” (which we now call babesiosis) in South Africa dating as far back as 1893, reported purebred dogs to be at a greater risk of infection than local dogs (Penzhorn, 2020). It has also been reported that dogs belonging to traditional fighting breeds (such as pit bull terriers) were overrepresented in a subgroup

of non-anaemic dogs which died because of babesiosis. This was thought to be due to a possible genetically endowed unusually reactive immune system resulting in an overwhelming inflammatory response (Reyers *et al.*, 1998). It may be possible that this suggested hereditary overwhelming inflammatory response in fighting breeds could be a further contributing factor to the poor regenerative response seen in the pit bull terrier group in our study sample. It is also a very well-known fact that pit bull terriers (along with American Staffordshire terriers), for an unknown reason, are overrepresented in cases of dogs infected with *B. gibsoni* in the USA (Macintire *et al.*, 2002). Further research is needed to determine if a specific host response is contributing to these breeds being more susceptible to *B. gibsoni* infection (Macintire *et al.*, 2002).

A survey conducted in 1993 to address several variables concerning canine babesiosis demonstrated that the majority of veterinary practitioners in South Africa consider ehrlichiosis as an important complicating factor in babesiosis cases. Of these practitioners, 89% made the diagnosis of a co-infection with *Ehrlichia* based on *E. canis* parasites being identified on a blood smear (Collett, 2000). The identification of *Ehrlichia* morulae on a peripheral blood smear can be very challenging and time-consuming. It has been reported to have varying sensitivity among investigators with values as low as 0% and as high as 84% (Mylonakis *et al.*, 2003). Diagnosis using peripheral blood smear examination is thus discouraged. The superimposition of platelets over monocytes and phagocytosed nuclear material within monocytes may be confused for *Ehrlichia* morulae and therefore careful observation and experience with their cytological morphology is of utmost importance to avoid false positive diagnosis of ehrlichiosis (Mylonakis *et al.*, 2003). Other cytological methods are more reliable for diagnosing ehrlichiosis. Examination of buffy coat smears alone had a sensitivity of 66% (Mylonakis *et al.*, 2003) however another study published this same technique to have a sensitivity of only 20.4% (Sainz *et al.*, 2000). Lymph node aspirates were found to have a sensitivity of 60.4% and morulae were detected with greater ease in dogs that presented with peripheral lymphadenopathy (Mylonakis *et al.*, 2003). The combined sensitivity of buffy coat smear and lymph node aspirate analysis was high at 74% and it is suggested that, at least in the acute phase of ehrlichiosis, its use for the detection and diagnosis of ehrlichiosis is justifiable. These techniques however are equally if not more time-consuming and the same limitations and errors might persist if experience is lacking.

Following on from the aforementioned survey, 72% of practitioners used monocytosis or cytological evidence of monocyte activation as an indicator of co-infection with ehrlichiosis, whilst 42% used thrombocytopenia and 23% a normal or low white cell count (Collett, 2000). Very low PPVs and high NPVs for thrombocytopenia (PPV: 2.1%; NPV: 100%) or leukopenia (PPV: 1.3%; NPV: 97.4%) have

previously been reported (Rautenbach, Schoeman and Goddard, 2018). We also found no statistically significant association between the specific population characteristics, haematological and biochemistry measures of interest we identified and an *E. canis* co-infection, and when testing dogs in our study population with a known 2% (95% CI: 0.4-5.7) prevalence of co-infection, for leukopenia, monocytosis, thrombocytopenia, non-regenerative ARC or band neutrophilia, the probability that a dog with babesiosis was co-infected, at most increased to only 3.5% (CI: 0.5-13) when a band neutrophilia was seen. Our data, along with results from a previous study (Rautenbach, Schoeman & Goddard, 2018), suggests that veterinary practitioners who base their diagnosis of a co-infection in dogs with babesiosis on haematology findings including monocytosis, thrombocytopenia or leukopenia (Collett, 2000) are likely to overestimate such co-infections.

