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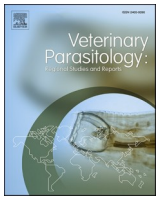

Original Article

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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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.

1. Introduction

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 et al., 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 diseases is possible and has been documented in individual animals. This is firstly due 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).

In South Africa, canine babesiosis is predominantly caused by *Babesia rossi* and to a lesser extent by *B. vogeli* (Matjila et al., 2008a).

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Babesia rossi is transmitted by the tick vector *Haemaphysalis elliptica* which has a wide distribution in South Africa and is present on large numbers of dogs in the Eastern Cape (Horak et al., 2009). *Babesia vogeli* has only been positively identified in the Gauteng, Free State, and Mpumalanga provinces of South Africa (Matjila et al., 2004; Kolo et al. 2014). In South Africa, *Rhipicephalus sanguineus* has been identified as the vector for *B. vogeli* (Uilenberg et al., 1989) and *E. canis* (Fourie et al., 2013). It was found to be the second most frequently collected tick species from ruminant hosts in the Eastern Cape Province (Iweriebor et al., 2017).

Ehrlichia canis is considered a disease 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 (Pretorius and Kelly, 1998). In poorer areas, the seroprevalence was significantly higher at 48% (Pretorius and Kelly, 1998). A seroprevalence of 41.7% has been reported in the uMkhanyakude district of KwaZulu-Natal (Mofokeng et al., 2020). *Ehrlichia ruminantium*, specifically the Pretoria North genotype, has been identified to infect dogs in southern Africa, however, the clinical manifestation of disease in dogs infected with *E. ruminantium* has not been described (Allsopp and Allsopp, 2001; Allsopp, 2010).

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 not been positively identified (Rosa et al., 2014). Theileriosis has not been reported in dogs in the Eastern Cape province. Dogs with theileriosis appear to present with similar clinical abnormalities to dogs with babesiosis (Matjila et al., 2008b; Rosa et al., 2014). The tick vector for theileriosis in dogs in South Africa has not been identified.

Reports focused on the prevalence of *Babesia* spp. infection with *Ehrlichia* spp. or *Theileria* spp. co-infections in dogs in South Africa are scarce. To the authors' knowledge, only three published studies (Du Plessis et al., 1990; Matjila et al., 2008a; Rautenbach et al., 2018) have aimed at investigating this relationship and only one of these (Matjila et al., 2008a) included data from the Eastern Cape Province, where co-infections were not reported. The first publication found a co-infection prevalence of 32% based on immunofluorescence antibody testing (IFAT) (Du Plessis et al., 1990). Veterinary practitioners rely heavily on serological methods to diagnose ehrlichiosis (Waner et al., 2001); however, in geographical regions where the disease is endemic the reliability of serological testing is doubtful (Waner et al., 1997). *Ehrlichia canis* antibody titres have 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% "co-infection prevalence" may thus reflect exposure rather than active infection. Two studies using PCR and 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., 2008b). 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 et al., 2018*)*. 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 (Suksawat et al., 2001; De Tommasi et al., 2013; Noden and Soni, 2015).

A survey conducted in 1993 that focused on canine babesiosis demonstrated that most veterinary practitioners in South Africa reported that they considered ehrlichiosis an important complicating factor in babesiosis infections (Collett, 2000). This belief likely stemmed from previous reports by several investigators regarding the importance of and the difficulties in diagnosing such co-infections (Van Heerden et al., 1983; Irwin and Hutchinson, 1991). Further to this, 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). 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%) (Rautenbach et al., 2018).

The objectives of this study were to a) 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 b) to identify host and environmental factors associated with co-infection.

2. Materials and methods

2.1. 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, 2018). One hundred and fifty samples were collected by the primary investigator from privately owned dogs positively diagnosed with babesiosis that were clinically ill and presented to the Mdantsane State Veterinary Clinic for treatment. The clinic is located 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 real-time quantitative PCR (qPCR) (Bhoora et al., 2010; Troskie et al., 2018; Nkosi et al., 2022). Inclusion criteria were dogs that were clinically ill, privately owned, weighed >3 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. 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, consent was obtained from the owner or the person responsible for the animal. Ethical approval for the study was granted by the Research Ethics Committee, Faculty of Veterinary Science, and the Animal Ethics Committee of the University of Pretoria (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)).

