Identification and molecular characterization of zoonotic rickettsiae and other vector-borne pathogens infecting dogs in Mnisi, Bushbuckridge, Mpumalanga Province, South Africa

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DECLARATION

I declare that this dissertation, which I hereby submit for the Master of Science degree in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, is my original work and has not been submitted by me for a degree to any other university.

SIGNED: _____ DATE: 16-02-2015

AGATHA ONYEMOWO KOLO

DEDICATION

To the Lord Almighty, the Most High God my Help, my Refuge, my Provider, my Maker, my greatest Love, my Redeemer, my ever Faithful God; to you I dedicate this work.

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LIST OF ABBREVIATIONS

16S rRNA	16S ribosomal Ribonucleic acid
18S rRNA	18S ribosomal Ribonucleic acid
AG	Ancestral group
ATBF	African tick bite fever
BLAST	Basic local alignment search tool
CME	Canine monocytic ehrlichiosis
^{o}C	Degrees Celsius
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
EDAC	1-ethyl-3- (3-dimethyl-amino-propyl) carbodimide
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBSF	Flea-borne spotted fever
HCl	Hydrogen chloride
HDSS	Health and demographic surveillance study
HGE	Human granulocytic ehrlichiosis
IFA	Immuno fluorescent assay

IFAT	Immuno fluorescent antibody test
IgG	Immunoglobulin G (Gamma)
IgM	Immunoglobulin M (Mu)
KCl	Potassium chloride
µl	Microlitre
µM	Micromolar
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MEGA	Molecular evolutionary genetics analysis
$MgCl_2$	Magnesium chloride
MIF	Microimmunofluorescence
MSF	Mediterranean spotted fever
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
omp A	Outer membrane protein A
omp B	Outer membrane protein B
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RFLO	*Rickettsia felis*-like organism

RFLP	Restriction fragment length polymorphism
RLB	Reverse line blot
RMSF	Rocky Mountain spotted fever
RNA	Ribonucleic acid
Rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
SFG	Spotted fever group
TG	Typhus group
TRG	Transitional group
Tris-HCl	Hydroxymethyl aminomethane hydrochloride
UDG	Uracil DNA glycosylase
USA	United States of America

RESEARCH OUTPUTS

Conferences

1. AO Kolo, KP Sibeko-Matjila, DL Knobel & PT Matjila. Molecular detection of haemoparasites in dogs in Mnisi Mpumalanga Province, South Africa presented at the 42nd Annual Congress of the Parasitological Society of Southern Africa (PARSA) 22–24 September 2013, Stonehenge in Africa, Parys, Free State, South Africa (Oral presentation).

2. AO Kolo, KP Sibeko-Matjila, DL Knobel & PT Matjila. Molecular detection of *Rickettsia africae* and *Rickettsia felis* from ticks and fleas collected from domestic dogs in Bushbuckridge, South Africa presented at the Joint 8th international Ticks and Tick-borne pathogens (TTP-8) and 12th Biennial Society for Tropical Veterinary Medicine (STVM) conference 24-29 August 2014, Capetown, South Africa (Poster presentation).

3. AO Kolo, KP Sibeko-Matjila, DL Knobel & PT Matjila. Molecular detection of *Rickettsia africae* and *Rickettsia felis* from ticks and fleas collected from domestic dogs in Bushbuckridge, South Africa presented at the Faculty day, Faculty of Veterinary science, University of Pretoria September 4 2014 (Poster presentation).

4. AO Kolo, KP Sibeko-Matjila, DL Knobel & PT Matjila. Molecular detection of an *Anaplasma* sp. strain in domestic dogs in Mpumalanga Province, South Africa presented at the Faculty day, Faculty of Veterinary science, University of Pretoria September 4 2014 (Oral presentation).

5. AO Kolo, KP Sibeko-Matjila, DL Knobel & PT Matjila. Molecular detection of an *Anaplasma* sp. strain in domestic dogs in Mpumalanga Province, South Africa presented at the Joint international congress of parasites of wildlife and the 43rd Annual Congress of the Parasitological Society of Southern Africa (PARSA) 14-18 September 2014, Kruger National Park, South Africa (Oral presentation).

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Identification and molecular characterization of zoonotic rickettsiae and other vector-borne pathogens infecting dogs in Mnisi, Bushbuckridge Mpumalanga Province, South Africa

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ABSTRACT

Dogs play an important role in the lives of humans, and because of this close contact they share with humans, they can serve as sentinels for infections to humans. Rickettsiae are small, obligate intracellular bacteria that are vector-borne and can cause mild to fatal diseases in humans worldwide. There is little information on the zoonotic rickettsial pathogens that may be harboured by dogs from rural localities in South Africa. Thus the primary aim of this study was to identify and characterise rickettsial pathogens infecting dogs using molecular techniques by screening blood samples and ectoparasites collected from domestic dogs in the Bushbuckridge area, Mpumalanga province of South Africa.

A total of 141 blood samples, 103 ticks and 43 fleas were collected from owned, free-roaming dogs in the Mnisi community area in Bushbuckridge Local Municipality, Mpumalanga Province, South Africa under the project "Health and Demographic Surveillance System (HDSS) in dogs" (protocol number: V033/11). Samples were collected from October 2011 through May 2012. Ectoparasites identified are as follows: the tick species include *Haemaphysalis elliptica*, *Amblyomma hebraeum*, *Rhipicephalus sanguineus*, *Rhipicephalus simus,* and an unspeciated *Ixodes* spp. Fleas includes *Ctenocephalides felis strongylus* and *Echidnophaga gallinacea.* DNA was extracted from blood and ectoparasites and screened for the presence of *Ehrlichia, Anaplasma*, *Theileria* and *Babesia* species infections using the reverse line blot (RLB) hybridization assay. Almost half of the blood samples reacted with the genus-specific probes for *Ehrlichia/Anaplasma* (49%), and 16% of the samples investigated were positive for *Ehrlichia canis* DNA. *Babesia rossi* (9%) and *Babesia vogeli* (4%) were detected among the 14% of samples that were positive for the genus-specific probe 1 of *Babesia*. Furthermore, 14% of the samples were positive for the genus-specific probes of *Theileria*/*Babesia*, 2% were positive for the genus-specific probe of *Theileria*, and 1% for the genus-specific probe 2 of *Babesia*. Haemoparasite DNA could not be detected in 36% of the samples investigated. Results of the RLB assay for ectoparasites showed that 30% of tick pools were positive for the genus-specific probes of *Ehrlichia*/*Anaplasma* species, *Babesia* 1 and 2 (30%) and *Theileria* (10%) while haemoparasite DNA could not be detected in 30% of the tick pools. Haemoparasite species DNA detected from tick pools

includes *Neoehrlichia* spp., *Ehrlichia ruminantium*, and *Babesia microti*. Flea pools were positive for the genus-specific probes of *Babesia* 1 and 2 (50%). Specific haemoparasite species were not detected from the flea pools.

Rickettsia genus and species-specific qPCR assays were performed to detect zoonotic rickettsiae from DNA obtained from blood and ectoparasite samples. Rickettsiae were not detected in blood samples but in 70% of tick pools analyzed using a genus-specific qPCR assay, based on the 17-kD antigen gene. Rickettsial infections were detected in tick pools, namely *Haemaphysalis elliptica*, *Amblyomma hebraeum*, *Rhipicephalus sanguineus*, *Rhipicephalus simus*, and *Ixodes*, and in flea pools *Ctenocephalides felis strongylus* and *Echidnophaga gallinacea*. Using the species-specific assays, three pools of ticks (two *Amblyomma hebraeum* pools and a *Haemaphysalis elliptica* pool) tested positive on a *R. africae* genotype-specific qPCR assay, while flea pools (*Ctenocephalides felis strongylus* and *Echidnophaga gallinacea)* were positive on the *R. felis* qPCR assay.

Blast analysis of nucleotide sequences of the V1 hypervariable region of the 16S rRNA gene of five selected *Ehrlichia*/*Anaplasma* positive samples on the RLB hybridization assay revealed an *Anaplasma* sp. South Africa dog strain 98% related to *Anaplasma phagocytophilum* in four of the five samples and an *Anaplasma phagocytophilum* strain in one sample. Furthermore, *Rickettsia massiliae* and *Orientia tsutsugamushi* sequences were also identified in two additional samples. Analysis of nucleotide sequences obtained from tick and flea pool DNA samples positive on *R. africae* and *R. felis* qPCR assays revealed *Rickettsia africae* and *Candidatus* Rickettsia asemboensis a new *Rickettsia felis*-like organism respectively. Analysis of nucleotide sequences of the 18S rRNA gene of three selected *B. rossi* positive samples on the RLB hybridization assay confirmed detection of *B. rossi* in all selected samples. The detection of zoonotic rickettsiae in canine blood and their ectoparasites suggests that dogs may play an important role in the lifecycle of these organisms; these data also provide preliminary information about the diversity of rickettsiae and other vector-borne pathogens circulating in domestic dogs and their ectoparasites in Bushbuckridge, South Africa. In conclusion the detection of zoonotic rickettsiales from blood samples of domestic dogs and their

ectoparasites in a rural community in South Africa highlights the potential risk of human infection that may occur from these pathogens.

CHAPTER 1

INTRODUCTION

A zoonosis is an infectious disease that may be transmitted between animals and humans. Transmission may be by direct contact, by arthropod vectors, or by fomites (Mantovani, 2001). Arthropod-borne rickettsial diseases have in recent times being heralded as standard paradigms for understanding emerging diseases worldwide (Rizzo et al., 2004; Raoult & Roux, 1997). There exists the likelihood that ectoparasites (i.e. fleas and ticks) that feed on dogs may also feed on humans and in the process transmit pathogens. Companion animals, specifically dogs can serve as vertebrate hosts for arthropod vectors and spotted fever group (SFG) *Rickettsia* (Mc Quiston et al., 2011). In addition some cases of rickettsioses in humans have been associated with cases in companion animals (Breitschwerdt et al., 1985). Dogs play an important role in the lives of humans, and because of this close contact they can serve as sentinels for human rickettsial infections (Labruna et al., 2007) as well as being possible sources of other infections.

Phylogenetically, rickettsiae are alphaproteobacteria, with closely related *Ehrlichia* and *Anaplasma* species (Dumler et al., 2001). *Rickettsia* is a genus of non-motile, gram-negative, non-spore forming, highly pleomorphic bacteria. In addition to the clinical features of rickettsial disease, diagnosis typically utilizes methods such as serological testing that do not differentiate among species. The species of the infecting *Rickettsia* is generally presumed, usually based upon typical disease manifestations and the geographic location in which the tick bite was encountered (Kidd et al., 2006). Advances in molecular techniques have contributed to the recognition and characterisation of classic and novel rickettsial species as disease causing agents in expanding geographic localities (Rizzo et al., 2004; La Scola & Raoult, 1997).

There is little information on the zoonotic rickettsial pathogens that dogs may harbour from rural localities in South Africa. Molecular characterization of rickettsial organisms isolated from dogs in the Mnisi community area, Bushbuckridge, Mpumalanga province will give insight on the epidemiology of rickettsial pathogens in this area and provide preliminary data about occurrence of these pathogens in South African rural communities. Data from this project will also add knowledge on the risk of zoonotic rickettsial infections in South Africa. This information may result in control measurements being put in to place for the prevention of infection between dogs and humans. This study will fill the knowledge gap that exists on the role of the domestic dogs in the epidemiology of rickettsial pathogens in South Africa. The findings of this study will also provide data on diversity of rickettsial pathogens found in the ectoparasites of dogs in the Mnisi community.

Therefore, the primary objective of this study was to identify and characterise rickettsial pathogens infecting dogs using molecular techniques by screening blood samples and ectoparasites collected from domestic dogs in the Mnisi community area, Bushbuckridge, Mpumalanga province of South Africa. The specific objectives were:

1. Screening blood samples collected from dogs for infections with rickettsial pathogens, particularly zoonotic pathogens using a species-specific real-time PCR assay.

2. Screening blood samples collected from dogs for infections with other blood parasites using the reverse line blot (RLB) hybridization assay.

3. Screening of tick and flea nucleic acid material for infections with rickettsial pathogens by RLB and qPCR.

4. Molecular characterization of identified rickettsial pathogens by sequencing of the 16S and 18S ribosomal RNA gene.

CHAPTER 2

LITERATURE REVIEW

2.1 Zoonotic tick and flea-borne infections

Ticks have been recognized as major vectors responsible for the transmission of disease agents in humans and animals worldwide (Jongejan & Uilenberg, 2004; Colwell et al., 2011). Zoonotic tick-borne infections include *Rickettsia africae* which causes African tick-bite fever and is transmitted by *Amblyomma hebraeum* and *A. variegatum* ticks and predominantly found in Africa and the Carribean Islands (Jongejan & Uilenberg, 2004; Piesman & Eisen, 2008). *Rickettsia conorii* causes Mediterranean spotted fever transmitted by *Rhipicephalus sanguineus* ticks and has occurred in Africa, Asia and Europe (Jongejan & Uilenberg, 2004, Piesman & Eisen, 2008). *Anaplasma phagocytophilum* causes human granulocytic anaplasmosis transmitted by *Haemaphysalis* ticks, *Ixodes ticks* and *Rhipicephalus bursa*. The disease is usually seen in Europe and North America (Jongejan & Uilenberg, 2004, Piesman & Eisen, 2008). *Ehrlichia chaffeensis* causes human monocytic ehrlichioses and is transmitted by *Amblyomma americanum* and predominantly found in North America (Jongejan & Uilenberg, 2004, Piesman & Eisen, 2008). *Borrelia burgdoferi* sensu lato causes lyme borreliosis transmitted by *Ixodes* spp. of ticks and is found in Asia, Europe and North America (Jongejan & Uilenberg, 2004, Piesman & Eisen, 2008). *Coxiella burnetti* causes Q-fever transmitted by different genera of ticks and is distributed worldwide (Jongejan & Uilenberg, 2004, Piesman & Eisen, 2008). *Babesia microti* and *B. divergens* cause zoonotic babesiosis transmitted by *Ixodes* spp. of ticks and the disease has occurred in North America and Europe (Jongejan & Uilenberg, 2004, Piesman & Eisen, 2008). *Babesia duncani* and *B. venatorum* have also been implicated in causing zoonotic babesiosis in the United States (Vannier & Krause, 2009).

Flea-borne zoonotic organisms include *Yersinia pestis* the cause of plague transmitted by *Xenopsylla* spp. of fleas, *Oropsylla montanus*, *Pulex irritans* and *Ctenocephalides felis*

(Stenseth et al., 2008). *Rickettsia typhi* is the cause of murine typhus, a worldwide zoonosis that is transmitted by *Xenopsylla cheopsis* (Traub & Wisseman, 1978). *Rickettsia felis* is the cause of flea-borne spotted fever transmitted by the cat flea *Ctenocephalides felis* and is an emerging zoonotic rickettsioses found worldwide (Perez-Osorio et al., 2008). *Anaplasma phagocytophilum*, *R. conorii* and *E. chaffensis* are associated with causing infection in dogs. Rickettsiae were the main focus of this project so a more detailed review of these fastidious organisms is given below.

2.2 Rickettsiae

Rickettsiae are small, gram-negative, aerobic, coccobacilli that are obligate intracellular parasites of eukaryotic cells associated with ticks, fleas, mites and lice (Raoult & Roux, 1997). They may live in the cytoplasm or within the nucleus of the cell that they invade (Todar, 2009). They divide by binary fission and metabolize host-derived glutamate via aerobic respiration and the citric acid cycle. They have typical gram-negative cell walls, and lack flagella. Rickettsiae are natural parasites of arthropod vectors that may transmit the organism to mammalian hosts. Rickettsiae have very small genomes of about 1.0-1.5 million bases (Todar, 2009).

The order Rickettsiales consists of two families, Rickettsiaceae and Anaplasmataceae. The family Rickettsiaceae consists of the genera *Rickettsia* and *Orientia*; while the family Anaplasmataceae consists of the genera *Anaplasma*, *Ehrlichia* and *Neorickettsia*. Common members of the order rickettsiales of companion animals are from the genera *Rickettsia, Orientia, Erhlichia, Neorickettsia* and *Anaplasma.* Previously members of the genus *Rickettsia* were classified into two groups, the spotted fever group (SFG) *Rickettsia*, and typhus group (TG) *Rickettsia* (Merhej & Raoult, 2011). The SFG group includes *Rickettsia conorii*, *R. rickettsii*, *R. parkeri* and *R. sibirica* and the TG group includes *Rickettsia prowazeckii* and *R. typhi* (Merhej & Raoult, 2011). However, recent studies on the genome of *Rickettsia* have determined that additional groups exist within the genus, namely the transitional group (TRG) *Rickettsia* and the ancestral group (AG) (Gillespie et al., 2007; Weinert et al., 2009). The TRG

group includes *Rickettsia felis* and the AG group incudes *Rickettsia bellii* and *Rickettsia canadensis* (Gillespie et al., 2007; Weinert et al., 2009). Common rickettsiae of humans and reservoirs are indicated in Table 2.1. *Rickettsia conorii*, *R. africae* and *R. aeschlimanii* are common rickettsiae found in South Africa (Pretorius & Birtles, 2002).

Table 2.1: Common rickettsiae of humans and reservoirs

Rickettsiae	Human disease	Animal reservoirs	Primary vectors	References
Rickettsia rickettsii	Rocky Mountain spotted fever	Dogs	*Dermacentor*, *Amblyomma* ticks	(Abramson & Givner, 1999; Parola et al., 2005)
R. conorii *R. massiliae* and *R. aeschlimanii*	Mediterranean spotted fever	Dogs, rodents	*Rhipicephalus sanguineus* *Haemaphysalis* ticks	(Parola et al., 2005; Levin et al., 2012)
R. africae	African tick-bite fever	Ruminants	*Amblyomma hebraeum*	(Parola et al., 2005)
R. felis	Flea borne rickettsiosis	Cats, opossums, rodents	*Ctenocephalides felis* flea	(Jensenius et al., 2004; Richards et al., 2010; Hii et al., 2011; Maina et al., 2012)

2.3 Epidemiology of rickettsial infection

Rickettsial infections have been found in every continent except Antarctica (Jensenius et al., 2004). The regions of the world where rickettsial infections has occurred is shown in Table 2.2.