The identification of clinical or clinicopathological associations with *Babesia* spp. and *Ehrlichia* spp. co-infections are complicated by how uncommon these co-infections are and the overlapping features that dogs with these conditions may present with. Therefore, it is recommended that further investigations should be performed when there is a suspicion of a co-infection with *Babesia* spp. and *Ehrlichia* spp. Serology which relies on antibody testing would largely be unreliable in the South African context based on the endemic status of the diseases here. The most sensitive test would be PCR to detect an active infection of ehrlichiosis. This would be considered the gold standard before initiating targeted therapy to practice responsible antimicrobial stewardship. The limitation of this technique is that it requires specialised equipment and expertise that might not always be accessible or affordable, especially when working in the primary animal healthcare sector.

Further testing for other *Ehrlichia* or *Theileria* species or the genus was not done, which is a limitation of this study. Future work should make use of catch-all probes. The prevalence of *E. ruminantium* infection in dogs is not well studied and documented, however from the available literature, it is unlikely that a greater prevalence of *Babesia* and *Ehrlichia* co-infections would have been seen if testing for *E. ruminantium* was included (Matjila *et al.*, 2008b; Allsopp, 2010; Rautenbach, Schoeman and Goddard, 2018). Additionally, it is not definitively known whether dogs can become clinically sick or remain asymptomatic carriers of *E. ruminantium* (Allsopp and Allsopp, 2001; Allsopp, 2010). *Theileria equi* has been found to cause clinical disease in dogs in South Africa (Rosa *et al.*, 2014). One study showed dogs to be infected with a *Theileria* sp. closely related to an unidentified *Theileria* sp. infecting antelopes (Matjila *et al.*, 2008b). However, there is no specific PCR primer available for this species and it has also not been identified in the Eastern Cape province before, thus making its presence in the dog population less likely. As reflected in our results, the study population, specifically

the group of co-infected dogs, was too small to detect statistically significant associations between certain variables of interest and a co-infection with *E. canis* and canine babesiosis. The findings of this study are limited to a population of dogs sampled at the Mdantsane State Veterinary Clinic in the Eastern Cape province. Variability in environmental factors, tick distribution, and host characteristics across different regions of the province may influence the prevalence and dynamics of tick-borne diseases. Future studies involving broader geographic sampling are necessary to better understand the epidemiology of these diseases across the province. Due to funding limitations, further disease testing to rule out co-morbidity with other non-tick-borne infectious diseases could not be done. Such co-morbidities were, however, considered unlikely based on the patient's history and a thorough clinical examination performed by the primary investigator.

Chapter 6: Conclusion

Co-infection with *E. canis* or *Theileria equi* is uncommon in *Babesia*-infected dogs from the Eastern Cape province. *Babesia rossi* was the most prevalent *Babesia* species infecting dogs with clinical signs of disease. The overall disease presentation of dogs infected with *B. rossi* mirrored previous descriptions. Pit bull terriers had significantly lower HCT and ARC values compared to crossbreed dogs and other purebred dogs. For all haematological measurands investigated for association with co-infections, the PPV (<4%) was very low and the NPV very high (>98%). This is explained by the interdependence between prevalence and predictive values (Rautenbach, Schoeman & Goddard, 2018; Rouvière, Souchon & Melodelima, 2018). It is recommended that further investigations on a larger sample size of dogs should be performed to document the true co-infection rate with *Babesia* spp. and *Ehrlichia* or *Theileria* species. A large cohort of co-infected animals will be required to establish clinical or clinicopathological associations with co-infections. Future work would also benefit from making use of catch-all probes to detect various species in the *Ehrlichia* or *Theileria* genus. Serology which relies on antibody testing would largely be unreliable in the South African context based on the endemic status of the diseases here. The most sensitive test would be PCR to detect an active *Ehrlichia* infection before initiating therapy to practice responsible antimicrobial stewardship.