2.2. 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 mL) and one serum vacutainer tube (3 mL) (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 anticoagulated 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 h of arrival at the laboratory and within 24 h 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.

2.3. Methodologies

2.3.1. 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 Nuclein 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* (TGGCTTTTTGCCTTATTA), *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 s and 40 cycles each of denaturing at 95 °C for one second and extension at 60 °C for 20 s. A control assay is routinely run by the laboratory and because the tests were previously validated, a control group was not included in the study.

2.3.2. Haematology

The samples were analysed with the ADVIA 2101 (Siemens) automated haematology analyser. Differential leukocyte counts and blood cell morphological evaluations were done by veterinary laboratory technicians. The presence of blood-borne parasites was visually noted, and the sample condition was assessed.

2.3.3. 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 (Glob = TSP - Alb).

2.4. 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 illness, leukocyte count, band neutrophil count, monocyte count, platelet count, ARC, and serum globulin 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 et al., 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 absolute reticulocyte count ($<80 \times 10^9$/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 $P < 0.05$ was used to assess significance.

3. Results

3.1. Study sample characteristics

All 150 dogs naturally infected with *Babesia* spp. and sampled in this cohort 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 <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. Further evaluation of the sample characteristics are detailed in the supplementary material (Appendix 1).

3.2. Co-infection prevalence

Quantitative real-time 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%) was solely infected with *B. vogeli*. None of the dogs were co-infected with *B. vogeli* and *E. canis*, or 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).

3.3. 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* (Table 1). There was no significant difference between the median leukocyte count, monocyte count, platelet count, haematocrit 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 white cell count. In the co-infected group, monocytosis was documented in 1/3 dogs, thrombocytopenia in 3/3 and a normal or low white cell count in 1/3.

An interesting finding was that Pit Bull Terriers, which represented the largest group of purebred dogs (25/150), had significantly lower

Table 1
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. Results reported as median (IQR).

Variable	Reference interval	*Babesia rossi* group ($n = 146$)	*Babesia vogeli* group ($n = 1$)	Co-infection group ($n = 3$)	*P*-value[a]
Haemoglobin	120–180 g/L	53 (39–80.75)	120	49 (48–67)	0.962
Red cell count	5.5–8.5 × 10^{12}/L	2.31 (1.67–3.66)	5.87	2.23 (2.1–2.99)	0.895
Haematocrit	0.37–0.55 L/L	0.18 (0.13–0.27)	0.42	0.18 (0.16–0.21)	0.927
White cell count	6–15 × 10^9/L	7.88 (5.54–13.19)	1.17	11.42 (2.92–12.88)	0.932
Segmented Neutrophils	3–11.5 × 10^9/L	5.23 (3.51–8.6)	0.44	7.31 (2.13–8.63)	0.729
Band Neutrophils	0–0.5 × 10^9/L	0.3 (0.13–0.82)	0	0.91 (0.03–1.42)	0.993
Lymphocytes	1–4.8 × 10^9/L	1.52 (1.03–2.31)	0.47	1.42 (0.26–2.63)	0.619
Monocytes	0.15–1.35 × 10^9/L	0.75 (0.44–1.3)	0.15	0.5 (0.46–1.42)	0.839
Platelet count	200–500 × 10^9/L	61 (39–86.75)	70	61 (43–75)	0.925
Nucleated red blood cells/ 100 White blood cells	0–9	4 (1–9)	0	2 (0–6)	0.477
Reticulocytes	% (NRR[b])	3.9 (1.8–9.2)	1.2	1.7 (1.1–7.8)	0.353
Absolute reticulocyte count	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

[a)] *P*-values comparing haematology and serum protein biochemistry results of the *B. rossi* infected group and the co-infected group
[b)] NRR = no reference range

Table 2
Haematocrit and bone marrow response comparison among crossbreed and purebred dogs, shown as median (IQR).