Table 1.2: Rickettsial infections and where they have occured worldwide

Rickettsial infections	Species	Regions of occurence	References
Mediterranean spotted fever	*R. conorii*	Southern Europe and Africa	Parola et al., 2005; Rovery & Raoult, 2008
Indian tick-typhus	Indian tick typhus rickettsia related to *R. conorii*	India	Jensenius et al., 2004
Israeli tick-typhus	Israeli tick typhus rickettsia related to *R. conorii*	Mediterranean countries	Jensenius et al., 2004
Thai tick-typhus	*R. honei*	Asia and Australia	Jensenius et al., 2004
Queensland tick-typhus and Australia spotted fever	*R. australis*	Eastern Australia	Jensenius et al., 2004
Tick-borne lymphadenopathy	*R. raoultii*	European countries	Jensenius et al., 2004
North Asian tick typhus	*R. sibirica*	China and Russia	Jensenius et al., 2004
Rocky mountain spotted fever	*R. rickettsii*	Americas	Jensenius et al., 2004
African tick-bite fever	*R. africae*	Africa and Carribeans	Jensenius et al., 2004
Flea-borne spotted fever	*R. felis*	Worldwide	Parola, 2011
Louse-borne epidemic typhus	*R. prowazekii*	Europe, Asia, Africa	Cowan, 2000
Murine typhus	*R. typhi*	Europe, Asia, Africa	Cowan, 2000

2.3.1 Mediterranean spotted fever (MSF)

Mediterranean spotted fever caused by *Rickettsia conorii* is prevalent in the Mediterranean countries and sub-saharan Africa (Parola et al., 2005; Rovery & Raoult, 2008). In Africa, *R. conorii* has been found in Tunisia, Algeria, Morocco, Libya, Egypt, Kenya, Central Africa, Zimbabwe and South Africa (Rovery et al., 2008). The brown tick *R. sanguineus* which transmits *R. conorii* is closely associated with canines. Thus, dogs stand to play an important role in the epidemiology of MSF. A study conducted by Kelly *et al*., (1992) about the re-isolation of a Zimbabwean strain of *R. conorii* from the blood of artificially inoculated dogs suggests that dogs may act as domestic reservoirs of infection for ticks and subsequently humans. Furthermore, research conducted by Levin *et al*., (2012), showed for the first time that the domestic dog (*Canis familaris*) may be a competent reservoir of *R. conorii*.

2.3.2 Flea-borne spotted fever

Flea-borne spotted fever (FBSF) caused by *R. felis* is found in Europe, Australia, North and South America, Africa and Asia (Azad et al., 1997). *Rickettsia felis* was originally designated as a human pathogen by (Schriefer et al., 1994), and has since been diagnosed worldwide including in sub-saharan Africa (Parola, 2011). Flea-borne spotted fever caused by *R. felis* has also shown endemic status in regions of Kenya in East Africa (Richards et al., 2010; Maina et al., 2012), however, the risk factors associated with flea-borne rickettsioses in humans have not been fully determined in these areas (Jiang et al., 2013). As a preferred host of *Ctenocephalides felis*, the flea that transmits the pathogen, dogs could potentially act as a reservoir for human flea-borne spotted fever (Hii et al., 2011). This is elucidated by a number of molecular studies which have shown *R. felis* in a variety of fleas and ticks collected from dogs (Kelly et al., 2004; Schloderer et al., 2006).

2.3.3 African tick-bite fever (ATBF)

African tick-bite fever is caused by *Rickettsia africae* and is transmitted by ticks of the *Ambloyomma* genus *Amblyomma hebraeum* and *A. variegatum* (Jensenius et al., 2004). *Rickettsia africae* is found in sub-Saharan Africa, North Africa, the west of Asia, the West Indies and Oceania (Jensenius et al., 2004). The organism is found in rural areas where domestic cattle and wildlife reside. In recent years, more than 350 travel related cases of ATBF have been reported from Europe, America, Australia, Argentina and Japan (Jensenius et al., 2003; Wesslen et al.,1999). Most of the cases seen were infected in South Africa which has many wildlife resorts and is endemic for *R. africae* (Jensenius et al., 2004).

2.3.4 *Rickettsia parkeri* infection

Rickettsia parkeri is an emerging tick-borne rickettsiosis in the Americas. It is transmitted mainly by *Amblyomma* spp. of ticks (Lado et al., 2014). Dogs have been shown to be exposed to this

human rickettsiosis in rural areas in Brazil, Bolivia and Uruguay by serological studies (Labruna et al., 2007; Tamekuni et al., 2011).

2.3.5 Murine typhus

Murine typhus also called endemic typhus is caused by *Rickettsia typhi* and transmitted by the rat flea *Xenopsylla cheopsis*. The main reservoirs are rodents, *Rattus norvegicus* and *R. rattus* (Parola & Raoult, 2006). Murine typhus is distributed worldwide and cases have been reported in Mexico, Europe and the USA (Parola & Raoult, 2006). Travel related cases have also been documented of tourists returning from Africa, South East Asia, Indonesia, India and China (Parola et al., 1998; Watt & Parola, 2003; Jensenius et al., 2004). In tropical areas a higher prevalence of murine typhus is seen in coastal regions with a high population of rodents (Dupont et al., 1995).

2.3.6 Epidemic typhus

Epidemic typhus is caused by *Rickettsia prowazekii* and transmitted by the body lice *Pediculus humanus humanus* or *P. humanus corporis* (Parola & Raoult, 2006). Sporadic outbreaks of the disease occurs when populations of the vector thrives in situations of natural disasters, war and famine (Raoult & Roux, 1999). Outbreaks have been documented in Nigeria, Ethiopia, Burundi, Eastern Europe, India, Central and South America (Parola & Raoult, 2006; Raoult et al., 1998).

2.4 The life cycle of Rickettsiae

The life cycle of *Rickettsia* involves a sequence of events including the transmission of the pathogen, entrance at a particular anatomic site, dissemination of the organism, escape from host defences, growth within a specific location in the body, and the sequence of injury caused to the cells, tissues, organs, and the rest of the host's body (Walker et al., 2003b).There are essentially two types of host cell cycles for *Rickettsia*. One involves hard ticks in the family

Ixodidae and a second involves a vertebrate host including mice, dogs and rabbits. The survival of *Rickettsia conorii* is dependent on both hosts. A sequence of events occurs in the vertebrate and invertebrate hosts for the successful transmission of rickettsial disease (Rudakov et al., 2003).

Infection of invertebrate host: Rickettsiae often infect ticks in the family *Ixodidae* (hard ticks). These ticks have four stages in their life cycle: egg, larva, nymph, and adult. Ticks are infected with rickettsiae in the larval, nymphal, or adult stage while feeding on blood from an infected host. In addition, male ticks can transfer the pathogen to female ticks through bodily fluids or spermatozoa during mating, and female ticks can transmit the bacteria to their eggs through transovarial transmission (Parola et al., 2005). Once infected with the pathogen, a tick can remain infected for life (Rudakov et al., 2003). In the tick the *Rickettsia* multiply in the epithelium of the intestinal tract; from there, it penetrates the body cavity of the tick where it continues to multiply (Popov et al., 2007). This is similar to what happens in fleas which get infected with rickettsiae upon feeding on an infected host. On entry into the midgut, epithelial cell rickettsiae multiply rapidly and spread to the entire midgut epithelial cells over a period of three to five days (Azad et al., 1997).

Infection of the vertebrate host: Once transmitted to vertebrate hosts, rickettsiae multiply through transverse binary fission. In unfavourable conditions, they stop dividing and grow into long filamentous forms; otherwise they grow into the typical short rod forms subsequent to many excessive, rapid divisions (McDade & Newhouse, 1986). Rickettsiae thrive in endothelial cells and upon entry into the cell there is a binding of outer membrane proteins Omp A and Omp B to a host cell receptor (Frean & Blumberg, 2002). Once in the cell interior, rickettsiae induce changes to the actin cytoskeleton and are engulfed. In the phagosomes, the bacteria escape into the cytosol by destroying the phagosomal membrane and avoid being destroyed by lysosymes (Olano, 2005). The cytosol provides the necessary nutrients, energy and growth factors for the bacteria. The rickettsiae move through the cytoplasm and penetrate surrounding

endothelial cells compromising vascular integrity due to the induction of oxidative stress on the host cells. These events lead to microvascular permeability and oedema and finally tissue and organ dysfunction (Olano, 2005; Walker et al., 2003b). Rickettsiae are excreted through faeces which are important in the transmission of the disease in situations where infection occurs by scratching infectious faeces into the skin as seen in the transmission *of Rickettsia typhi* or inhaling dust contaminated with dry faeces (Azad & Beard, 1998). Infection with *R. felis* can be maintained vertically in cat flea populations through both transovarial and transstadial transmission for several generations without requiring an infectious vertebrate blood meal for the fleas (Reif & Macaluso, 2009). Most rickettsiae are transmitted by arthropod vectors (ticks, lice, fleas and mites), thus humans are considered to be accidental hosts and hence they are not fundamental in the rickettsial cycle (Frean & Bloomberg, 2002).

2.4.1 Vectors important in the transmission of rickettsial infection

Common genera of invertebrate vectors that transmit rickettsial infection worldwide include *Dermacentor*, *Rhipicephalus*, *Amblyomma*, *Haemaphysalis*, *Ixodes* ticks and *Ctenocephalides* fleas (Azad & Beard, 1998). *Dermacentor* spp of ticks are implicated in the transmission of *Rickettsia rickettsii* and *R. slovaca* in the Americas and Europe (Parola et al., 2005). *Ixodes* spp ticks are the main vectors known in the transmission of *R. helvetica* and *Anaplasma phagocytophilum* in the Americas and Europe (Parola et al., 2005; Woldehiwet, 2010). Species of *Rhipicephalus, Haemaphysalis,* and *Amblyomma* are common tick vectors transmitting rickettsial infection in Africa (Kelly et al., 1996). The ticks, *Rhipicephalus sanguineus* and *Haemaphysalis elliptica* are the two most common species of tick-vectors of domestic dogs and wild felids in South Africa (Horak & Matthee, 2003).These ticks feed on many vertebrates such as dogs, medium-sized mammals, and small rodents (Horak & Matthee, 2003). *Rhipicephalus sanguineus* transmits *R. conorii* to dogs and humans (Levin et al., 2012). *Rickettsia conorii* has also been detected in *H. elliptica* in Zimbabwe (Beati et al., 1995). The cat flea *Ctenocephalides felis* is the only defined biological vector *of R. felis* (Reif & Macaluso, 2009). It has also been shown that dogs may act as important reservoir host for *R. felis* which is the

cause of flea-borne spotted fever in humans (Hii et al., 2011). *Amblyomma maculatum* transmits an emerging tick-borne rickettsioses *R. parkeri* (Toledo et al., 2011).

2.4.2 Rickettsial infections in South Africa

Spotted fever rickettsiosis has been recognized in South Africa since the beginning of the 20th century (McNaught, 1911). *Rickettsia conorii* the agent of Mediterranean spotted fever has long been associated with human disease in South Africa (Pretorius & Birtles, 2002). *Rickettsia aeshlimannii* which causes a Mediterranean spotted fever-like illness has also been identified in South Africa (Beati et al., 1997) and African tick-bite fever caused by *R. africae* is prevalent in the country (Fournier et al., 1998). Game hunting and travelling to Southern Africa from November through April increases the risk for African tick-bite fever in travellers. Contact with tick-infested cattle and game in areas endemic for certain spotted fever group rickettsiae may increase the risk of disease (Jensenius et al., 2004). *Rickettsia mongolotimonae* which causes a lymphangitis-associated rickettsiosis has also been reported in South Africa (Pretorius & Birtles, 2004). *Rickettsia felis* has not yet been reported in South Africa although recognized as an emerging pathogen (Reif & Macaluso, 2009).

2.5 Pathogenesis of rickettsial infection and the disease syndrome

2.5.1 Pathogenesis in dogs

The pathogenesis of rickettsioses in the dog is usually associated with a vasculitis caused by the multiplication and growth of rickettsial organisms in the endothelial lining of small capillaries, arteries and venules (Varela, 2003). The proliferation of rickettsiae in the vessels causes damage to the cells. Vasculitis is progressive and it leads to an increase in vascular permeability of the vessels (Olano, 2005) resulting in the extravasation of blood cells and plasma which subsequently leads to oedema, hypotension, haemorrhage and shock (Olano, 2005; Walker et al., 2003b). Pulmonary oedema is usually associated with signs of respiratory distress whereas when oedema occurs in the central nervous system, neurologic signs are

seen (Varela, 2003). Ocular damage occurs due to infiltration of inflammatory cells and petechial haemorrhages on the retina, and obstruction of the vessels results in tissue loss and organ damage (Varela, 2003). Dogs infected with *R. rickettsii* have a rapid and severe disease course. Fever occurs 4 - 5 days after the tick bite. Petechiae and ecchymoses develop on mucous membranes due to decreased platelet numbers. Clinical signs progress to oedema and eventually to multisystemic organ disorders. Leukopenia may be present early in the disease and change to a leukocytosis at a later stage (Varela, 2003). Ocular haemorrhage is a consistent finding. Scrotal swelling, hemorrhage and testicular pain are often observed in male dogs. Neurologic signs including pain, loss of balance, tilting of the head, stupor, seizures and coma may also occur (Warner & Marsh, 2002). The duration of illness with *R. rickettsii* generally lasts about 2 weeks, and occurs seasonally in the warmer months of the year. The clinical presentation of rickettsial infection may vary with the causative agent. Common clinical signs in dogs resulting from infections with *R rickettsii* include fever, malaise and sometimes vomiting and nausea (Labruna et al., 2009). Clinical signs observed in dogs, resulting from infections by *R. conorii* are reported to include anorexia, lethargy, intermittent lameness and intermittent vomiting (Solano-Gallego et al., 2006).

2.5.2 Pathogenesis in humans

Pathogenesis of rickettsioses in humans is similar to what is seen in dogs. Upon transmission, rickettsiae multiply in vascular endothelial cells of arteries, veins and capillaries resulting in vasculitis and thrombosis of many organs including the heart, lungs, gastrointestinal tract, skeletal muscles, skin, brain and kidneys. The vasculitis results in the clinical signs usually associated with rickettsioses (Raoult & Roux, 1997; Walker et al., 2003b). In humans, common clinical features for Rocky Mountain spotted fever (RMSF) include fever, headache, rash, confusion, and myalgia (Abramson & Givner, 1999). The onset of the disease may be gradual or abrupt, beginning from approximately 1 week (range, 2-14 days) following a bite from an infected tick. As many as 40% of patients may be unaware of the tick bite, which is usually painless and may go unnoticed or may be easily forgotten. The headache is usually persistent and intense. Neurologic signs include agitation, insomnia and tremor. This may progress to

mental confusion and delirium. Gastro intestinal symptoms (abdominal pain and diarrhoea) are common during early stages of the illness. Conjunctival infection may also be seen (Abramson & Givner, 1999). In humans, MSF has an acute onset with high fever (above 39°C), headache, malaise and arthromyalgias. Headaches are less frequent in children (Rovery & Raoult, 2008). Unusual presentations such as acute pancreatitis have also been described (Rombola, 2011). The incubation period is usually 6 days ranging from 1-16 days. An eschar or cutaneous necrosis caused by a rickettsial vasculitis at the tick-bite site of inoculation, known as *tache noire* ("black spot"), is usually seen. This lesion heals slowly over 10-20 days without leaving a scar.

African tick-bite fever has similar clinical signs to those of MSF, but has a milder presentation. It differs from other similar rickettsioses in that it produces a painful lymphadenopathy, multiple eschars, nuchal myalgia, and occasionally, a sparse vesicular rash (Walker, 2007; Parola et al., 2005). Clinical signs of infection with *R. felis* in humans include fever, headache, malaise, joint pain, back pain, enlarged lymph nodes and maculopapular rash (Richter et al., 2002; Richards et al., 2010).

2.6 Diagnosis

Rickettsial diseases in humans and dogs are difficult to diagnose clinically because of the similarity in clinical signs and symptoms of many infectious diseases. Therefore, routine laboratory investigations help in ascertaining the cause of rickettsial diseases (Parola & Raoult, 2006). Current methodologies used in the laboratory include serological and molecular assay.

2.6.1 Cell culture

The cultivation of rickettsiae in reference laboratories remains the most definitive way of isolating rickettsiae from clinical samples but it requires the research being conducted in biosafety level 3 laboratories (Parola & Raoult, 2006). Embryonated chicken egg yolk sacs, laboratory animals, and cell cultures using Vero or L929 cells have been successfully used in the isolation of rickettsiae (La Scola & Raoult, 1997).

2.6.2 Serological assays

Serological tests are the most valuable techniques used in the diagnosis of rickettsial infection (La Scola & Raoult, 1997). Serological assays are used to to detect seroconversion in dogs; the major limitation of these assays, however, is wide antigenic cross-reactions among SFG rickettseae (Raoult & Roux, 1997). Furthermore, serological tests are often negative early in the disease due to the time it takes for antibodies to develop and the low concentration of the organisms in the blood stream (Frean & Blumberg, 2002). The Weil-Felix test is one of the earliest serologic assays used in the diagnosis of rickettsial infection and the test is based on the detection of antibodies to *Proteus* species antigens that cross-react with rickettsias (Parola & Raoult, 2006) however, it has the limitations of lacking both sensitivity and specificity (Parola & Raoult, 2006) but is still used as a first line test in tropical countries with limited laboratory facilities (La Scola & Raoult, 1997).

The immunofluorescent assay (IFA) is regarded as the reference technique for serologic diagnosis of rickettsioses (La Scola & Raoult, 1997) and has the advantages of being readily commercially available and also of simultaneously detecting antibodies to a number of rickettsial antigens with the same drop of serum in a single well. One of the drawbacks is that it requires the use of rheumatoid factor absorbent before IgM antibody determination (La Scola & Raoult, 1997). The indirect microimmunofluorescence (MIF) assays is a micro-method adaptation of the IFA (Phillip et al., 1976) and is viewed as the most sensitive serological test that detects IgM and IgG antibodies and is a widely used test in the diagnosis of SFG

rickettsioses. It is also used to detect seroconversion in dogs with infections by *R. rickettsii* (Sykes, 2013). ELISA on the other hand is more sensitive than the IFA allowing detection of low levels of antibody and is useful for diagnosis of acute cases of infection for seroepidemiology. However, ELISA has the disadvantage of requiring a complex and slow antigen purification procedure (La Scola & Raoult, 1997). The Western immunoblot assay has the advantage of being the most specific and sensitive serologic test for detecting early antibodies in rickettsial infection and is a useful tool to differentiate true-positives from false-postives results caused by antibodies of cross-reactive rickettsias but the procedure is time consuming (La Scola & Raoult, 1997).

2.6.3 Molecular biological assays

Since rickettsial diseases are often incorrectly diagnosed and detectable antibody levels are not seen until after day ten of the illness (Raoult et al., 1986), a more rapid means of diagnosis is desired. Technological advances in the detection and identification of microorganisms using the polymerase chain reaction (PCR) has led to a new phase in the diagnosis of rickettsial infection (Fenollar & Raoult, 2004). A PCR that targets a portion of the omp A gene has been shown to detect 160 copies of *Rickettsia* DNA (Fournier & Raoult, 2004). PCR-based assays have been used to detect rickettsiae responsible for typhus, spotted fever and scrub typhus (Tzianabos et al., 1989). PCR can also demonstrate *Rickettsia* infection in seronegative patients, making it a likely important tool in the acute diagnosis of disease (Choi et al., 2005). A number of PCR assays, focusing directly at a number of different genes (glt A, omp B and 17kD antigen gene) have been used to detect *Rickettsia* in clinical specimens (Stenos et al., 2005; Henry et al., 2007; Jiang et al., 2012).