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Appendix I:



Faculty of Veterinary Science
Animal Ethics Committee

8 October 2020

Approval Certificate
New Application

AEC Reference No.: REC061-20
Title: The prevalence of Ehrlichia or or Theileria co-infections in free roaming owned dogs with babesiosis
Researcher: Mr HPP Cloete
Student's Supervisor: Prof JP Schoeman

Dear Mr HPP Cloete,

The **New Application** as supported by documents received between 2020-08-23 and 2020-10-02 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-10-02.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number
Dogs (Canine) Privately owned dogs	150
Samples	
Whole blood	150 (6 ml each)
Peripheral blood smear	150

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-10-08.
3. Please remember to use your protocol number (REC061-20) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. As part of your approval, the committee requires that you record a **short video footage** of major animal procedures approved in your study. **The committee may request them for monitoring purposes at any later point.**

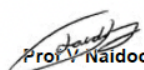
Ethics approval is subject to the following:

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

Room 6-13, Arnold Theiler Building, Onderstepoort
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Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseense tša Bongakadiruiwa

We wish you the best with your research.
Yours sincerely



Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Appendix II:

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



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Owner:	
Owner contact no:	
Species:	
Sex:	
Age:	
Breed:	
Weight:	
Patient name:	
Patient number:	
Patient photographed:	

History

Chief complaint
Duration of illness
When did the dog last eat?
Treatment received after diagnosis and collection of samples

Physical examination

Parameter	At initial presentation		
Habitus (1 – 4+)			
Vital Signs	T:	P:	R:
Mucous Membranes and CRT			
Peripheral Lymph nodes			
Tick infestation	Present	Absent	
Tick position on body			

Appendix III:

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



UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

Project title: The prevalence of Babesia and Ehrlichia co-infections in free roaming owned dogs in the Eastern Cape, South Africa

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

Please encircle Yes or No where necessary

1. Has your dog received any treatment for *Babesiosis* or *Ehrlichiosis* within the last week? Yes / No
2. Have you read the information sheet on these two tick-borne diseases? Yes / No
3. Have you had the opportunity to ask questions about the research project? Yes / No
4. Have you received satisfactory answers to your questions? Yes / No
5. Have you received enough information about this study? Yes / No
6. Do you grant consent that blood samples may be drawn from your dog? Yes / No

I, _____ (name and surname),
hereby give permission that my dog _____ (patient's name),
a _____ (breed of dog) may participate in
this clinical study conducted at the Mdantsane State Veterinary Clinic under the authority of the
University of Pretoria.

I understand that this study will in no way harm my dog. I also understand that I will not be liable for the costs of the additional tests as part of the clinical study but that I will be liable for costs pertaining to the treatment of *Babesiosis* and *Ehrlichiosis* and any complications that may arise as a result thereof.

Signed at Mdantsane on this _____ day of _____ 20_____

Signature Owner/authorised agent _____

Home Tel: _____

Work Tel: _____

Cell No: _____

Appendix IV:

Babesia, Ehrlichia, Theileria
co-infection study (Cloete)

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



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FACULTY OF VETERINARY SCIENCE

Owner:
Species:
Age:
Sex:
Breed:
Patient number:

Laboratory Number

Clin Path Number

Clinician: HPP Cloete

Provisional Diagnosis: Babesia, Ehrlichia, Theileria co-infection study	
Cost point number: N1B344001L. D-Lab Info - Owner: 368562, Client/Acc: 368561	
Date:	
Laboratory Tests Requested	CBC, 1 smears archived
	TSP, Alb, Glob
Was bleeding easy?	
Was animal excited before or during?	
Clinician initials MANDATORY	
<i>Serum and EDTA pellet to be stored in biobank see lab SOP.</i>	

Appendix V:

MSc project title: The prevalence of Ehrlichia and Theileria co-infections in Babesia infected dogs

University of Pretoria Ethics: REC061-20

Cost point: N1B344001L

Student: Dr. HPP Cloete

Research supervisors: Prof JP Schoeman, Dr. Y Rautenbach

Data collection time period: 07-12-2020 – 31-05-2021

SOP for the samples to be submitted to the Clinical Pathology Laboratory:

EDTA-anticoagulated whole blood sample and two fixed blood smears	<ol style="list-style-type: none"> 1. Perform Complete Blood Count 2. Spin down and aliquot the red cell pellet into a cryovial to be stored at -80°C
Serum collection tube	<ol style="list-style-type: none"> 1. Perform the requested biochem panel (TSP, Albumin and Globulin) 2. Divide the remaining serum between 2 cryovials and store at -80°C