Parameter	Pit Bull Terriers ($n = 25$)	Crossbreed dogs ($n = 90$)	*P*-value[a]	Purebred dogs excluding Pit Bull Terriers ($n = 35$)	*P*-value[b]
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 ($x10^9$/L)	59.9 (29.2–107.6)	107.85 (55.55–205.35)	0.004	140.2 (52.9–175.1)	0.009

[a)] *P*-values comparing the HCT and ARC of pit bull terriers and crossbreed dogs.
[b)] *P*-values comparing the HCT and ARC of pit bull terriers and other purebred dogs.

median (IQR) haematocrit 0.14 L/L (0.11–0.2) and ARC values 59.9 × 10^9/L (29.2–107.6) compared to crossbreed dogs and other purebred dogs (Table 2).

3.4. Serum protein results

Serum protein results were available for 140 dogs in the *B. rossi* group and for all three dogs in the co-infected group and the one infected with B. *vogeli* (Table 1). The median total serum protein, albumin and globulin concentrations for the *B. rossi* group were not significantly different from the co-infected group.

3.5. Associations with co-infection and predictive values

No significant association with co-infection was found for any of the population characteristics and haematological and biochemistry measures of interest. This is further detailed in the supplementary material (Appendix 2). For all parameters investigated the PPV was very low (<4%) (Table 3).

4. Discussion

The study results demonstrated a low prevalence of co-infections with multiple tick-borne diseases in the Eastern Cape, 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. *Babesia rossi* mono-infections accounted for almost all dogs infected and clinically ill, with 146/150 (97.33%) dogs testing positive using qPCR. *Babesia vogeli* infection accounted for only 1/150 dogs. This is the first known study to definitively confirm the presence of *B. vogeli* using PCR in the Eastern Cape. Previous studies that investigated tick-borne disease prevalence and the prevalence of co-infections with multiple tick-borne diseases in the South African context have yielded similarly low co-infection results to what was found in this study (Matjila et al., 2008a; Rautenbach et al., 2018). 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). A cytokine profile indicative of a T-helper 1-like response, with high levels of interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-*α*) has been observed in *Babesia* spp. infection in mouse models (Hemmer et al., 2000). These cytokines are intended to stimulate immunity but also play

Table 3
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 × 109/L	2.2% (0.1–11.5)	98.0% (93.0–99.8)
Monocytosis	> 1.35 × 109/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)

a pivotal role in the pathogenesis of the disease (Day, 2011; Leisewitz et al., 2019b). 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 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.

The overall disease presentation of dogs infected with *B. rossi* 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 et al., 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.

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. This low PPV, along with results from a previous study (Rautenbach et al., 2018), suggests that veterinary practitioners who base their diagnosis of co-infection in dogs with babesiosis on haematology findings including monocytosis, thrombocytopenia or leukopenia (Collett, 2000) are likely to overestimate the prevalence of such co-infections.

Initial reports on "bilious fever" (which we now call babesiosis) in South Africa dating as far back as 1893, reported purebred dogs 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 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 are overrepresented in cases of dogs infected with *B. gibsoni* in the USA (Macintire et al., 2002).

Further testing for other *Ehrlichia* or *Theileria* species or for 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. Additionally, it is not definitively known whether dogs can become clinically ill 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. Another potential limitation of our study was that, 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.

5. Conclusion

Co-infection with *Ehrlichia 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 haematocrit and ARC values compared to crossbreed dogs and other purebred dogs. It is recommended that further investigations on a larger sample size of dogs 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 *Ehrlichia* infection before initiating therapy to practice responsible antimicrobial stewardship.

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Ethics statement

Prior to the commencement of the study, ethical approval was obtained from the following ethical review board: the Research Ethics Committee and the Animal Ethics Committee of the University of Pretoria, Faculty of Veterinary Science (REC061–20). The authors declare that this submission is in accordance with the principles laid down by the Responsible Research Publication Position Statements as developed at the 2nd World Conference on Research Integrity in Singapore, 2010.