Nested PCR have been shown to be more sensitive than conventional PCR assay and cell cultures (Fournier & Raoult, 2004). One study in dogs experimentally infected with *R. rickettsii* showed that a single tube nested PCR detected *Rickettsia* earlier and for longer compared to cell culture (Breitschwerdt et al., 1999). However given the fact that nested PCR reaction tubes

have to be opened to add the second set of primers and additional polymerase, laboratory contamination is a big drawback of this technique. The risk of contamination is reduced in suicide-nested PCR, which makes use of nested PCR, but the primers are used only on a limited number of assays in a laboratory and then removed. A suicide-nested PCR detecting sequences from several different genes was more sensitive than cell culture for detecting *Rickettsia* in skin biopsy specimens (Fournier & Raoult, 2004). A suicide-nested PCR targeting non-protruding portions of the omp A gene was also found to be more sensitive than culture for skin biopsy specimens but not for serum (Fournier & Raoult, 2004). The development of a real-time PCR assay specific for SFG and TG rickettsiae is significant to overcoming the limitations of serology and conventional PCR (Stenos et al., 2005). Real-time PCR assays have been developed to detect *Rickettsia typhi*, *Rickettsia felis* and differentiate rickettsiae from orientiae using primers targeting omp B, 47kDA and glt A genes (Henry et al., 2007; Paris et al., 2008).

2.7 Characterization of *Rickettsia*

Advances in molecular techniques have changed the ability to detect and characterize infection with *Rickettsia*. Polymerase chain reaction combined with DNA sequencing, restriction fragment length polymorphism (RFLP) has increased sensitivity and offers the potential aid of identifying the species of the infecting *Rickettsia* even without obtaining an isolate (Choi et al., 2005).Many genes have been sequenced from *Rickettsia* isolates, and as a result, species classifications of various isolates and phylogenetic relationships between species have switched quickly in recent years. The standard for the definition of a species using gene sequencing approach has come under investigation for bacteria in general, and *Rickettsia* in particular (Parola et al., 2005). The number of base pair differences that describes a strain or species has not been determined. Confirmatory strains or species classifications based on genotype with traceable phenotypic differences have been recommended, along with sequencing of at least five different genes. Sequences from the genes glt A (citrate synthetase), rrs (16S rRNA), the 5’ region of omp A (outer membrane protein A), gene D and omp B (outer membrane protein B) have been used to establish gene sequence based standards for the identification of new *Rickettsia* species (Parola et al., 2005). Since the protein omp A is an antigen that contributes to the serologic differences used to phenotypically

differentiate *Rickettsia* species (Xu & Raoult, 1997) and its genotypic variability can differentiate among species (Fournier et al., 2003; Roux et al., 1996) the gene encoding Omp A protein has become a logical target for PCR in diagnosing clinical specimens. In fact, sequence analysis of a 632 bp amplicon produced from amplification using primers Rr190.70 and Rr190.701 demonstrated 98.8% sequence differences among *Rickettsia* species, and these variations have been routinely used to identify the species of infecting SFG *Rickettsia* in clinical specimens (Fournier et al., 2003; Raoult & Roux, 1997). A spotted fever quantitative PCR (SQ-PCR) was developed for the detection and differentiation of *R. rickettsii* and other closely related SFG species (Eremeeva et al., 2003), however, a flaw of this assay is a lack of sensitivity to diagnose infection when low numbers of rickettsiae are present in blood mononuclear cells. Other PCR assays with a demonstrated limit of detection superior to the assay using the Rr190.70 and Rr190.701 primers, either have not been established for the purpose of differentiating SFG species, or they differentiate a limited number of species (Choi et al., 2005; Leitner et al., 2002; Eremeeva et al., 2003, Stenos et al., 2005).

2.8 Control and treatment of rickettsial Infection

Prevention of rickettsial infections is aimed at limiting exposure of dogs to potentially infected arthropods. Dogs should be maintained year round on tick control products (e.g. collars impregnated with amitraz) to prevent attachment and feeding of ticks and other ectoparasites (Warner & Marsh, 2002). Ectoparasite control can also be aided by removal of parasite habitats around homes by trimming brush, removal of leaf litter and clipping grass and also limiting contact with infested areas by preventing dogs from roaming (Warner & Marsh, 2002). Means of tick control for dogs include the use of systemic or topically applied acaricides containing permethrin or fipronil (Shaw & Day, 2005).

Doxycycline remains the antibiotic of choice in treatment of RMSF in dogs (Warner & Marsh, 2002), but newer macrolides like azithromycin may be of interest because they have been used successfully for the treatment of acute infection (Rovery & Raoult, 2008). Fluoroquinolones and

enrofloxacin may be used as alternative antibiotics. Supportive therapy with electrolyte and fluid maintenance are also effective to the management of human patients with rickettsial diseases, especially if there are signs of low blood pressure, electrolyte imbalance, and blood clotting problems (Rovery & Raoult, 2008). Development of subunit vaccines, containing outer membrane proteins, has been shown to be effective against challenge infection in experimental animals (Shaw & Day, 2005).

2.9 Other canine vector-borne disease pathogens

2.9.1 *Babesia*

Babesia is an intraerythrocytic parasite that causes babesiosis. *Babesia canis*, *B. rossi*, *B. vogeli*, and *B. gibsoni* are species implicated in causing canine babesiosis. The disease has a worldwide distribution and in warm climates like Africa the organism is usually transmitted by Ixodid ticks *Rhipicephalus sanguineus* and *Haemaphysalis elliptica* (Irwin, 2010). Canine babesiosis is the most predominant tick-borne protozoal infection of dogs in South Africa (Shakespeare, 1995; Collett, 2000), with *B. rossi* and *B. vogeli* most commonly associated with the disease (Matjila et al., 2004).

Clinical signs of babesiosis include anaemia, weakness, icterus, fever and varying degrees of haemoglobinuria and bilirubinuria (Irwin, 2010). In acute cases, babesiosis diagnosis is usually by microscopy to detect intraerythrocytic piroplasms, serology using immunofluorescent antibody test (IFAT) and molecular diagnosis using PCR. Treatment involves use of drugs like imidocarb dipropionate, diminazene aceturate and clindamycin (Shaw & Day, 2005). The disease can be prevented by keeping dogs free of tick exposure by use of systemic or topically applied acaricides (Shaw & Day, 2005).

2.9.2 *Ehrlichia*

Ehrlichia canis is the cause of monocytic ehrlichiosis in dogs and is the most common species affecting dogs. Other species include *E*. *chaffensis* and *E*. *ewingii*. *Ehrlichia* is transmitted by the ticks *R*. *sanguineus* and *Amblyomma americanus* which are identified as primary vectors. *Ehrlichia canis* has a worldwide distribution (Dumler & Walker, 2001), and is an important tick-transmitted infection causing clinical disease in dogs in South Africa (Rautenbach et al., 1991; Van Heerden, 1982). *Ehrlichia chaffensis* infecting dogs and humans has also been documented in South Africa although the study was based on serological diagnosis (Pretorius and Kelly, 1998; Pretorius et al., 1999). Clinical signs of ehrlichiosis include depression, anorexia, pyrexia, petechiae of the skin and mucous membranes and epistaxis (Shaw & Day, 2005). Diagnosis is usually by (IFAT) and treatment is achieved with the use of doxycycline, imidocarb dipropionate and oxytetracycline (Kelly, 2000). However, dogs remain chronic carriers of the infection. Tick control is the most effective measure of preventing the infection (Shaw & Day, 2005).

2.9.3 *Anaplasma*

Anaplasma phagocytophilum is a zoonotic obligate intracellular parasite that causes granulocytic anaplasmosis in dogs and is transmitted transtadially by ticks of *Ixodes* spp. (Cockwill et al., 2009). The disease has been reported in Europe and North America. A new *Anaplasma* sp. strain closely related to *Anaplasma phagocytophilum* has also been detected using molecular techniques in canine blood samples in South Africa (Inokuma et al., 2005). Clinical signs of granulocytic anaplasmosis include lethargy, anorexia, fever, diarrhea and lameness due to polyarthritis (Little, 2010). Diagnosis is by the use of microscopy, serological tests and PCR (Shaw & Day, 2005). The disease can be prevented by tick control.

Anaplasma platys is the cause of infectious canine cyclic thrombocytopaenia. The disease has a worldwide distribution and has occurred in the USA, Europe, Middle East, Africa, Asia and Australia (Sykes, 2013). *Anaplasma platys* is thought to be transmitted by the brown dog tick *R.*

sanguineus but this has not been conclusively demonstrated (Little, 2010). Clinical signs include cyclical fever, bleeding disorders and lymphadenopathy (Harrus et al., 1997). Diagnosis is usually by microscopic identification of morulae in platelets, IFAT, and PCR (Shaw & Day, 2005). Treatment involves use of tetracyclines and prevention is mainly by vector control using acaricides with a long duration of activity (Brown et al., 2001).

2.9.4 *Theileria*

Theileria spp. are ampicomplexan parasites closely related to *Babesia* spp. Members of the genus implicated in causing disease in dogs include *T. annae, T. annulata, T. equi*, and *Theileria*-like group (*Babesia conradae*) (Criado-Fornelio et al., 2003; Criado et al., 2006; Dixit et al., 2010; Simões et al., 2011). *Theileria annae* is endemic in Spain, but has also been identified in Portugal, Croatia and North America (Sollano-Gallego & Baneth, 2011; Simões et al., 2011). In South Africa Matjila *et al*., (2008 a), detected a *Theileria* sp. closely related to *Theileria* sp. (sable) in one out of three dogs that presented with an immune mediated condition and severe thrombocytopenia in the absence of *Ehrlichia* infections. Vectors proposed as transmitting *Theileria* spp. to dogs are *Ixodes hexagonus* (Camacho et al., 2003) and a variety of tick species from genera *Hyalomma*, *Dermacentor* and *Rhipicephalus* (Beck et al., 2009).

Clinical characteristics of dogs infected with *T. annae* include fever, lethargy, tachycardia, hemoglobinuria, tachypnea, severe regenerative anaemia, thrombocytopenia, splenomegaly, and peripheral lymphadenomegaly (Falkenö et al., 2013; Garcia 2006; Solano-Gallego & Baneth, 2011). Diagnosis is usually by microscopic identification of the parasite in wet blood smears and PCR (Solano-Gallego & Baneth, 2011). Treatment involves use of epoximicin and artesunate (Aboulaila et al., 2010; Goo et al., 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study area

A Health and Demographic Surveillance System in Dogs (HDSS-Dogs) was established in Hluvukani settlement in the Mnisi community situated in the north-eastern corner of Bushbuckridge local municipality in the Ehlanzeni district of Mpumalanga in 2011, with funding from the Morris Animal Foundation, USA. A rural community, the study area falls within the savannah region and is adjacent to the Andover and Manyaleti provincial game reserves. The GPS coordinates are S -24* 39´, E 31* 20´. The mean annual rainfall is about 600 mm. The maximum temperature in the summer months is about 29 °C and the minimum temperature averages 12 °C while in the winter months the highest temperature is about 23 °C and the minimum temperature averages 6 °C. Figure 3.1 shows the map of the study area.

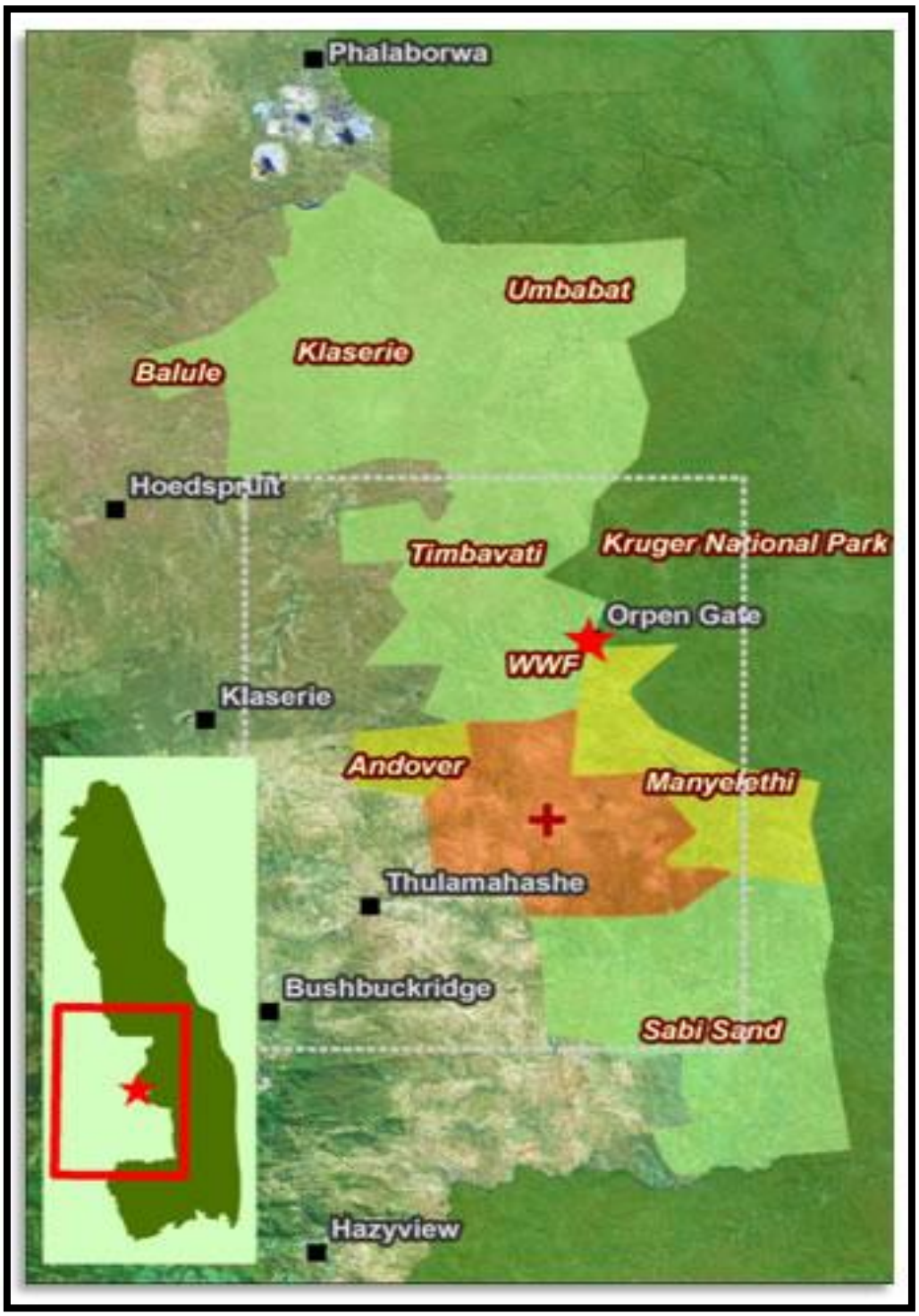

Figure 3.1: Map of study site Mnisi Community area Bushbuckridge, Mpumalanga Province, South Africa

3.1.1 Blood sample collection

Blood samples were collected from dogs present at households visited by the HDSS-Dogs field team during periods when the team was accompanied by undergraduate veterinary students from the University of Pretoria and Utrecht University, The Netherlands. Samples were collected from 21 dogs in October 2011, 64 dogs in December 2011, and 56 dogs in April/May 2012, making a total of 141 samples. Dog-owning households were not systematically selected for inclusion in the study. An attempt was made to collect blood samples from all dogs present at the household at the time of the visit, but samples were not collected from dogs that owners were unwilling or unable to handle.

During the first two sample collection periods (October and December, 2011), blood was collected directly in capillary tubes from the cephalic vein and stored on FTA filter paper. During the third period (April/May 2012), blood was collected in EDTA and plain vacutainer tubes. In addition, blood from the EDTA tube was stored on FTA cards. Blood samples stored on FTA cards were then sent to the Department of Veterinary Tropical Diseases, University of Pretoria (UP) for analysis. All samples were collected under the HDSS project protocol which was approved by UP Animal Ethics Committee (protocol number V033/11).

3.1.2 Ectoparasite collection

During the visits to the households through the HDSS-Dogs project, dogs were also inspected for the presence of ectoparasites. The full body of the animals was brushed with a plastic comb or brush and white paper to brush off fleas for collection. Live adult, unengorged ticks were removed manually from the animals using forceps. A total of 103 ticks and 43 fleas were collected. All ectoparasites were preserved in a solution of 70% ethanol and identified to species level under a stereomicroscope, according to standard morphological identification guides (Soulsby, 1982; Urquhart et al., 1996; Walker et al., 2003a). Ticks and fleas were pooled into groups of 10 and 2 respectively based on their species (Table 3.1).

Table 2.1: Ectoparasite pools and identified species

Pool number	Number per pool	Ectoparasite	Species name
T1	Tick pool (n=10)	Tick	*Haemaphysalis elliptica*
T2	Tick pool (n= 10)	Tick	*H. elliptica*
T3	Tick pool (n=10)	Tick	*H. elliptica*
T4	Tick pool (n=13)	Tick	*Amblyomma hebraeum*
T5	Tick pool (n=14)	Tick	*A. hebraeum*
T6	Tick pool (n=1)	Tick	*Ixodes*
T7	Tick pool (n=13)	Tick	*Rhipicephalus sanguineus*
T8	Tick pool (n=14)	Tick	*R. sanguineus*
T9	Tick pool (n=9)	Tick	*Rhipicephalus simus*
T10	Tick pool(n=9)	Tick	*R. simus*
F1	Flea pool (n=23)	Flea	*Ctenocephalides felis strongylus*
F2	Flea pool (n=20)	Flea	*Echidnophaga gallinacea*

3.2 DNA extraction

3.2.1 DNA extraction from blood spotted on FTA filter cards

DNA was extracted from 141 blood samples spotted on FTA filter cards using the QIAamp DNA mini kit® (Qiagen, Hilden, Germany). Three pieces of approximately 3 mm of a dried blood spot placed onto a cutting mat were cut out for individual samples using a sterile lancet and placed into a 1.5 ml microcentrifuge tube. Buffer ATL (180 µl) was added and the tubes were incubated at 85ºC for 10 minutes then centrifuged briefly to remove drops from the lid. Twenty microlitres of proteinase K solution was added and mixed by vortexing, following which the samples were incubated at 56ºC for 1 hour. The samples were centrifuged briefly to remove drops from inside the lid and 200 µl of AL buffer was then added and mixed by vortexing before incubation at 70ºC for 10 minutes. Incubation was again followed by brief centrifugation. Two hundred µl of ethanol was then added to each sample, mixed thoroughly by vortexing and spun down by brief centrifugation. The mixture was then transferred to the QIAamp Mini spin column, and centrifuged at 8000 rpm for 1 minute. The QIAamp mini spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded.

The AW1 buffer (500 µl) was added to the QIAamp mini spin column and centrifuged at 8000 rpm for 1 minute. The filtrate was discarded and the spin column was placed in a clean 2 ml collection tube. The AW2 buffer (500 µl) was added to each spin column and centrifuged at 14000 rpm for 3 minutes. The filtrate was discarded and the spin columns were then placed in a new 2ml collection tube and further centrifuged at 14000 rpm for 1 minute. The QIAamp mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and the DNA was eluted by adding 150 µl of elution buffer AE, incubating at room temperature for 3 minutes, and then centrifuging at 8000 rpm for 1 minute. Extracted DNA was stored at -20 ºC until further analysis.