- All results should be archived for a minimum of five years.
- All samples stored in the -80°C freezer should be stored in labelled cryovials in the upright -80°C freezer, door 3, rack 4.
- The cryovials should be labelled with the patient reference/ identification number (as on the Clin Path submission form) (i.e. BET001 up to BET150), the date of submission and the sample type
- Please e-mail each individual case results as completed also include ADVIA printout (henrypp.cloete@gmail.com)

Appendix VI:

Population characteristics for the various groups within the cohort at the time of presentation.

Where applicable numerical values reported as median (IQR)

Characteristic	Entire population	<i>Babesia rossi</i> group	<i>Babesia vogeli</i> group	Co-infection group
Age (months)	12 (8 – 30)	12 (8 – 30)	3	18 (8 - 24)
Weight (kg)	18 (12 – 25)	18 (12 – 25)	3	27.4 (15.1 - 36.7)
Sex	Male (84) Female (66)	Male (80) Female (66)	Male (1)	Male (3)
Breeds (Number of animals)	Mixed breed (90) Pit bull (25) Boerboel (15) Greyhound (5) Rottweiler (5) German Shepherd (4) Jack Russell Terrier (2) Coonhound (2) Pug (1) Husky (1)	Mixed breed (88) Pit bull (25) Boerboel (14) Greyhound (5) Rottweiler (4) German Shepherd (4) Jack Russell Terrier (2) Coonhound (2) Pug (1) Husky (1)	Mixed breed (1)	Mixed breed (1) Rottweiler (1) Boerboel (1)
Primary complaint (Number of animals)	Inappetence (137) Lethargy (77) Haemoglobinuria (16) Weight loss (12) Vomiting (6) Diarrhoea (1) Epistaxis (1) Aborted (1)	Inappetence (133) Lethargy (74) Haemoglobinuria (16) Weight loss (11) Vomiting (6) Diarrhoea (1) Epistaxis (1) Aborted (1)	Inappetence (1) Weight loss (1)	Inappetence (3) Lethargy (3)
Duration of sickness (days)	3 (2 – 5)	3 (2 – 5)	3	2 (1 – 14)
Rectal temperature (°C)	40 (39.9 – 40.4)	40 (39.9 – 40.4)	<35	39.4 (39.3 – 40.4)
Peripheral lymph node (LN) enlargement (Number of animals)	Nonpalpable (53) 1-3 LN palpable (50) >3 LN palpable (47)	Nonpalpable (51) 1-3 LN palpable (48) >3 LN palpable (47)	Nonpalpable (1)	Nonpalpable (1) 1-3 LN palpable (2)

Appendix VII:

Peer-reviewed journal publication:

Cloete, H.P.P., Rautenbach, Y., Leisewitz, A.L., Mellanby, R.J., Thompson, P.N., & Schoeman, J.P., 2024, 'Prevalence of co-infections with *Ehrlichia* spp. or *Theileria* spp. in dogs naturally infected with babesiosis in the Eastern Cape province', *Veterinary Parasitology: Regional Studies and Reports* 54. DOI: 10.1016/j.vprsr.2024.101092.

Veterinary Parasitology: Regional Studies and Reports 54 (2024) 101092



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Veterinary Parasitology: Regional Studies and Reports

journal homepage: www.elsevier.com/locate/vprsr



Original Article

Prevalence of co-infections with *Ehrlichia* spp. or *Theileria* spp. in dogs naturally infected with babesiosis in the Eastern Cape province

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ARTICLE INFO

Keywords:
Babesia rossi
Ehrlichia canis
Co-infection
protozoa
Canine
Predictive value

ABSTRACT

Background: Canine babesiosis and ehrlichiosis are tick-borne infections of great significance in South Africa. Theileriosis in dogs in South Africa is still poorly understood. Co-infection with multiple tick-borne diseases has been documented and is perceived as a common occurrence in South Africa.