Animal welfare statement

Prior to the commencement of the study, ethical approval was obtained from the following ethical review board: the Research Ethics Committee and the Animal Ethics Committee of the University of Pretoria, Faculty of Veterinary Science (REC061-20). The authors declare that this submission is in accordance with the principles laid down by the Responsible Research Publication Position Statements as developed at the 2nd World Conference on Research Integrity in Singapore, 2010.

CRediT authorship contribution statement

Henry P.P. Cloete: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yolandi Rautenbach:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Andrew L. Leisewitz:** Writing – review & editing, Resources, Methodology, Conceptualization. **Richard J. Mellanby:** Writing – review & editing, Resources, Methodology, Conceptualization. **Peter N. Thompson:** Writing – review & editing, Validation, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Johan P. Schoeman:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper: Yolandi Rautenbach reports financial support was provided by Health and Welfare Sector Education and Training Authority (HWSETA). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Appendix

Appendix 1
Population characteristics for the various groups within the cohort at the time of presentation. Where applicable numerical values reported as median (IQR).

Characteristic	*Babesia rossi* group	*Babesia vogeli* group	Co-infection group
Age (months)	12 (8–30)	3	18 (8–24)
Weight (kilograms)	18 (12–25)	3	27.4 (15.1–36.7)
Sex	Male (80) Female (66)	Male (1)	Male (3)
Breeds (Number of animals)	Crossbreed (88) Pitbull (25) Boerboel (14) Greyhound (5) Rottweiler (4) German Shepherd (4) Jack Russell Terrier (2) Coonhound (2) Pug (1) Husky (1)	Crossbreed (1)	Crossbreed (1) Rottweiler (1) Boerboel (1)
Primary complaint (Number of animals)	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 illness (days)	3 (2–5)	3	2 (1–14)
Rectal temperature (°C)	40 (39.9–40.4)	<35	39.4 (39.3–40.4)
Peripheral lymph node (LN) enlargement (Number of animals)	Nonpalpable (51) 1–3 LN palpable (48) >3 LN palpable (47)	Nonpalpable (1)	Nonpalpable (1) 1–3 LN palpable (2)

Appendix 2
Variables of interest selected for further investigation of possible association with co-infections.

Variable	n	*B. rossi* only (%)	Co-infection (%)	*P*-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%)	
Crossbreed	89	88 (99%)	1 (1%)	
Age				0.633
< 12 months	55	54 (98%)	1 (2%)	
12–24 motnhs	53	51 (96%)	2 (4%)	
> 24 months	41	41 (100%)	0	
Duration of illness				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

(*continued on next page*)

Appendix 2 (*continued*)

Variable	n	*B. rossi* only (%)	Co-infection (%)	*P*-value
Leukopenia (<6 × 109/L)	46	45 (98%)	1 (2%)	
Normal (6–15 × 109/L)	74	72 (97%)	2 (3%)	
Leukocytosis (>15 × 109/L)	27	27 (100%)	0	
Band neutrophil count				0.295
Normal (0–0.5 × 10^9/L)	94	93 (99%)	1 (1%)	
High (> 0.5 × 10^9/L)	53	51 (96%)	2 (4%)	
Monocyte count				0.524
Monocytosis (> 1.35 × 10^9/L)	32	31 (97%)	1 (3%)	
Normal or low (< 1.35 × 10^9/L)	115	113 (98%)	2 (2%)	
Platelet count				0.959
Normal or high platelet count (> 200 × 109/L)	2	2 (100%)	0	
Thrombocytopenia (< 200 × 109/L)	145	142 (98%)	3 (2%)	
Absolute reticulocyte count				0.506
Mild to marked ARC (>80 × 109/L)	63	62 (98%_	1 (2%)	
Inadequate ARC (<80 × 109/L)	64	62 (97%)	2 (3%)	
Globulin concentration				0.596
Low (< 20 g/L)	7	7 (100%)	0	
Normal (20–41 g/L)	106	104 (98%)	2 (2%)	
High (> 41 g/L)	30	29 (97%)	1 (3%)	

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