3.2.2 DNA extraction from ticks and fleas

Ticks and fleas were washed in distilled water and air-dried overnight. Grouped ticks according to their species were cut into four smaller pieces and placed into a sterile tube containing glass beads. The pooled fleas were also placed in sterile tubes containing glass beads. DNA extraction was done using QIAamp DNA mini kit® (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Three hundred µl of ATL buffer was added into each tube containing ticks and fleas and the tubes were placed on ice. The tick and flea samples were homogenized using the Tissue Lyser®instrument (Qiagen) for 3 minutes at 7000 rpm and then the tubes were again placed on ice. Twenty microlitre of proteinase K was added into each tube and the tubes were incubated overnight at 56ºC for lysis. The following day the samples were vortexed and 200 µl of supernatant was transferred into a 2 ml eppendorf tube. Two hundred µl of buffer AL was added to the supernatant and the mixture was vortexed thoroughly before incubation at 70ºC for 10 minutes. Ethanol (200µl) was added to each tube and the samples were vortexed and subsequently transferred into marked QIAamp mini spin columns. The spin columns were then centrifuged at 8000 rpm for 1minute after which the flow through was discarded. Five hundred µl of AW1 buffer was added to the columns followed by centrifugation at 8000 rpm for 1 minute. The flow through was then discarded and 500 µl of AW2 was added to the columns. The spin columns were centrifuged for 3 minutes at 14000 rpm, and the flow through was discarded. The column was then placed in a new collection tube and centrifuged for 1 minute at 14000 rpm to remove residual AW buffer. The column was placed in a marked 2

ml tube and 100 µl elution buffer AE was added directly onto the QIAamp membrane of the spin column. The columns were incubated at room temperature for 3 minutes and then centrifuged for 1 minute at 8000 rpm to elute DNA. DNA was then stored at -20ºC until further analysis.

3.3.1 Polymerase chain reaction

Polymerase chain reaction was conducted with a set of primers that amplified the V4 hypervariable region of the 18S ribosomal RNA gene of *Theileria* and *Babesia* species and the V1 hypervariable region of the 16S rRNA gene of *Ehrlichia* and *Anaplasma* species (Gubbels et al., 1999; Bekker et al., 2002; Matjila et al., 2004). Table 3.2 shows the primer sequences used for the *Theileria*/*Babesia*, and *Ehrlichia*/*Anaplasma* PCR. Vaccine strains of *Anaplasma centrale* and *Babesia bovis* (Onderstepoort Biological Products) were used as positive controls and PCR grade water was used to replace the DNA template in a negative control reaction.The PCR reaction comprised 5 µl of DNA template, primers (forward and reverse) (Table 3.2) at a final concentration of 0.1 µM and 12.5 µl of Platinum Quantitative PCR SuperMix-UDG® (Invitrogen) which contains 60 U/ml platinum *Taq* DNA polymerase, 40 mM Tris-HCl (pH 8.4), 100 mM KCl, 6 mM $MgCl_2$, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 400 µM dUTP, 40 U/ml UDG, and stabilizers. Seven µl of PCR grade water was added to make a total reaction volume of 25 µl. The PCR was performed on the Gene Amp® PCR system 9700 (Applied Biosystems) in a programme that included an enzyme activation cycle at 37ºC for 3 minutes, initial denaturing at 94ºC for 10 minutes, followed by 10 cycles of 20 sec at 94°C, 30 sec at 67ºC and 30 sec at 72ºC. The annealing temperature was lowered by 2ºC, every second cycle for the stepdown PCR followed by 40 cycles of denaturation at 72ºC for 30 sec and annealing at 57ºC for 30 sec. The final extension was performed at 72ºC for 7 minutes.

Table 3.2: Primer sequence for PCR

Genus	Primer	Primer Sequence(5'-3')
Babesia and *Theileria*	RLB-F2 RLB-R2	GACACAGGGAGGTAGTGACAAG biotin-CTAAGAATTTCACCTCTGACAGT
Erhlichia and *Anaplasma*	Ehr-F Ehr-R	GGA ATT CAG AGT TGG ATC MTG GYT CAG CGG GAT CCC GAG biotin-TTT GCC GGG ACT TYT TCT

3.3.2 Reverse line blot (RLB) hybridization assay

Reverse line blot hybridization assay was performed on the PCR products as described by Gubbels *et al*., (1999). A blotting membrane was activated by 10 min incubation in 10 ml of 16% 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide (EDAC) at room temperature. The membrane was washed for 2 minutes with distilled water and placed in an MN45 miniblotter (Immunetics, Cambridge, Massachusetts). In 200 µl labeled PCR tubes specific oligonucleotide probes 0.25 pmol /µl (Table 3.3) were diluted to a final concentration of 2 pmol /150 µl by adding 142 µl of 0.5 M $NaHCO_3$ (pH 8.4) and were subsequently covalently linked to the membrane with the amino linker. The membrane was then incubated for 2 minutes at room temperature after which the oligonucleotide solution was aspirated. The membrane was inactivated by incubation in 100 ml of a 100 mM NaOH solution for 8 minutes at room temperature on a shaker and subsequently in 100 ml of 2 x SSPE/0.1% sodium dodecyl sulfate (SDS) for 5 minutes at 60°C in a shaker incubator.

Before use, the membrane was washed for 5 minutes at 42°C with 50 ml of 2 x SSPE/0.1% SDS under gentle shaking and placed in the miniblotter with the slots perpendicular to the previously applied specific oligonucleotides. PCR products were diluted by adding 130 µl of 2 x SSPE/0.1%, and denatured for 10 minutes at 99.9ºC on the Gene Amp® PCR system 9700 (Applied Biosytems) thermal cycler. Denatured PCR products were applied to slots and hybridized in an incubator at 42ºC for 60 minutes and then aspirated. The membrane was

washed twice in preheated 2 x SSPE/0.5% SDS for 10 minutes at 50ºC in the incubator under gentle shaking, and then incubated with 10 ml of 2 x SSPE/0.5% SDS and 12.5 µl streptavidin-POD (peroxidase labeled) conjugate (Roche Diagnostics, South Africa) for 30 minutes at 42ºC on a shaker. The membrane was then washed twice in preheated 2 x SSPE/0.5% SDS for 10 minutes at 42ºC and again washed twice with 2 x SSPE for 5 minutes at room temperature under gentle shaking. Ten millilitres of ECL detection fluid (Perkin Elmer, U.S.A) was added onto the membrane and incubated for 1 minute at room temperature before exposure to an X-ray film and subsequent development for final visualization by chemiluminescence. The film was placed in a grid and lanes of sample reaction were viewed against oligonucleotide probes. The PCR products were stripped from the membrane by washing it twice with 200 ml pre-heated 1% SDS for 30 minutes at 80ºC; under gentle shaking followed by a 2nd wash with 200 ml 20 mM EDTA for 15 minutes at room temperature. The membrane was then sealed in a plastic container with 50 ml 20 mM EDTA and stored at 4ºC for re-use (Gubbels et al., 1999). Table 3.3 shows probe sequences used on the RLB hybridization assay membrane.

Table 3.3: Probe sequences for specific detection of parasite species

Genus/Species Target	Probe Sequence 5^1-3^1	Source
Ehrlichia/Anaplasma genus-specific	GGG GGA AAG ATT TAT CGC TA	Bekker et al., 2002
A. centrale	TCG AAC GGA CCA TAC GC	RLB manual, Isogen
A. marginale	GAC CGT ATA CGC AGC TTG	Bekker et al., 2002
A. phagocytophilum	GRA TAR TTA GTG GCA GAC GGG T	Bekker et al., 2002
E. ruminantium	AGT ATC TGT TAG TGG CAG	RLB manual, Isogen
A. bovis	CTT GCT ATG AGA AYA ATT AGT GGC	Bekker et al., 2002
E. chaffiensis	ACC TTT TGG TTA TAA ATA ATT GTT	RLB manual, Isogen
Anaplasma sp. omatjenne	CGG ATT TTT ATC ATA GCT TGC	Bekker et al., 2002
E. canis	TCT GGC TAT AGG AAA TTG TTA	Bekker et al., 2002
Theileria/Babesia genus-specific	TAA TGG TTA ATA GGA RCR GTT G	Gubbels et al., 1999
Theileria genus-specific	ATT AGA GTG CTC AAA GCA GGC	Nijhof[a] (unpublished)
Babesia genus-specific 1	ATT AGA GTG TTT CAA GCA GAC	Nijhof (unpublished)
Babesia genus-specific 2	ACT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
B. felis	TTA TGC GTT TTC CGA CTG GC	Bosman et al., 2007
B. divergens	TGA CTA ATG TCG AGA TTG CAC TTC	Nijhof et al., 2003
B. microti	GRC TTG GCA TCW TCT GGA	Nijhof et al., 2003
B. bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels et al.,1999
B. bovis	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels et al.,1999
B. rossi	CGG TTT GTT GCC TTT GTG	Matjila et al., 2004
B. canis	TGC GTT GAC CGT TTG AC	Matjila et al., 2004
B. vogeli	AGC GTG TTC GAG TTT GCC	Matjila et al., 2004
B. major	CGCTGTGGCTTATCCTTTTAC	Georges et al., 2001
B. bicornis	TTGGTAAATCGCCTTGGTCG	Nijhof et al., 2003
B. caballi	GTG TTT ATC GCA GAC TTT TGT	Butler et al., 2008
B. gibsoni	TAC TTG CCT TGT CTG GTT T	Nijhof et al., 2003

Babesia sp. (*sable*)	GCG TTG ACT TTG TGT CTT TAG C	Oosthuizen et al., 2008
Neoehrlichia genus-specific	GGAATAGCTGTTAGAAATGACAGG	Schouls et al., 1999
Candidatus Neoehrlichia mikurensis	GCTGTAGTTTACTATGGGTA	Schouls et al., 1999
T. bicornis	GCG TTG TGG CTT TTT TCT G	Nijhof et al., 2003
T. annulata	CCT CTG GGG TCT GTG CA	Georges et al., 2001
T. buffeli	GGC TTA TTT CGG WTT GAT TTT	Gubbels et al., 1999
T. mutans	GCGGCTTATTTCGGACTYG	Gubbels et al., 1999
T. parva	GGA CGG AGT TCG CTT TG	Nijhof et al., 2003
T. taurotragi	TCT TGG CAC GTG GCT TTT	Gubbels et al., 1999
T. velifera	CCT ATT CTC CTT TAC GAG T	Gubbels et al., 1999
T. equi	TTC GTT GAC TGC GYT TGG	Butler et al., 2008
T. lestoquardi	ATTGCTTGTGTCCCTCCG	Schnittger et al., 2004
T. ovis	GCATTGCTTTTGCTCCTTTA	Matjila et al., 2004
T. annae	CCG AAC GTA ATT TTA TTG ATT TG	Matjila et al., 2004

3.4 Real-time PCR for *Rickettsia*

A *Rickettsia* genus-specific and two species-specific qPCR assays detecting *R. africae* and *R. felis* were used to further screen specifically for *Rickettsia* infections from blood and ectoparasite DNA samples. Oligonucleotide primers and Taqman probes used for the *Rickettsia* genus-specific, *R. africae* and *R. felis* assays are presented in Table 3.4. For the *Rickettsia* genus and *R. felis* assay amplification reaction mix, primers and probes as indicated in Table 3.4 were used with 10 µl of Platinum® quantitative PCR supermix-UDG (Invitrogen) which contains Platinum *Taq*® DNA polymerase, HCl, KCl, 6mM $MgCl_2$, 400µM dGTP, 400µM dATP, 400µM dCTP, 800µM dUTP, uracil DNA glycosylase (UDG), and stabilizers. Four microlitres of DNA was used as a template and 2.2 µl of PCR grade water added to get a final reaction volume of 20 µl. For the *R. africae* assay (Maina et al., 2014), primers and probes as indicated in Table 3.4 were used with 10 µl of Platinum Quantitative PCR Supermix-UDG® (Life Technologies), 4 µl of DNA template and 2.4 µl of water to give a final reaction volume of 20 µl.

PCR amplification and product detection was performed on the Light cycler 2.0® (Roche) when using FAM-labeled probes and the Step one plus® real-time PCR system (Applied biosystems) when using BHQ-labeled probes. Cycling conditions included a UDG incubation at 50°C for 2 minutes, denaturation at 95°C for 2 minutes, and 40 cycles of amplification at 95°C for 15 sec, annealing at 60°C for 30 sec and a final holding step at 40°C. Specific plasmid DNA (*Rickettsia* genus, *R. africae* and *R. felis)* was used as positive controls for each assay and PCR grade water replaced the DNA template in negative control reactions.

Table 3.4: Concentration and sequences of primers and probes for qPCR targets

Assay qPCR Target	**Primer/Probe name**	**Sequence(5'-3')**	**Primer/probe/$MgCl_2$ Concentration**
Rickettsia genus assay	R17K202TaqP	FAM-CCGAATTGAGAACCAAGTAATGC-BHQ	0.4 µM
	R17K128F2	GGGCGGTATGAAYAAACAAG	0.5 µM
	R17K238R	CCTACACCTACTCCVACAAG	
	Mg Cl_2		5 mM
R. felis	RF1396F	ACCCAGAACTCGAACTTTGGTG	0.5 µM
	RF1524R	CACACCCGCAGTATTACCGTT	
	RF1448BP-FAM	FAM-CGCGACTTACAGTTCCTGATACTAAGG TTCTTACAGGTCGCG-BHQ-1	0.4 µM
	$MgCl_2$		5 mM
R .africae	Raf1797F	TTGGAGCTAATAATAAAACTCTTGGAC	0.7 µM
	Raf1915R	GAATTGTACTGCACCGTTATTTCC	
	Raf1879Probe	6-FAM-CGCGATGTTAATAGCAACATCACCGCC ACTATCGCG-BHQ-1	0.3 µM
	$MgCl_2$		4 mM

3.5 Sequencing and sequence data analysis

3.5.1 Sanger sequencing of the variable region of 16S and 18S rRNA genes

Eight samples that tested positive with genus-specific probes for *Anaplasma*, *Ehrlichia*, *Babesia*, or *Rickettsia* spp., when subjected to the (RLB) hybridization assay or qPCR assay

analyses were further analyzed by DNA sequencing to determine the specific species. Samples were prepared for sequencing by first subjecting them to the RLB PCR amplification for the 16S and 18S rRNA genes as described above with a slight modification of using primers without biotin labels. The amplicons were examined on a 1.25% agarose gel stained with ethidium-bromide and the presence of amplicons on the gel was visualized using an ultra-violet transilluminator. PCR products with single strong bands on agarose gel were selected for sequencing which was performed by INQABA biotechnologies (South Africa).

The 16S and 18S rRNA sequences obtained from PCR amplicons were prepared for assembly using the PreGap4 program of the Staden package (version 2.0 for Windows) (Bonfield et al., 1995). Sequences were then assembled, edited and consensus sequences generated using the Gap4 program of the same analysis package. Sequence identification and comparison with similar published 16S and 18S rRNA gene sequences was performed using MegaBlast from the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). MUSCLE alignment tool (Edgar, 2004) was used to create a multiple sequence alignment with consensus sequences from PCR amplicons against selected sequences of the same genes obtained from the GenBank.

Sequence alignments were further examined for phylogenetic relationships by generating phylogenetic trees using the Neighbour-joining (Saitou & Nei, 1987), Maximum parsimony, Minimum evolution and Maximum-likelihood methods using MEGA 6 (Tamura et al., 2013). A bootstrap analysis was performed to investigate the stability of the trees obtained. Bootstrap values were obtained from a consensus tree based on 1000 randomly generated trees. The Genbank accession numbers of the gene sequences used to construct the phylogenetic trees are shown in Appendix 1.

3.5.2 Sequence analysis of 16S rRNA gene using DNA barcoding

To further characterise specific *Rickettsia* species infections from samples infected with *Anaplasma*, *Ehrlichia* and *Rickettsia* spp. - five samples were subjected to next generation sequencing using DNA bar coding. The 16S rRNA gene was amplified from genomic DNA of selected samples (106, 116, 125, T 8, and F 1) using universal primers 340F and 805R modified with Illumina specific adapters (Klindworth et al., 2013). Barcodes with specific oligonucleotide sequences were added to each individual sample so they can be differentiated during the analysis of the data generated. Sequencing was performed on the Illumina's MiSeq platform using a MiSeq v3 kit. Sequences had an approximate coverage/depth of ˜30, 000X for each amplicon pool (20Mb of data/sample). Next generation sequencing using the Illumina platform was performed by INQABA biotechnologies (South Africa).

Sequence data was analyzed using the CLC Genomics Workbench 5.1 software. Sequences were assembled using the *de novo* assembly algorithm of the CLC workbench to create simple sequence contigs which were then updated based on mapped reads. BLAST searches were performed on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.hih.gov/) to detect the identity of sequence contigs. Sequence alignments and phylogenetic analyses were performed following the same methods as described above.

CHAPTER 4

RESULTS

4.1 Detection of haemoparasites using reverse line blot hybridization assay

4.1.1 Detection of haemoparasites in blood

The results of the RLB hybridization analysis for DNA prepared from the 141 blood samples spotted on FTA filter cards showed the presence of *Ehrlichia*, *Anaplasma*, *Theileria* and *Babesia* species detected either as single or mixed infections. *Ehrlichia*/*Anaplasma* species was detected in 70 samples (49%), *Babesia* genus-specific 1 in 31 samples (14%), and *Babesia* genus-specific 2 in two samples (1%). *Theileria* species was detected in three samples (2%) and *Theileria*/*Babesia* species in 21 samples (14%). Specific species include *Ehrlichia canis* detected in 23 samples (16%), *Babesia rossi* in 14 samples (9%) and *Babesia vogeli* in 6 samples (4%) while 51 samples (36%) were negative. RLB hybridization analysis also revealed a variety of co-infections in the blood samples examined: *Theileria*/*Babesia*, *Ehrlichia*/*Anaplasma*, *Babesia* 1 and *B. vogeli* co-infection was detected in four samples (2%), *Theileria*/*Babesia, Ehrlichia*/*Anaplasma*, *Babesia* 1 and *B. rossi* in three samples (2%), *Theileria*/*Babesia*, *Ehrlichia*/*Anaplasma*, *Babesia* 1, *B. rossi* and *E. canis* was detected in two samples (1%). The following co-infections were detected in single samples (0.7%): *Ehrlichia*/*Anaplasma*, *E. canis* and *Babesia* 1, another of *Ehrlichia*/*Anaplasma*, *Theileria*/*Babesia*, *Babesia* 1 and *E. canis*. *B. rossi*, *Ehrlichia*/*Anaplasma and Theileria* were also detected, another of *Ehrlichia*/*Anaplasma* and *Theileria*/*Babesia*, and finally *Ehrlichia*/*Anaplasma*, *Babesia* 1 and *Babesia* 2 species respectively (Table 4.1). A representative RLB result is shown in Figure 4.1. Figure 4.2 shows the summary of the results of RLB hybridization assay on blood samples.