Objectives: The main objective of this study was to determine the prevalence of co-infections with *Ehrlichia canis* or *Theileria equi* in dogs with babesiosis in the Eastern Cape province. There is a lack of data on canine tick-borne disease distribution in this region. Possible associations of population characteristics and haematological and biochemistry measures with a co-infection of *E. canis* or *T. equi* in these dogs were also investigated.

Method: The study population included 150 dogs naturally infected with babesiosis that presented to the Mdantsane State Veterinary Clinic between January 2021 and November 2021. Quantitative polymerase chain reaction was used to confirm the *Babesia* spp. that the dogs were infected with and to identify co-infections. Association with co-infection for the following parameters were evaluated: sex, breed, age, duration of illness, leukocyte count, band neutrophil count, monocyte count, platelet count, ARC, and serum globulin concentration. Positive and negative predictive values of monocytosis, leukopenia, band neutrophilia, thrombocytopenia, and non-regenerative absolute reticulocyte count for co-infection were also calculated.

Results: *Babesia rossi* was identified in 149/150 samples and *B. vogeli* in only 1/150 samples. A co-infection prevalence of 2.0% (3/149; 95% CI: 0.4–5.7) with *B. rossi* and *E. canis* was found. No other co-infections were reported. No investigated variables showed significant associations with co-infections. Monocytosis, in particular, was not associated with co-infection.

Conclusion: Co-infection with other tick-borne diseases in dogs with babesiosis is uncommon in the Eastern Cape province. These findings raise the possibility that *B. rossi* may have a protective effect against other tick-borne diseases.

Appendix VIII:

Poster Abstract Presentation:

ECVIM-CA 34th Annual Congress, 5-7 September 2024, La Cité Centre de Congrès, 50 quai Charles de Gaulle, 69006, Lyon, France.



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Prevalence of co-infections with *Ehrlichia* spp. or *Theileria* spp. in dogs naturally infected with babesiosis in the Eastern Cape province

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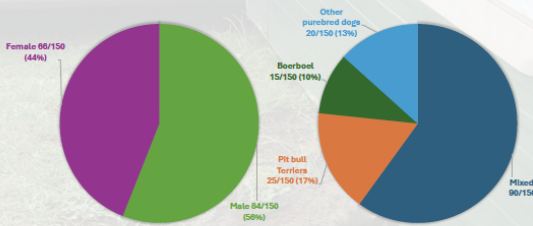
Introduction

Canine babesiosis and ehrlichiosis are tick-borne infections of great significance in South Africa; both may result in severe clinical disease. In South Africa, babesiosis is predominantly caused by *Babesia rossi* and to a lesser extent by *B. vogeli*. *Ehrlichia canis* causes clinical disease in dogs and *E. ruminantium* has been identified in infected dogs in southern Africa. The clinical manifestation of disease in dogs infected with *E. ruminantium* has not been described. Theileriosis in dogs in South Africa is poorly understood. Apart from *Theileria equi* which causes the disease known as equine piroplasmosis in equids, the *Theileria* species known to cause disease in dogs in South Africa has yet to be positively identified. Co-infection with multiple tick-borne diseases has been documented in individual animals. This is primarily due to single tick species having the ability to act as a vector for multiple pathogens and due to heavy tick infestations.^{1,2} *Babesia* and *Ehrlichia* co-infections are perceived as common in South Africa, with veterinary practitioners directing treatment at both pathogens. Various haematological variables are also perceived to be associated with such co-infections.³ Little is known regarding the vector-borne diseases of companion animals elsewhere in sub-Saharan Africa and global reports of co-infections are often limited to smaller case series.