Table 4.1: Co-infections detected from the RLB hybridization assay from filter card DNA obtained from dog blood samples

Types of co-infections	Number of samples
Theileria/Babesia, *Ehrlichia/Anaplasma*, *Babesia* probe 1, *B. vogeli*	4
Theileria/Babesia, *Ehrlichia/Anaplasma*, *Babesia* probe 1, *B. rossi*	3
Theileria/Babesia, *Ehrlichia/Anaplasma*, *Babesia* probe 1, *B. rossi* and *E. canis*	2
Ehrlichia/Anaplasma, *E. canis*, *Babesia* probe 1	1
Ehrlichia/Anaplasma, *Theileria/Babesia*, *E. canis*, *Babesia* probe 1	1
B. rossi, *Ehrlichia/Anaplasma, Theileria* probe	1
Ehrlichia/Anaplasma, *Theileria/Babesia*	1
Ehrlichia/Anaplasma, *Babesia* probe 1, *Babesia* probe 2	1

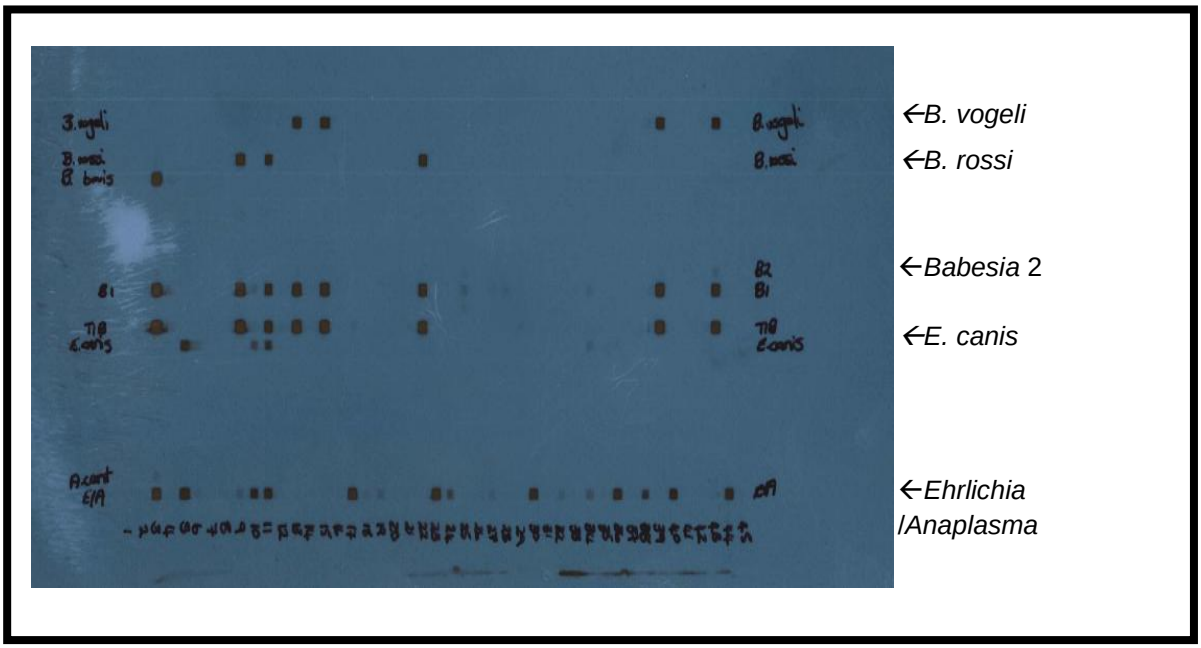

Figure 4.1: An x-ray film from RLB hybridization analysis of 141 blood samples collected from dogs in Mnisi area at the Bushbuckridge district of Mpumalanga, South Africa showing simultaneous detection of *Ehrlichia*, *Anaplasma*, *Theileria* and *Babesia* species.

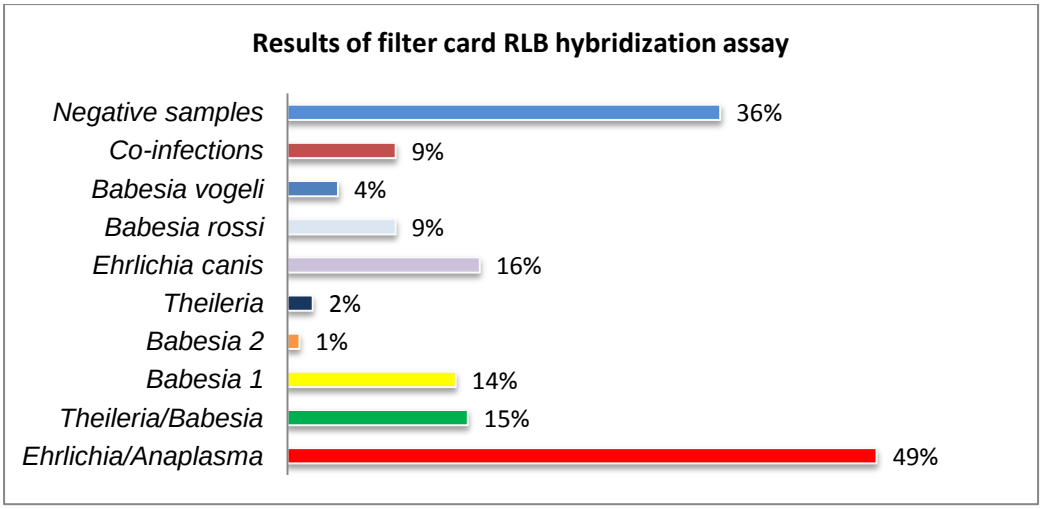

Figure 4.2: Summary of results of blood samples RLB hybridization analysis

4.1.2 Detection of haemoparasites in ticks and fleas

Tick species identified included *Haemaphysalis elliptica* (29%), *Amblyomma hebraeum* (26%), *Rhipicephalus sanguineus* (26%), *Rhipicephalus simus* (17%), and one unspeciated *Ixodes* (0.9%). The fleas included *Ctenocephalides felis strongylus* (53%) and *Echidnophaga gallinacea* (46%). The results of the RLB hybridization analysis of DNA from pooled tick and flea samples also showed the presence of *Ehrlichia*, *Anaplasma*, *Theileria* and *Babesia* species from ectoparasite DNA. *Ehrlichia/Anaplasma* species were detected in *H. elliptica*. *Ehrlichia/Anaplasma* species, *Babesia* 1, *Babesia* 2 species and *E. ruminantium* were detected in *A. hebraeum*. *Theileria* species was detected in the *Ixodes* tick. *Ehrlichia/Anaplasma*, *Babesia* 1, *Babesia* 2 and *Neoehrlichia* species were detected in *R. sanguineus*. *Babesia* 1 species and *B. microti* were detected in *R. simus* while *Babesia* 1 and *Babesia* 2 species were detected in *C. felis strongylus*. Haemoparasite DNA could not be detected in one *R. sanguineus* pool, one *R. simus* pool, two *H. elliptica* pools and the *E. gallinacea* pool (Table 4.2). A representative RLB hybridization analysis result for the ectoparasite samples is shown in Figure 4.3. A summary of the results of RLB hybridization analysis of ectoparasites is shown in Figure 4.4.

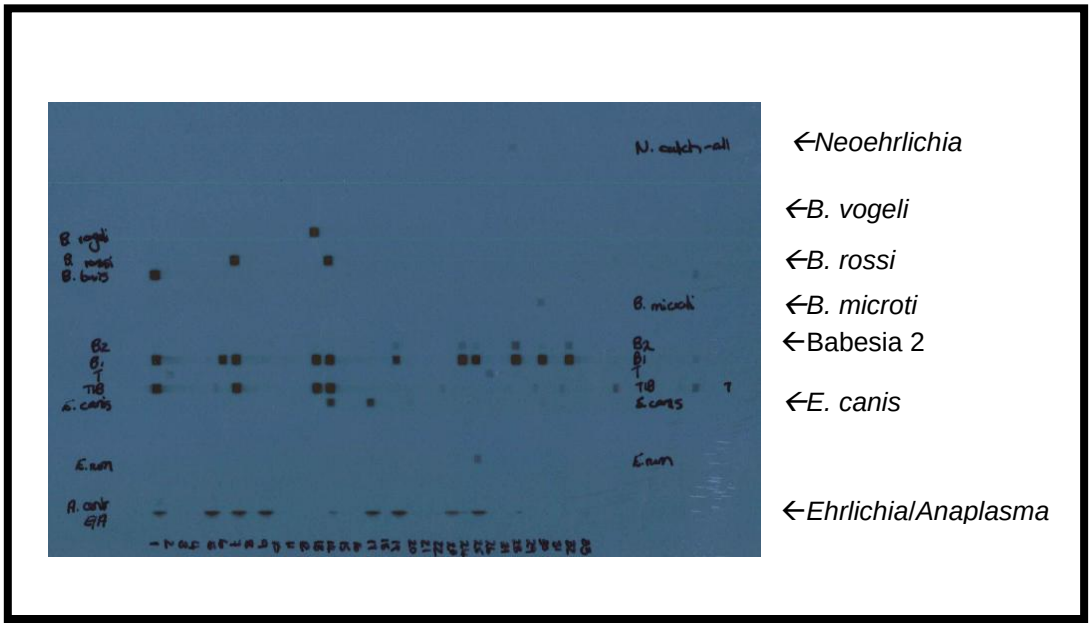

Figure 2.3: An x-ray film from RLB hybridization analysis of a representation of ticks and fleas collected from domestic dogs in Mnisi area at the Bushbuckridge district of Mpumalanga Province, South Africa showing simultaneous detection of *Ehrlichia*, *Anaplasma*, *Theileria* and *Babesia* species.

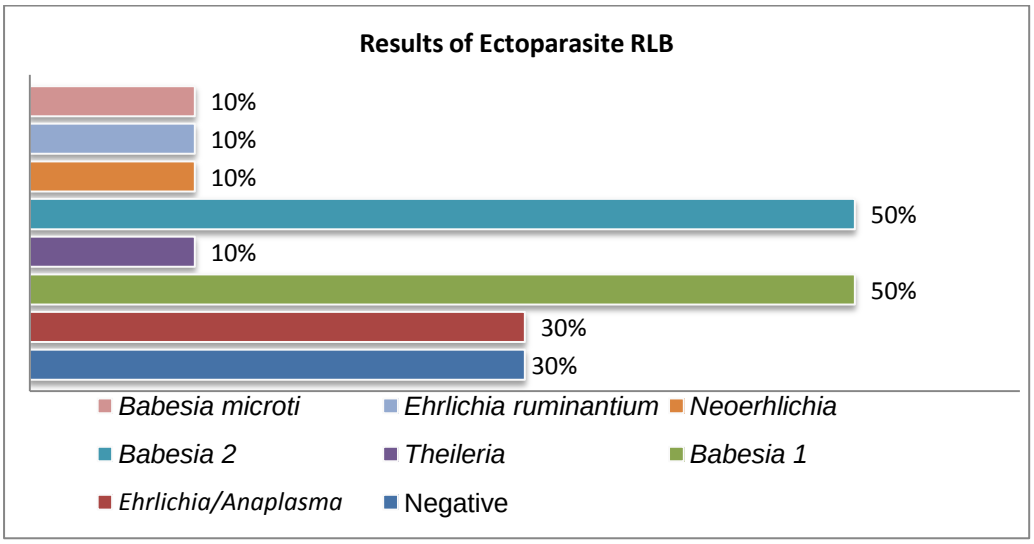

Figure 4.4: Summary of results of RLB hybridization assay of ectoparasites.

Table 4.2: RLB hybridization assay results from ticks and fleas

Pool number	Ectoparasite	Specie name	Haemoparasite detected by RLB analysis
T1	Tick	*Haemaphysalis elliptica*	negative
T2	Tick	*H. elliptica*	negative
T3	Tick	*H. elliptica*	*Ehrlichia/Anaplasma* genus-specific probe
T4	Tick	*Amblyomma hebraeum*	*Babesia* genus-specific probe 1 and 2
T5	Tick	*A. hebraeum*	*Ehrlichia/Anaplasma*, *E. ruminantium*, *Babesia* genus-specific probe 1 and 2
T6	Tick	*Ixodes*	*Theileria* genus-specific probe
T7	Tick	*Rhipicephalus sanguineus*	negative
T8	Tick	*R. sanguineus*	*Ehrlichia/Anaplasma*, *Babesia* probe 1 and 2, *Neoehrlichia* genus-specific probe
T9	Tick	*Rhipicephalus simus*	negative
T10	Tick	*R. simus*	*Babesia* genus-specific probe 1, *Babesia microti*
F1	Flea	*Ctenocephalides felis strongylus*	*Babesia* genus-specific probe 1 and 2
F2	Flea	*Echidnophaga gallinacea*	negative

4.2 Detection of *Rickettsia* by real-time PCR

DNA samples from blood and ectoparasites were subjected to a *Rickettsia* genus-specific qPCR assay for detection of rickettsial infections prior to testing with species-specific real-time PCR assays which identify *R. africae* and *R. felis* DNA. *Rickettsia* DNA was not detected from any of the DNA samples prepared from blood when using the quantitative *Rickettsia* genus-specific assay. By contrast, 7/10 (70%) of ticks and both flea pools tested positive for *Rickettsia* DNA using the same assay (Table 4.3). *Rickettsia* DNA was not detected in three (30%) tick pools. Analysis with species-specific real time PCR assays revealed that 30% of all tick pools tested positive for *R. africae* and the two flea pools tested positive for *R. felis*. Representative amplification curves for the *Rickettsia* genus-specific qPCR assay and species-specific assays are shown in the Figures 4.5 (*Rickettsia* genus), 4.6 A (*R. africae*) and 4.6 B (*R. felis*). Table 4.3 shows a summary of the results of the *Rickettsia* qPCR assays.

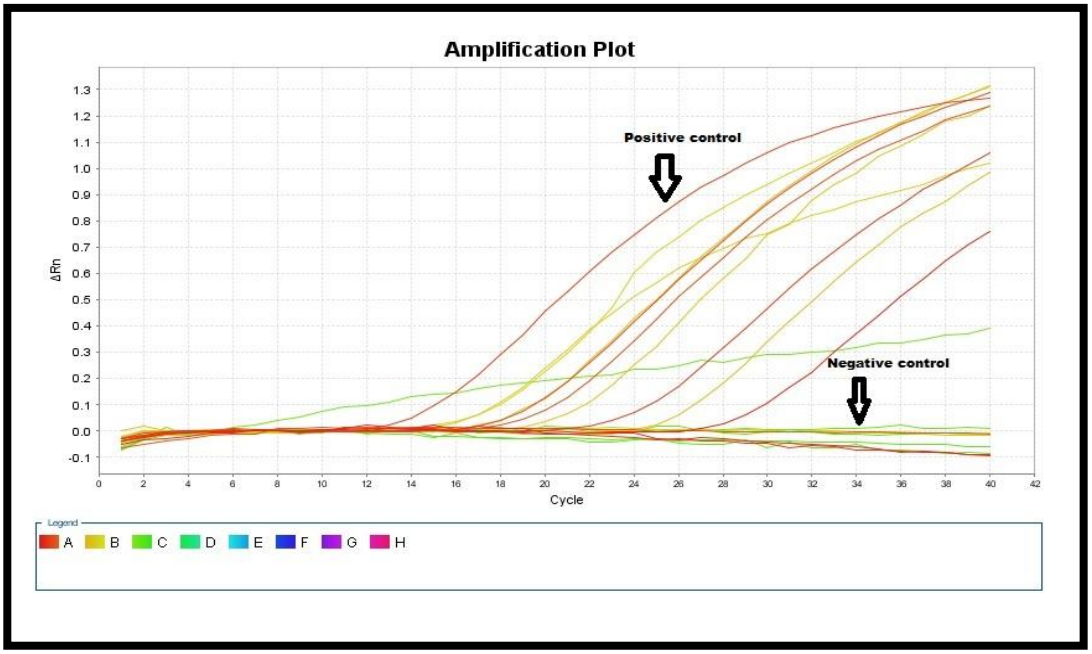

Figure 4.5: The amplification curves showing detection of rickettsial infection in ectoparasites using *Rickettsia* genus-specific qPCR assay performed on the Step one plus® real time PCR system (Applied biosystems).

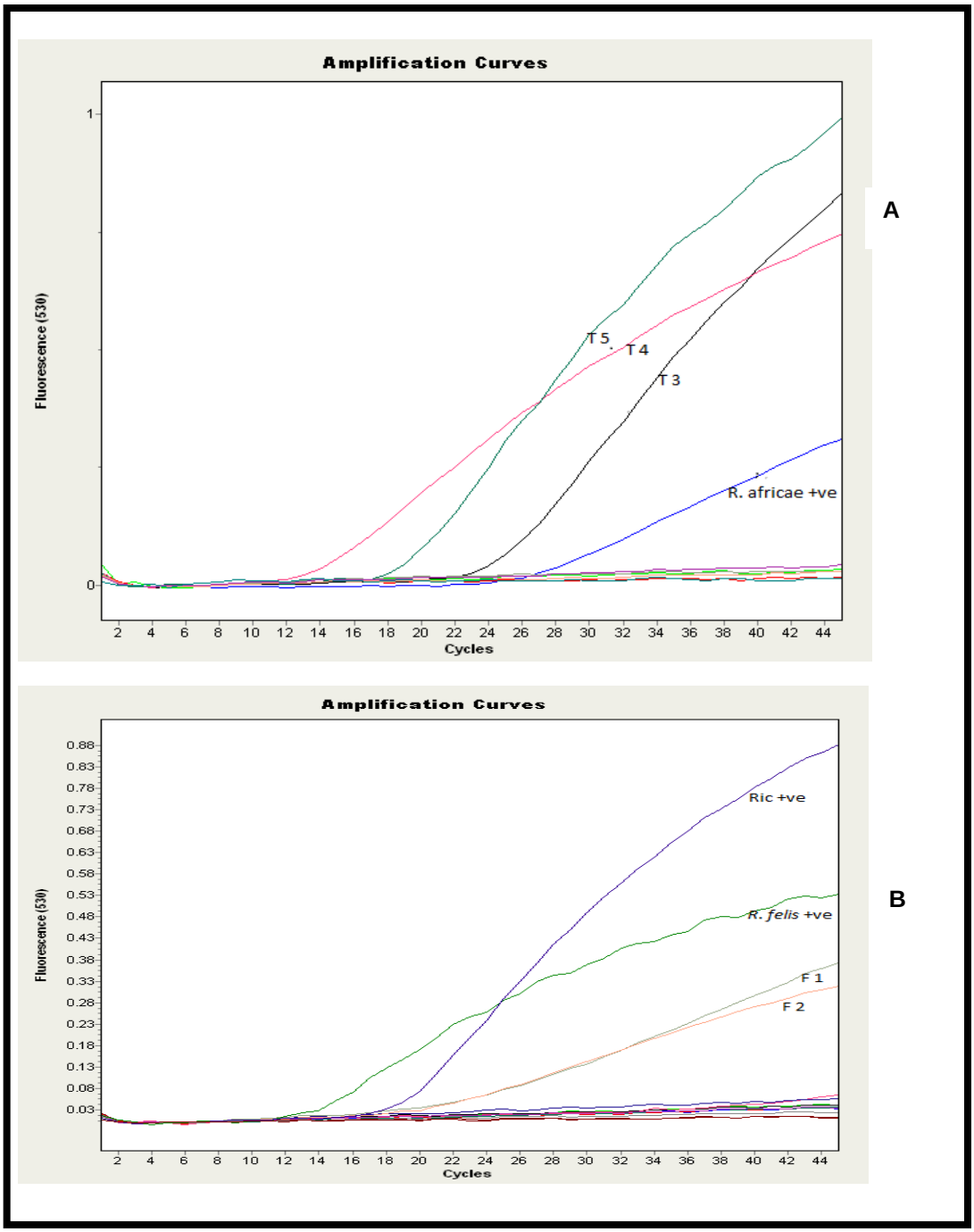

Figure 4.6: (A) the amplification curves of *R. africae* infection in ticks using *Rickettsia africae* genotype-specific assay. (B) The amplification curves showing detection of *R. felis* infection in fleas using *Rickettsia felis* species-specific assay performed on the Light cycler 2.0® (Roche).

Table 4.3: Summary of real-time PCR assays

Sample ID	Species	Results		
		Rickettsia genus assay	*R. africae* assay	*R. felis* assay
1-141	Canine	Negative (100%)	ND	ND
T1	*Haemaphysalis elliptica*	+	-	-
T2	*Haemaphysalis elliptica*	-	+	-
T3	*Haemaphysalis elliptica*	+	+	-
T4	*Amblyomma hebraeum*	+	+	-
T5	*Amblyomma hebraeum*	+	+	-
T6	*Ixodes* spp.	+	-	-
T7	*Rhipicephalus sanguineus*	-	-	-
T8	*Rhipicephalus sanguineus*	+	-	-
T9	*Rhipicephalus simus*	+	-	-
T10	*Rhipicephalus simus*	+	-	-
F1	*Ctenocephalides felis strongylus*	+	-	+
F2	*Echidnophaga gallinacea*	+	-	+

+: qPCR positive; -: qPCR negative; ND: Not done

4.3 Sequence analysis

4.3.1 Sanger sequence analysis

Five samples that were positive for the *Ehrlichia*/*Anaplasma* genus-specific probe on the RLB hybridization assay were sequenced for the 16S rRNA gene using Sanger sequencing. The megablast homology searches on the sequences revealed four out of the five sequences to have 99% sequence homology to an *Anaplasma* sp. South African dog strain (AY57038-AY57040) (Table 4.5). A sequence obtained from DNA of *H. elliptica* pool (T 3) which had tested positive for *Ehrlichia*/*Anaplasma* genus-specific probe on the RLB assay and *Rickettsia africae* on qPCR was confirmed to be 99% similar to *R. africae* (JF949783) 16S rRNA gene

sequences. Three blood samples (sample numbers 8, 22 and 26) that tested positive for *Theileria/Babesia*, *Babesia* 1 and *Babesia rossi* DNA on the RLB hybridization assay were sequenced for the 18S rRNA gene and were confirmed to be *Babesia rossi* by sequence analysis.

Table 4.4: Summary of Sanger sequence analysis

		16S rRNA sequence analysis	
Sample number	**Length of amplicon (bp)**	**Specie identified**	**% Identity**
97	481	*Anaplasma* sp. South African dog strain	99
98	483	*Anaplasma* sp. South African dog strain	99
107	482	*Anaplasma* sp. South African dog strain	99
115	482	*Anaplasma* sp. South African dog strain	99
T3 (*Haemaphysalis elliptica*)	480	*Rickettsia africae*	99
		18S rRNA sequence analysis	
8	414	*Babesia rossi*	99
22	416	*Babesia rossi*	99
26	308	*Babesia rossi*	99

4.3.2 Next generation sequence analysis

Five samples were selected for 16S rRNA gene metagenomic analysis using next generation sequencing. These included three DNA samples prepared from blood (sample numbers 106, 116 and 125) which had tested positive for the *Ehrlichia*/*Anaplasma* genus-specific probe on the RLB hybridization assay. A DNA sample from one of the two flea pools *C. felis strongylus* (F1) which had tested positive for *Rickettsia* genus and *R. felis* qPCR assays was selected. As well as a DNA sample from *R. sanguineus* (T8) this was positive on the Rickettsia genus-specific qPCR assay. The summary of *de novo* assembly report on the sequenced samples is shown in Table 4.5. Analysis of 16S rRNA gene sequences using megablast homology

searches revealed a diverse number of organisms from the different samples (Table 4.6). Identified organisms of interest highlighted in red in Table 4.6 included *Rickettsia helvetica*, *R. massiliae*, *R. peacockii*, *Orientia tsutsugamushi*, *Rickettsia conorii*, *Candidatus* Rickettsia asemboensis, *Candidatus* Rickettsia kotlanii and *Anaplasma phagocytophilum*.

Table 4.5: Summary of *de novo* assembly report

Sample number	Total no. of reads	No. of matched reads	No. of unmatched reads	No. of contigs	Average length of contigs	Total bases of contigs
106	157,536	77,861	79,685	37	335	12,417
116	88,332	48,298	40,034	17	337	5,629
125	78,096	38,570	39,526	16	330	5,294
F1	20,390	9,630	10,760	17	281	4,792
T8	13,396	6,473	6,923	9	283	2,554

Table 4.6: Organisms identified from next generation sequencing of 16S rRNA gene

Sample number / sample type	Fragment length	Organisms found on BLAST search
106 / blood sample	265 letters	*Rickettsia helvetica* *Rickettsia massilae* *Candidatus* Rickettsia asemboensis Uncultured bacterium clone *Bacillus subtilis* *Wolbachia pipentis* *Weisella fabaria* *Leuconostoc pseudomesenteroides* strain *Amborella trichopoda* *Papilio xuthus* *Nicotiana tabacum* *Attacus ricini* *Weisella confusa* *Chryseobacterium* sp. *Cacoecimorpha pronubana* *Klebsiella oxytoca* *Tritymba* sp.
116 / blood sample	257 letters	*Rickettsia helvetica* *Rickettsia peacockii* *Candidatus Rickettsia kotlanii* *Rickettsia massilae*

		Orientia tsutsugamushi strain Kawasaki *Orientia tsutsugamushi* strain Gilliam *Geobacillus stearothermophilus* *Bacillus subtilis* *Nicotiana tabacum* *Atacus ricini* *Cacoecimorpha pronubana* *Pachliopta aristolochiae* *Pantoea* sp. *Papilio xuthus* *Bacillus megaterium* *Wolbachia pipientis*
125 /blood sample	258 letters	*Anaplasma phagocytophilum* strain *Anaplasma platys* isolate 3ax1 *Anaplasma phagocytophilum* strain ApGDr1 *Anaplasma phagocytophilum* strain ApGDr2 Uncultured *Klebsiella* sp. *Geobacillus stearothermophilus* *Bacillus* sp. *Nicotiana confusa* *Cacoecimorpha pronubana* *Acteris braunana* *Papilio xuthus* *Trityma* sp. *Canis lupus familaris* *Galleria mellonela* *Bombyx mori*
F1 (*Ctenocephalides felis strongylus*)	264 letters	*Candidatus* Rickettsia asemboensis *Rickettsia helvetica* *Geobacillus stearothermophilus* *Wolbachia pipientis* *Anteraea pernyi* *Bacillus* sp. *Pieris napi* *Tritymba* sp. *Bombyx mori* *Exophiala lecanii* *Ascomycete* sp. *Candida sojae* strain *Candida tropicalis strain* *Uncultured fungus clone*
T 8 (*Rhipicephalus sanguineus*)	303 letters	*Rickettsia conorii* *Rickettsia helvetica* *Rickettsia peacockii*

		Rickettsia massilae *Bacillus subtilis* *Geobacillus stearothermophilus* *Prevotella* sp. Uncultured fungus clone 1D5 *Exophiala lecanni-corni* strain Uncultured bacterium clone FAIR SPR 10A

4.4 Phylogenetic analysis

Phylogeny of all sequenced samples including Sanger sequences and next generation sequences against reference sequences was determined using neighbor joining method, maximum likelihood, minimum evolution and maximum parsimony analyses. Longer sequences were trimmed to the length of the smallest fragments using Bioedit. Phylogenetic analyses of the 16S rRNA gene of *Rickettsia* species (Figures 4.7 & 4.8) showed that the 16S rRNA sequence of T 3 (*H. elliptica* pool) was in the same clade with *Rickettsia africae* 95.2 (accession number: JF949783). All other spotted fever group (SFG) and ancestral group *Rickettsia* clustered together. The 16S rRNA sequence of flea DNA F 1 (*C. felis strongylus* pool) formed a cluster with *Candidatus* Rickettsia asemboensis F82 (accession number: JN315973), and sequence of blood sample 106 was in the same clade as *Rickettsia massiliae* 8 (accession number: KJ459344). The 16S rRNA sequence of tick DNA T 8 (*R. sanguineus* pool) was placed between the typhus group of *Rickettsia* and the *Orientia* genus while sequence of blood sample 116 was in the same clade as *Orientia tsutsugamushi* kawasaki strain (accession number: D38625).

Phylogenetic analyses of the *Anaplasma* species (Figures 4.9 & 4.10) 16S rRNA sequences from blood samples 97, 98, 107 and 117 (Sanger sequenced) showed that these sequences clustered together with sequences of *Anaplasma* sp. South Africa dog 1076, 1108, and 1245 (accession numbers: AY570539, AY570538 and AY570540 respectively). The sequence of blood sample 125 fits in the same clade with sequences of *Anaplasma phagocytophilum*

strains ApGDr1, ApGDr2 and GDR4 (accession numbers: KC800963, KC800964 and KC455366). Phylogenetic analyses showed that the 18S rRNA sequences of the samples 8, 22 and 28 (Figures 4.11 & 4.12) fit in the same clade with 18S rRNA sequences of *Babesia rossi* (accession number: HM585429), *B. rossi* isolate 44 (accession number: DQ111760) and *B. rossi* NGR clone 22 (accession number: JN982344). Other related *Babesia* and *Theileria* species grouped into different clusters respectively. There was no significant difference in the topology of the *Rickettsia*, *Anaplasma* and *Babesia* phylogenetic trees generated using different analyses algorithms.

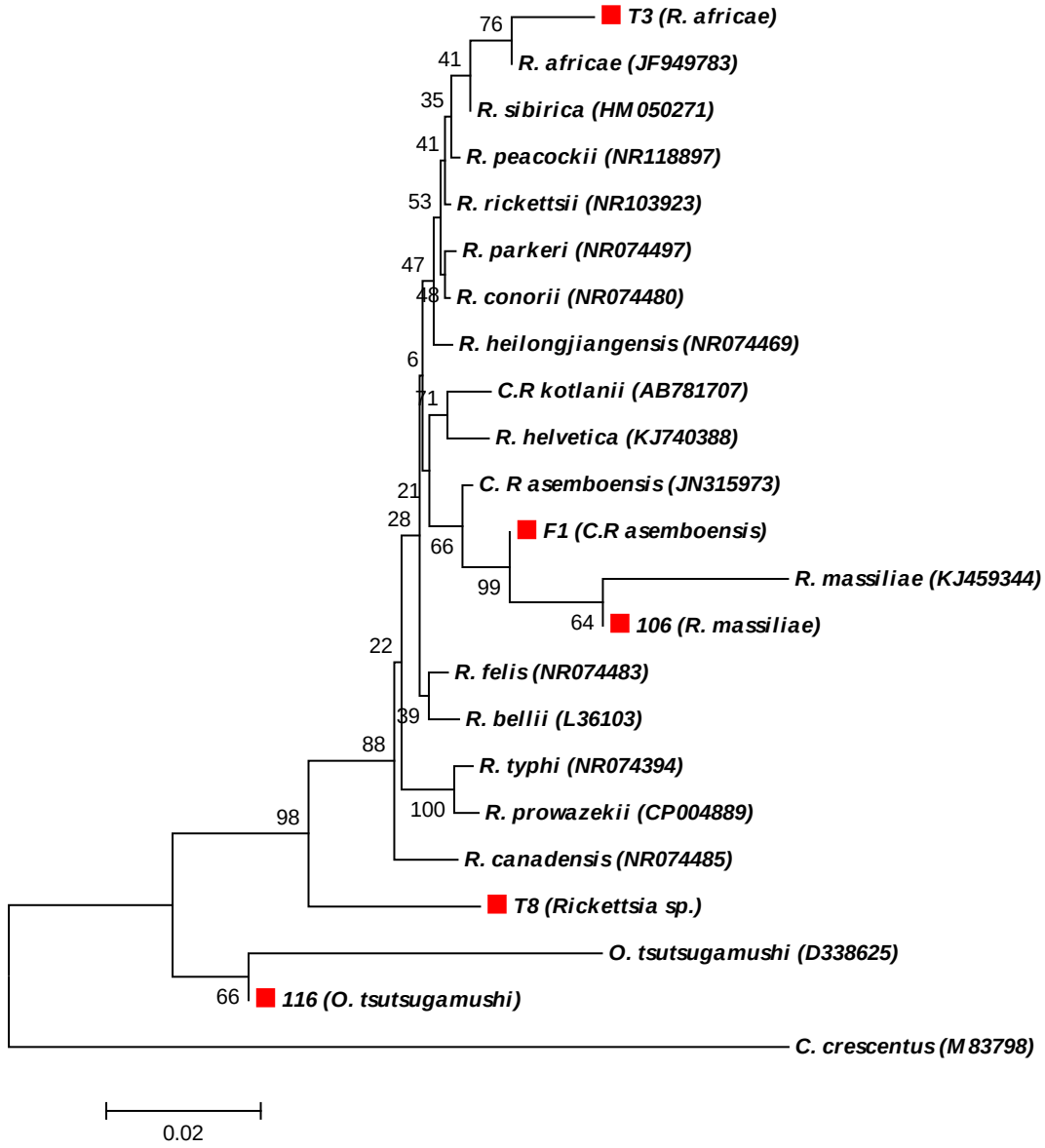

Figure 4.7: Neighbour-joining phylogenetic tree of the 16S rRNA gene sequences of *Rickettsia* species generated from this study together with homologous sequences from Genbank. Bootstrap analyses were performed with a 1000 replications (MEGA software version 6). Red square indicates sequences obtained from the study.

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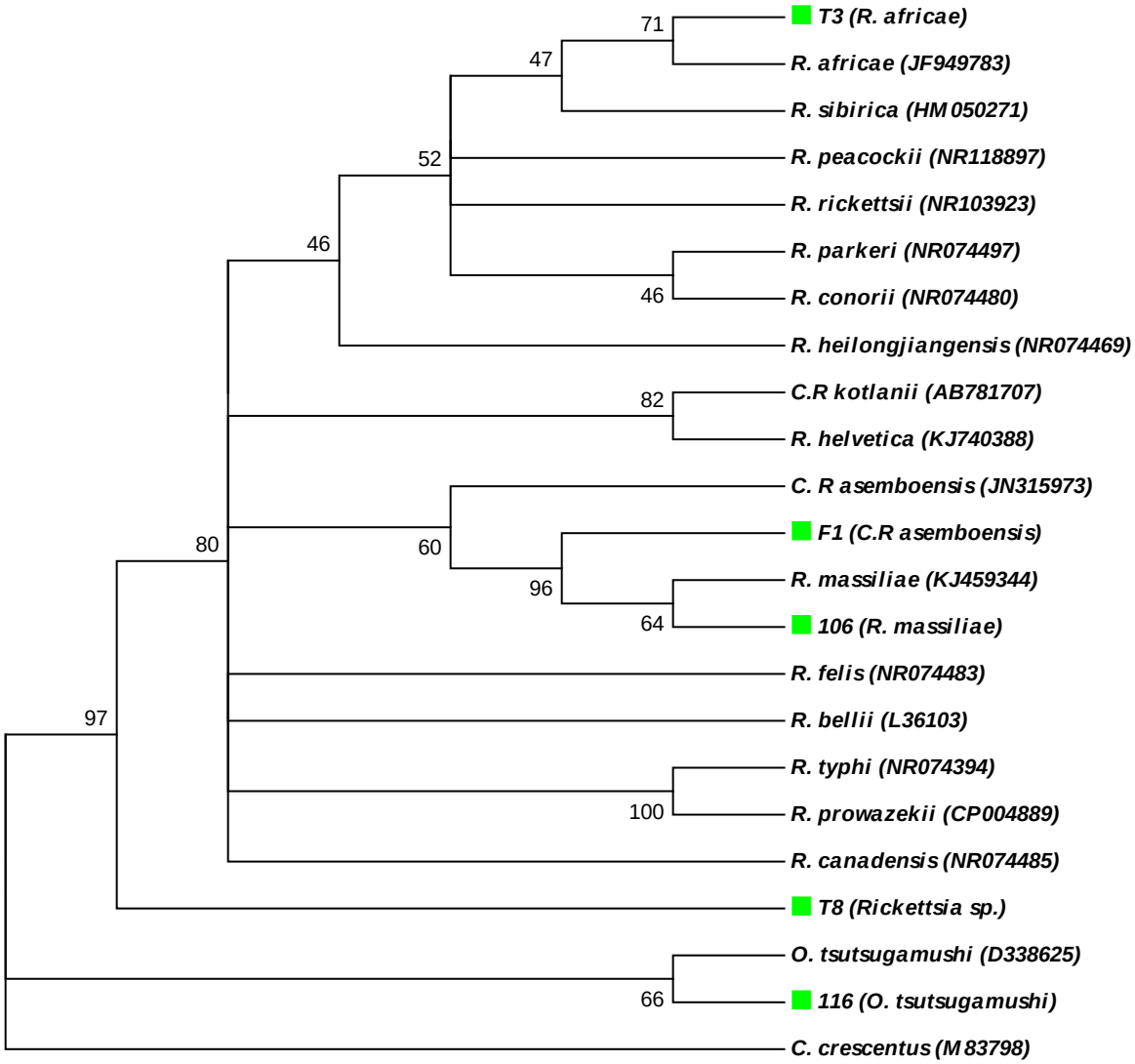

Figure 4.8: Minimum evolution phylogenetic tree of the 16S rRNA gene sequences of *Rickettsia* species generated from this study together with homologous sequences from Genbank. Bootstrap analyses were performed with a 1000 replications (MEGA software version 6). Green square indicates sequences obtained in the study.