Methodology

1. Diagnosis of babesiosis was made by visualisation of piroplasm on a peripheral blood smear. A total of 150 dogs from the Eastern Cape province that were naturally infected with babesiosis were included in the study.
2. In accordance with ethical considerations, to be included in the study, the dogs had to be clinically ill, privately owned, weigh more than 3kg and be older than 12 weeks of age.
3. Dogs were excluded if there was a clinical suspicion of comorbidity with other non-tick-borne infectious diseases or if they had received treatment for a suspected tick-borne disease in the four weeks prior to presentation.
4. Peripheral venous blood was collected at presentation and before any treatment. The collected blood was divided into one EDTA vacutainer tube (3mL) and one serum vacutainer tube (3mL).
5. Haematology and serum protein concentration measurement was performed within 24 hours following sampling.
6. Whole blood EDTA pellets were submitted for quantitative real-time PCR using probes specific for *B. rossi*, *B. vogeli*, *E. canis* and *T. equi* according to standard operating procedures described for each of these pathogens.^{4,5,6}
7. Cases were categorised based on the *Babesia* species with which they were infected and the presence or absence of co-infection with *Ehrlichia canis* or *Theileria equi* to calculate the co-infection prevalence.
8. Specific population characteristics, haematology and biochemistry measures of interest were identified to assess associations with infection status. This included sex, breed, age, duration of illness, leukocyte count, band neutrophil count, monocyte count, platelet count, absolute reticulocyte count (ARC) and serum globulin concentration.
9. The positive predictive values and their 95% confidence intervals (CIs) for leukopenia, monocytosis, thrombocytopenia, band neutrophilia and non-regenerative ARC were calculated.

Results



- *Babesia rossi* was identified in 149/150 samples and *B. vogeli* in only 1/150 samples. A co-infection prevalence of 2.0% (3/149; 95% CI: 0.4 – 5.7) with *B. rossi* and *E. canis* was found. No other co-infections were reported.
- No investigated variables showed significant associations with co-infections.
- When looking at the positive predictive values in our study population with a known 2% (95% CI: 0.4-5.7) prevalence of co-infection, for leukopenia, monocytosis, thrombocytopenia, non-regenerative ARC or band neutrophilia, the probability that a dog with babesiosis was co-infected at most increased to only 3.5% (CI: 0.5-13) when a band neutrophilia was seen.
- An interesting finding was that Pit bull terriers, which represented the largest group of purebred dogs, had significantly lower median (IQR) haematocrit and ARC values compared to mixed-breed dogs and other purebred dogs.

Table 1: Positive and negative predictive values of haematological findings for co-infection in *B. rossi* infected dogs

Variable	Cut-off	PPV (%) (95% CI)	NPV (%) (95% CI)
Leukopenia	< 6 × 10 ⁹ /L	2.2% (0.1 - 11.5)	98.0% (93.0 - 99.8)
Monocytosis	> 1.35 × 10 ⁹ /L	3.1% (0.1 - 16.2)	98.3% (93.9 - 99.8)
Thrombocytopenia	< 200 × 10 ⁹ /L	2.1% (0.4 - 5.9)	100% (15.8 - 100)
Non-regenerative ARC	< 80 × 10 ⁹ /L	2.9% (0.3 - 9.9)	98.7% (93.0 - 99.97)
Band neutrophilia	> 0.5 × 10 ⁹ /L	3.5% (0.5 - 13.0)	98.9% (94.2 - 99.97)

Table 2: Haematocrit and bone marrow response comparison among mixed-breed and purebred dogs

Parameter	Pit bull terriers (n=25)	Mixed-breed dogs (n=90)	P-value ¹	Other purebred dogs (n=35)	P-value ²
Haematocrit (L/L)	0.14 (0.11 - 0.2)	0.2 (0.13 - 0.28)	0.0057	0.18 (0.14 - 0.25)	0.042
ARC (x10 ⁹ /L)	59.9 (29.2 - 107.6)	107.85 (55.55 - 205.35)	0.0036	140.2 (52.9 - 175.1)	0.0087

Conclusions

- Co-infection with other tick-borne diseases in dogs with babesiosis is uncommon in the Eastern Cape province.
- *Babesia rossi* was the most prevalent *Babesia* species infecting dogs.
- Monocytosis and thrombocytopenia in dogs with babesiosis are not predictive of an *Ehrlichia* spp. co-infection.
- It is theorised that the marked cytokine derangements seen with *B. rossi* infection may inhibit other pathogens from establishing themselves in the host environment.^{7, 8, 9, 10}
- *Babesia vogeli* was confirmed in the Eastern Cape province for the first time.

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