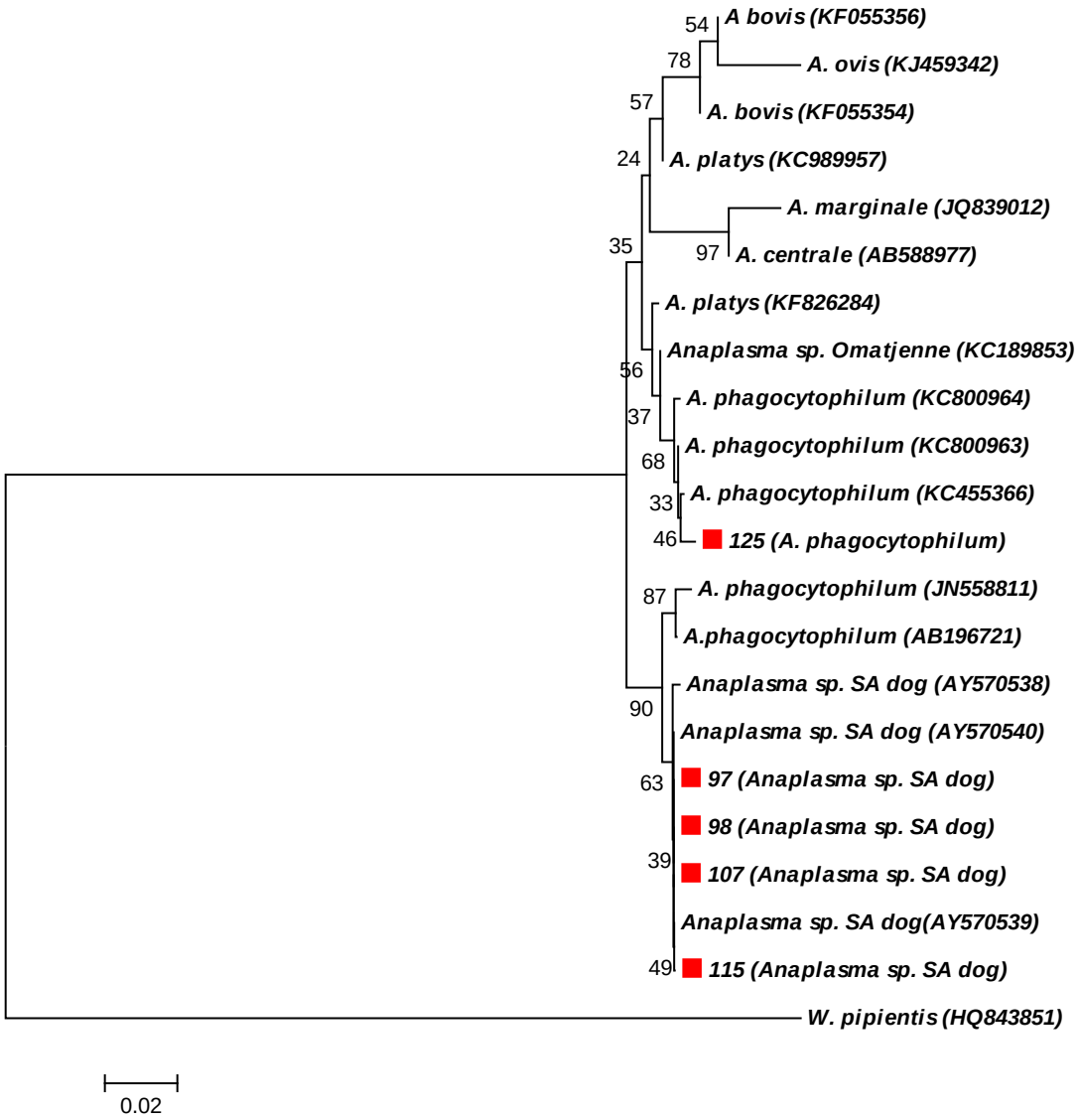

Figure 4.9: Neighbour-joining phylogenetic tree of the 16S rRNA gene sequences of *Anaplasma* species generated from this study together with homologous sequences from Genbank. Bootstrap analyses were performed with a 1000 replications (MEGA software version 6). Red square indicates sequences obtained in the study.

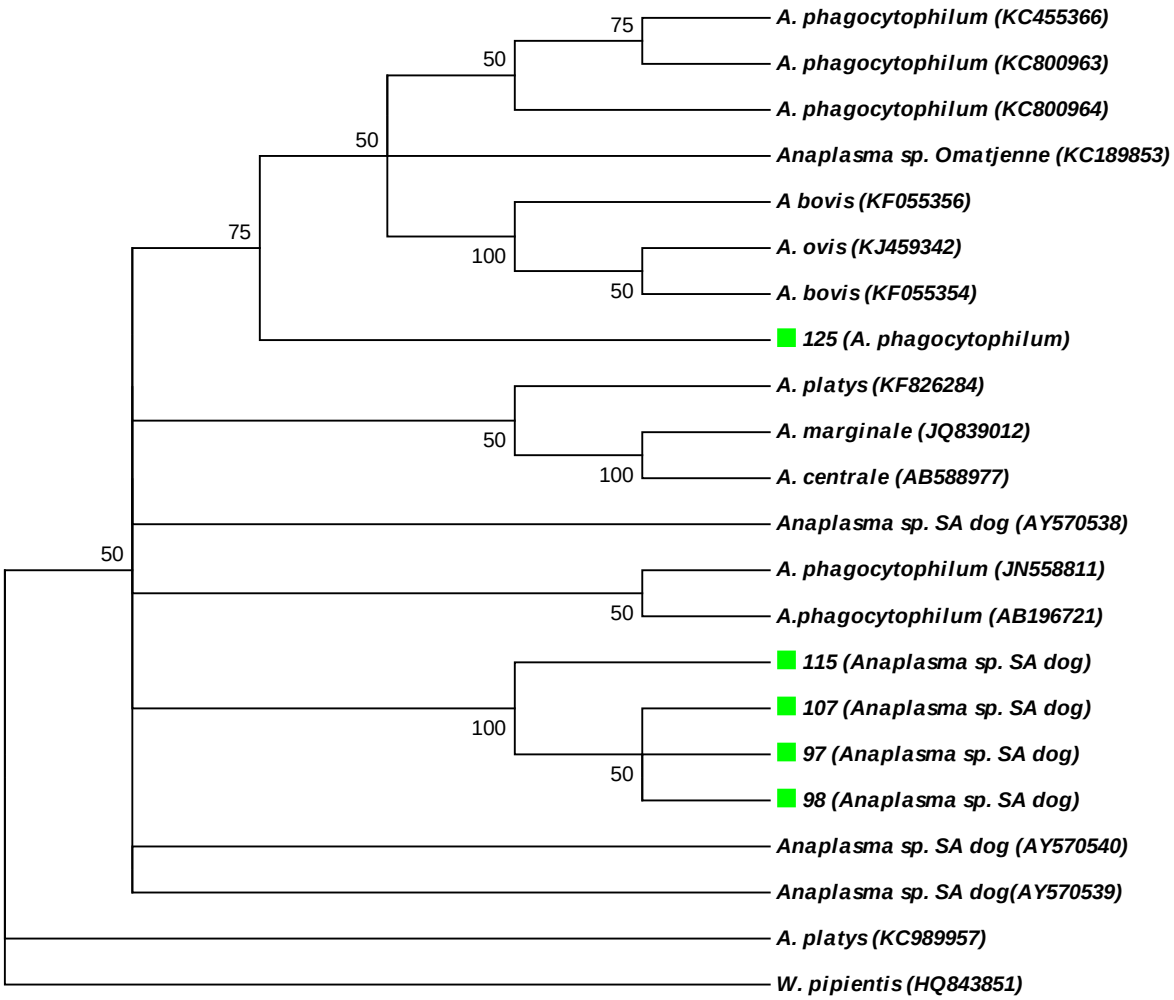

Figure 4.10: Maximum parsimony phylogenetic tree of the 16S rRNA gene sequences of *Anaplasma* species generated from this study together with homologous sequences from Genbank. Bootstrap analyses were performed with a 1000 replications (MEGA software version 6). Green square indicates sequences obtained from the study.

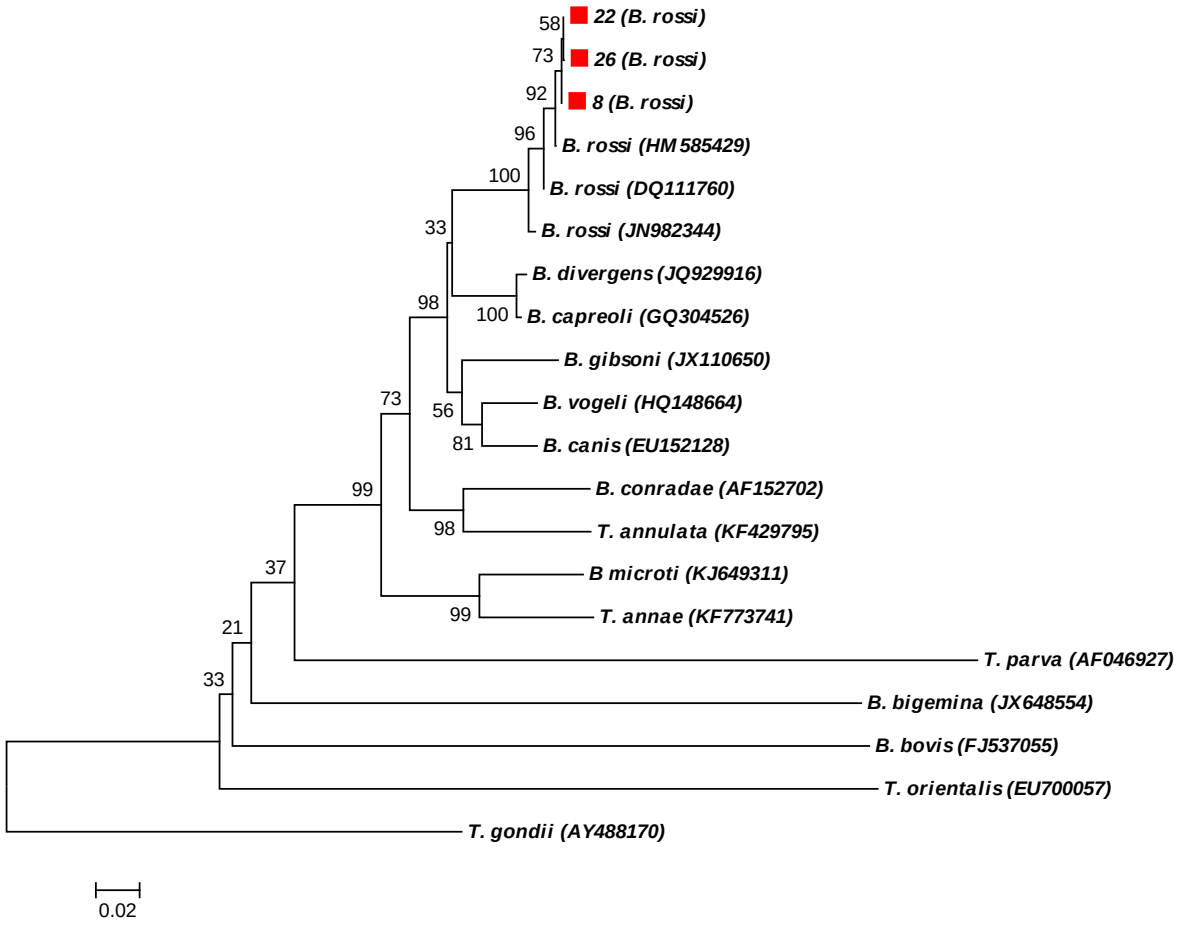

Figure 4.11: Neighbour-joining phylogenetic tree of the 18S rRNA gene sequences of *Babesia* species generated from this study together with homologous sequences from Genbank. Bootstrap analyses were performed with a 1000 replications (MEGA software version 6). Red square indicates sequences obtained in the study.

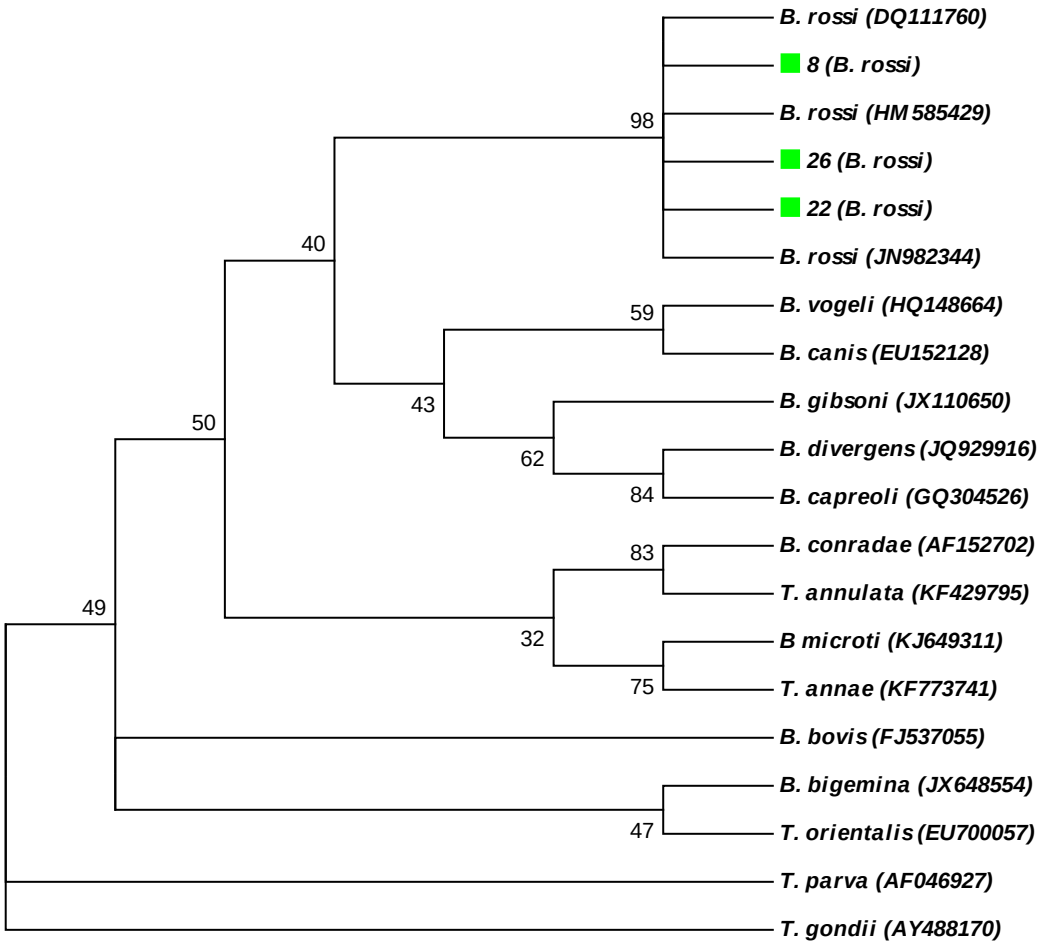

Figure 4.12: Maximum likelihood phylogenetic tree of the 18S rRNA gene sequences of *Babesia* species generated from this study together with homologous sequences from Genbank. Bootstrap analyses were performed with a 1000 replications (MEGA software version 6). Green square indicates sequences obtained from the study.

CHAPTER 5

DISCUSSION

5.1 Detection of *Rickettsia* infections

5.1.1 Detection of rickettsial infection in blood samples

Rickettsial DNA was not detected in any of the blood samples examined in this study. The inability to amplify rickettsial DNA using *the Rickettsia* genus-specific qPCR and species-specific qPCR assays in this study can be attributed to several factors. First, it is possible that rickettsiaI DNA was present in the samples tested but at a copy number that was below the detection limit of the assays used (Hawley et al., 2007). It is also probable that the dogs were infected with rickettsial pathogens but the organism was enclosed in other tissues like the vascular endothelium, dermis or spleen (Hawley et al., 2007; Bayliss et al., 2009). Lastly it can also be related to sampling times, since rickettsial DNA can be cleared from the blood of dogs by a fast and effective immune response, as hypothesized by Bayliss *et al*., (2009). However there are documented reports where rickettsial DNA was successfully detected from canine blood samples (Hii et al., 2011; Sollano-Gallego et al., 2008). In the study by Hii *et al*., (2011) they were able to detect *Rickettsia felis* in 9% of pound dogs tested in Australia by conventional PCR using primers targeting a 297-bp region of the outer membrane protein (ompB) gene of SFG rickettsiae while Sollano-Gallego *et al*., (2008) detected rickettsial DNA in 1% of sick dogs sampled in Italy by qPCR.

5.1.2 Detection of *Rickettsia africae*

Rickettsia africae was successfully detected in 30% of tick pools tested with the *R. africae* genotype-specific qPCR assay; this is consistent with previous molecular studies in Africa where the organism was detected in Chad, Burundi, Ethiopia, Senegal and in southern Africa (Parola et al., 2001; Stephany et al., 2009; Mediannikov et al., 2010). *Rickettsia africae* is the cause of African tick-bite fever (ATBF), an emerging infectious disease of humans transmitted by ticks (Althaus et al., 2010).

Many cases of human infections with *R. africae* have been reported from South Africa which has many wildlife tourist centres usually situated in areas that are endemic with tick vectors transmitting the organism (Chmielewski et al., 2013). In South Africa, *R. africae* is transmitted mainly by *Amblyomma hebraeum* (Kleinerman et al., 2013). Other ticks in the *Amblyomma* genus such as *A. variegatum* also serve as a reservoir and vector of this organism (Jensenius et al., 2003). This is consistent with our results where *R. africae* was detected in both tick pools of *A. hebraeum*. The *Amblyomma* tick has a particularly aggressive hunting strategy which results in ticks frequently biting humans that are in close proximity. This also explains the usual presence of multiple eschars at the inoculation sites in patients (Althaus et al., 2010). It is also important to note that one of the positive tick pools (T 3) on the *R. africae* genotype-specific qPCR assay from the *Haemaphysalis elliptica* pool confirmed the detection of *R. africae* based on sequence analysis. The pathogen has been detected in other tick genera as reported from other African countries such as Egypt, Liberia, Guinea and Turkey where *R. africae* was detected in *Hyalomma* sp., *Rhipicephalus* sp. and *Haemaphysalis paraleachi* (Abdel-Shafy et al., 2012; Gargili et al., 2012; Mediannikov et al., 2012). This is however the first time that the organism has been detected in *H. elliptica* thereby extending the known host range of this organism. Clinical signs and symptoms of ATBF are reported to include fever, headaches, lymph node enlargement, myalgia, and a cutaneous rash that presents either as a vesicular or maculopapular rash or is absent in some cases (Jensenius et al., 2003; Althaus et al., 2010; Chmielewski et al., 2013). Treatment is usually effective on administration of doxycycline (Chmielewski et al., 2013). The detection of *R. africae* from *A. hebraeum* a tick known for readily biting humans, and *H. elliptica*, one of the commonest ticks infesting domestic dogs in South Africa (Horak & Mathee, 2003), underpins the potential risk of human infection with this pathogen in the study area.

5.1.3 Detection of *Rickettsia felis* and closely related organisms

This is the first report of *R. felis* in South Africa. It was detected from the flea species *Ctenocephalides felis strongylus,* and *Echidnophaga gallinacea* using the *R. felis* species-specific assay developed by Henry *et al*., (2007). *Rickettsia felis* is an emerging zoonotic disease pathogen that has been found in several countries globally and causes flea-borne spotted fever (FBSF) in humans (Perez-Osorio et al.,

2008). The cat flea, *C. felis*, is the only defined biological vector and reservoir of *R. felis* known so far (Reif & Macaluso, 2009). Previous reports have detected *R. felis* infection in many parts of the world in humans and arthropods (Parola, 2011). In Africa *R. felis* has been detected in Gabon (Rolain et al., 2005), Tunisia (Znazen et al., 2006), Egypt (Parker et al., 2007), Congo (Sackal et al., 2008), Algeria (Bitam et al., 2009) and in Kenya where substantial work on the organism has been carried out (Richards et al., 2010; Maina et al., 2012; Jiang et al., 2013). In the research carried out by Richards *et al*., (2010), they were able to identify *R. felis* in 3% of human patients presenting with febrile conditions over a 23 month period while Maina *et al*., (2012) showed a 7% prevalence of FBSF in febrile patients in Asembo, Kenya. Clinical signs of FBSF in humans include pyrexia, headache, malaise, rash, myalgia and eschar (Richards et al., 2010).

Sequence analysis of the 16S rRNA gene amplicon from one of the positive flea pools *Ctenocephalides felis strongylus* (F 1) revealed a 100% sequence homology to *Candidatus* Rickettsia asemboensis.This is a *R. felis*-like organism (RFLO) previously identified from fleas in Kenya (Jiang et al., 2013). In that study *Candidatus* R. asemboensis was detected in 60% of flea pools tested. This shows that the *R. felis* species-specific qPCR assay (Henry et al., 2007) that was used in this project was not specific to *R. felis* alone but was able to detect other RFLOs. However there has been a recent development and validation of a new assay, the Rfel B qPCR assay, which is specific for the detection of *R. felis* (Odhiambo et al., 2014). Further work will be needed to unravel the pathogenicity, prevalence and characterization of the new rickettsia *Candidatus* R. asemboensis.

5.1.4 Detection of other rickettsial infections

Sequence analysis of 16S rRNA gene using the high throughput sequencing technology the Ilumina platform detected rickettsiae sequences from DNA from two dog blood samples (106 and 116). Both samples were positive for the *Ehrlichia*/*Anaplasma* genus-specific probe only on the RLB hybridisation assay but were negative on the *Rickettsia* genus-specific qPCR assay based on the 17-kD antigen gene (Jiang et al., 2012). Further demonstrating that the assay used in this study may not be specific to detect all species of *Rickettsia* as previously thought. Sequence analysis from sample

106 sequences revealed a 99% identity to *Rickettsia massiliae* and *Rickettsia helvetica*. *Rickettsia massiliae* was also detected in sequences of sample 116 and T 8 (a *Rhipicephalus sanguineus* pool) with a 96% identity to other published sequences of this organism.

Rickettsia massiliae is in the spotted fever group of *Rickettsia* and has been found in different parts of the world in association with *Rhipicephalus* ticks (Beati et al., 1992; Dupont et al., 1994). *Rickettsia massiliae* was first isolated from a *Rhipicephalus turanicus* tick in the 1990s from a horse in France (Beati et al., 1992). As far as we can question, three cases of human infection with *R. massiliae* have been reported, from Italy, France and Argentina (Vitale et al., 2006; Parola et al., 2008; Garcia-Garcia et al., 2010). Dogs in California, USA have also been shown to be seropositive for *R. massiliae* but rickettsial DNA was not detected by PCR of the blood samples (Beeler et al., 2011). Clinical signs of *R. massiliae* infection in humans include fever, maculopapular rash, hepatomegaly and eschar (Vitale et al., 2006, Parola et al., 2008; Garcia-Garcia et al., 2010). Treatment is usually effective on administration of doxycycline (Garcia-Garcia et al, 2010). Phylogenetic analysis of the sequence from sample 106 placed it in the same clade as *R. massiliae* sequence published in the Genbank confirming this to be the first report of the detection of this organism in canine blood from South Africa.

Rickettsia helvetica was detected in blood samples 106 and 116 and tick pool T 8, and confirmed by sequence analysis of 16S rRNA gene to have 99% identity to *R. helvetica* strain IR-698 9-AF (KJ740388) and 96% sequence homology to *R. helvetica* strain IR-37.3-BAT (KJ577822). A member of the spotted fever group of rickettsiae, it has been isolated from *I. ricinus* ticks in many European countries (Parola et al., 2005). In Africa *R. helvetica* has been detected in *I. ricinus* ticks from Morocco, Tunisia and Algeria (Sarih et al., 2008; Sfar et al., 2008; Kernif et al., 2012). *Rickettsia helvetica* has been reported as a cause of human infection in France, Thailand, Denmark and Sweden (Fournier et al., 2000; Parola et al., 2001; Nilsson, 2009; Nilsson et al., 2010). Infection with *R. helvetica* in humans presents as a mild disease with fever, headache and myalgia (Nilsson et al., 2010); or as a more serious condition where septicaemia, fever, headaches, neck stiffness, arthalgia,

myalgia, photophobia and macular rash were observed (Fournier et al., 2000; Nilsson, 2009). Treatment has been effective on administration of doxycycline (Nilsson et al., 2010). The detection of *R. helvetica* in canine blood and in a *R. sanguineus* pool underpins the potential risk of human infection to this zoonotic organism in the study area.

Candidatus Rickettsia kotlanii was detected in the sequence of blood sample 116 with a 96% homology to published 16S rRNA gene sequences of this organism. A relatively new organism, it was first detected in *I. ricinus*, *Dermacentor reticulatus* and *Haemaphysalis concinna* ticks collected from red foxes (*Vulpes vulpes*) in Hungary, Europe (Sreter-Lancz et al., 2006). The pathogenicity of this organism and its geographic distribution are not yet known, and it is possible that it may be the cause of yet-to-be-identified infections in humans and animals (Sreter-Lancz et al., 2006). In the phylogenetic analysis carried out on the sequences in this study, this organism clustered in the same clade with *R. helvetica* suggesting that they are genetically closely related and may require further studies to distinguish them.

Rickettsia peacockii another spotted fever group rickettsiae was detected in the sequences of blood sample 116 and *R. sanguineus* tick pool (T8) with a 96% identity to published 16S rRNA gene sequences of this organism. The organism was first detected in *Dermacentor andersoni* ticks in Montana, USA (Niebylski et al., 1997). *Rickettsia peacockii* is generally considered to be a nonpathogenic SFGR and is mainly transmitted transovarially from one tick generation to the next (Simser et al., 2001). An endosymbiont of *D. andersoni* ticks, its presence in the tick is related to the reduced prevalence of *Rickettsii rickettsii* the cause of RMSF in dogs and humans in the Americas (Felsheim et al., 2009).

Orientia tsutsugamushi (formerly *Rickettsia tsutsugamushi*) was detected in blood sample 116 with a 16S rRNA gene sequence of 96% homology to published sequences of *O. tsutsugamushi* strain Kawasaki (D38625) and *O. tsutsugamushi* strain Gilliam (D38622). Phylogenetic analyses revealed

sample 116 sequence to cluster in the same clade with *O. tsutsugamushi* strain Kawasaki. *Orientia tsutsugamushi* causes scrub typhus a febrile disease transmitted by the larval stage of the mites *Leptotrombidium akamushi* and *L. deliense* commonly called “chiggers” (Silpapojakul, 1997; Parola & Raoult, 2006). The disease has occurred in the Asia-Pacific region of the world which includes the north of Australia, India, Korea, Japan, Papua New Guinea and Afghanistan (Kelly et al., 2009). Humans are accidental hosts in scrub typhus while mice, rats and bigger mammals are the final hosts. Infected mites serve as the vector and reservoir of this organism (Jiang et al., 2004). The mortality rate for untreated cases of scrub typhus can be low or high depending on the physical condition of the patient and the virulence of the infecting strain (Jiang et al., 2004; Parola & Raoult, 2006). Clinical signs of the infection in humans include fever, headaches, maculopapular rash, eschar, lymphadenopathy and neurological signs, cough and interstitial pneumonia in some cases (Silpapojakul, 1997; Richards et al., 1997; Parola & Raoult, 2006). There is a recent case report of *O. tsutsugamushi* infection in a lethargic dog in an area of Japan showing that dogs can be naturally infected and play a role as a host of the organism (Namikawa et al., 2014). The detection of *O. tsutsugamushi* in sample 116 sequence confirms the first report of the organism in canine blood in South Africa. To our knowledge this is the first time *O. tsutsugamushi* has been identified in an area outside the scrub typhus area in Asia-Pacific. As a known zoonotic agent, the detection of *O. tsutsugamushi* in canine blood also highlights the potential risk of human infection that may occur from this pathogen in the study area.

The 16S rRNA gene sequence obtained from the *Rhipicephalus sanguineus* pool (T 8) had a 96% sequence identity to *Rickettsia conorii* Malish 7 strain (NR074480). *Rickettsia conorii* subsp. *conorii* is the aetiological agent of Mediterranean spotted fever (MSF), one of the earliest recognized vector-borne diseases (Parola et al., 2009). It is mainly transmitted through the bite of an infected *R. sanguineus* tick (Raoult & Roux, 1997) or the accidental inoculation of the organism from homogenized ticks via mucous membranes (Nicholson et al., 2010). MSF is endemic in the Mediterranean regions of the world and case reports have occurred in central Europe, central Africa and southern Africa (Parola et al., 2005). The disease in humans is similar to other spotted fever rickettsial infections with signs like fever, maculopapular rash, flu-like symptoms and inoculation

eschar having been reported (Renvoisé et al., 2009). Clinical improvement is usually seen within 48 hours of antibiotic administration (Renvoisé et al., 2009). Dogs play an important role as biological hosts of the tick and bridge the gap between the infected tick populations and humans (Nicholson et al., 2010). The infection in dogs is usually sub-clinical but reports have shown clinical signs of *R. conorii* infection to include anorexia, lethargy, lameness and vomiting (Solano-Gallego et al., 2006). Dogs are also capable of acquiring *R. conorii* from infected *R. sanguineus* ticks and of transmitting the infection to uninfected ticks (Levin et al., 2012). Phylogenetic analysis of 16S rRNA gene sequence obtained from the *R. sanguineus* tick pool against other homologous rickettsial sequences published from the Genbank showed a close relationship to the typhus group of rickettsiae and the *Orientia* genus suggesting that it may potentially be a new species of rickettsiae. Further characterization will however be needed to determine if this is a genetic variant of *R. conorii* or a new species of *Rickettsia* and wether this organism is pathogenic to dogs and/or humans.

5.2 Detection of *Anaplasma* infections

The results of the reverse line blot hybridization assay showed that 49% of the blood samples and 30% of tick pools were positive for the genus-specific probes of *Ehrlichia*/*Anaplasma* species. Sequence analysis of the 16S ribosomal RNA gene of four blood samples positive for the *Ehrlichia*/*Anaplasma* genus-specific probes revealed a 99% sequence homology to an *Anaplasma* sp. (South African dog strain) closely related to *Anaplasma phagocytophilum* (Inokuma et al., 2005). In another blood sample positive for *Ehrlichia*/*Anaplasma* genus-specific probes, sequencing analysis of the 16S rRNA gene revealed a 99% sequence identity to *A. phagocytophilum* strains ApGDr1 (KC800963), ApGDr2 (KC800964) and GDR4 (KC455366). *Anaplasma phagocytophilum* was first detected in dogs in the United states in the 1980s (Madewell & Gribble, 1982) with several genetic variants subsequently described based on molecular typing. The genetic variant (Ap-*ha*) appears to be the strain most attributed to causing disease in humans and dogs in North America (Bakken & Dumler 2008; Morissette et al., 2009). *Ixodes* spp. ticks are the vectors implicated for transmitting *A. phagocytophilum* (Woldehiwet, 2010). However in this study only one out of 103 ticks collected from the domestic dogs was identified as an (unspeciated) *Ixodes* sp. This suggests that other genera of ticks may be responsible for the transmission of *Anaplasma* spp. to dogs in Bushbuckridge. In this

study *Theileria* sp. was detected in the *Ixodes* pool on RLB hybridization assay and it was positive on the *Rickettsia* genus-specific qPCR assay.

Anaplasma phagocytophilum is a known zoonotic pathogen causing human anaplasmosis (formerly known as human granulocytic ehrlichiosis or HGE) with disease in humans characterized by fever, myalgia, headache, an increase in liver function enzymes, reduced platelets in circulation and disorientation (Dumler et al., 2007; Bakken & Dumler, 2008). Similar to what is seen in dogs, the disease in humans is usually transmitted by tick vectors (Little, 2010) and treatment is effective upon the administration of doxycycline. The disease can be prevented by tick control on pets in the household and the environment and public enlightenment on ticks and tick bite prevention (Nicholson et al., 2010). Phylogenetic analyses showed the *Anaplasma* sp. sequences in the study clustered close to previous South African *Anaplasma* sequences reported by Inokuma *et al*., (2005), suggesting that they are genetically related strains of *Anaplasma* sp. Since almost half of the dog blood samples on the reverse line blot hybridization assay were positive for the *Ehrlichia*/*Anaplasma* genus-specific probes and since the sequenced samples all revealed either an *Anaplasma* sp. South Africa strain closely related to *A. phagocytophilum or A. phagocytophilum* itself, it seems there is a potential risk of human infection with this rickettsial pathogen.

5.3 Detection of *Ehrlichia* infections

Ehrlichia canis was detected in 16% of blood samples on the RLB hybridization assay. *Ehrlichia canis* is the first ehrlichial agent that is associated with severe, sometimes fatal illness described from dogs worldwide (Harrus & Waner, 2011). *Ehrlichia canis* is the cause of canine monocytic ehrlichiosis (CME) usually presented in the acute phase with thrombocytopenia, leukopenia, fever, depression and anorexia. In South Africa, previous studies have shown *E. canis* to be among the most common pathogen species detected from domestic dogs (Matjila et al., 2008c). In some dogs a fatal form of chronic ehrlichiosis can develop after some months to years after initial infection and signs of weight loss, bleeding disorders and neurologic signs like ataxia, seizures and nystagmus have been described (Neer & Harrus, 2006). Following adequate treatment, the majority of dogs recover from the

acute phase of the disease (Neer & Harrus, 2006). Doxycycline, imidocarb, and rifampin have been shown to be effective in the treatment of ehrlichiosis caused by *E. canis* in dogs (Litte, 2010). Dogs are reservoir hosts for *E. canis* and also serve as the maintainance host for the vector, the brown dog tick *R. sanguineus* (Little, 2010). Populations of *R. sanguineus* can establish and thrive inside homes and can maintain *E. canis* providing an almost persistent source of infection to dogs in infested surroundings (Dantas-Torres, 2008).

Ehrlichia canis has also been known to cause human infection in rare circumstances. Human monocytic ehrlichiosis can be quite severe with a fatality rate of 3% in Venezuela (Perez et al., 2006; Dumler et al., 2007). Since Mnisi is a rural community with a sub-tropical climate that favours populations of tick vectors like *R. sanguineus* it is possible that *E. canis* may be the cause of febrile conditions of unknown causes in the human population. Dogs are preferred hosts for *R. sanguineus* but it is also capable of feeding on humans (Parola et al., 2008). In a study conducted by Diniz *et al*., (2007) in southeastern Brazil, 198 sick dogs were screened for vector-borne pathogens and it was found that an *E. canis* sequence of the Brazilian strain was identical to the strain responsible for causing human ehrlichiosis in Venezuela (Perez et al., 2006); therefore, the possibility exists that human infection with *E. canis* in communities such as Mnisi can occur.

Another *Ehrlichia* species, *Ehrlichia ruminantium* was detected in an *Amblyomma hebraeum* pool (T 5) from the RLB hybridization assay. *Ehrlichia ruminatium* is transmitted by ticks of the genus *Amblyomma* and is the cause of heartwater in ruminants a disease that poses a serious obstacle to livestock production in sub-saharan Africa (Bekker et al., 2002). Adult *A. hebreum* are usually found on large mammals like cattle, bufallo and other large wild life ruminants, but they can also be found on sheep and goats (Walker et al., 2003). The larvae and nymphs have a wider host range feeding on birds, smaller mammals as well as the large mammals (Walker et al., 2003). Since 26% of the ticks collected from the domestic dogs used in our study were *A. hebraeum*, this shows the host diversity of this important tick species.

Neoehrlichia sp. was detected in a *R. sanguineus* tick pool (T 8) on RLB hybridization assay. *Candidatus* Neoerhlichia mikurensis is one species of *Neoehrlichia* that is an emerging zoonotic pathogen causing human infection (Palomar et al., 2014). The organism has been detected in ticks and rodents in Europe, Asia and Africa over the last decade (Li et al., 2012; Kamani et al., 2013). In Europe the organism has been detected mostly in *I. ricinus* ticks (Palomar et al., 2014) while Li *et al*., (2012) detected the pathogen in 4% of rodents, 2% of ticks and 7 humans in north eastern China. In Africa *C.* Neoehrlichia mikurensis was detected in 5% of ticks sampled in Nigeria (Kamani et al., 2013). Further investigation needs to be carried out to determine the species of *Neoehrlichia* detected in this study.

5.4 Detection of *Babesia* and *Theileria* infections

Babesia rossi was detected in 9% of blood samples on the RLB hybridization assay. It is the most virulent *Babesia* species of dogs in South Africa (Schoeman, 2009). *Babesia rossi* has been detected in previous molecular studies (Matjila et al., 2008b) and is regarded as one of the most important diseases affecting dogs in the country (Penzhorn, 2011). Clinical signs include fever, depression, pale mucous membranes, anorexia, tachycardia, tachypnea and splenomegaly (Schoeman, 2009) with a case-fatality rate of about 12% (Schoeman, 2009). In sub-Saharan Africa, *B. rossi* is mainly transmitted by *H. elliptica* (Penzhorn, 2011) and in this study 30% of the ticks collected from domestic dogs in Mnisi were identified as *H. elliptica*. The results of sequence analysis of the 18S rRNA gene of three samples (8, 22 and 26) that were positive for *B. rossi* on the RLB hybridization assay were confirmed as *B. rossi* with sequence and phylogenetic analyses. The detection of *B. rossi* in canine blood samples in our study is consistent with previous studies in South Africa that have reported an average of 10% of dogs presented in veterinary clinics nationwide being diagnosed with canine babesiosis (Collett, 2000). It is noteworthy that the dogs sampled in this study were not selected because they were sick whereas those presented to veterinary clinics usually are; therefore the fact that these percentages are similar shows that the dogs in Mnisi community which are of the Africanis breed may have developed some sort of resistance or tolerance to *B. rossi* (Penzhorn, 2011).

Another *Babesia* sp. *B. vogeli* was detected in 4% of the blood samples on the RLB hybridization assay. This parasite is found in South Africa, USA, France, Australia, Japan and Brazil and is transmitted by the tick vector *R. sanguineus* (Dantas-Torres, 2008; Yisaschar-Mekuzas et al., 2013). It is less virulent than *B. rossi* and is reported to cause a mild syndrome in adult dogs with low parasitaemia (Schoeman, 2009) but a serious illness in some young puppies (Matjila et al., 2004). Again results from this study are consistent with previous molecular studies in South Africa which have detected *B. vogeli* in 4% of dogs sampled (Matjila et al., 2004).

One of the tick pools (T 10), a *Rhipicephalus simus* pool was positive for *Babesia microti* on the RLB hybridization assay. *Babesia microti* is a parasite associated with microtine rodents and has been reported in humans in the United States, Germany, Japan and Taiwan (Gray et al., 2010) and in dogs in Spain and Portugal (Camacho et al., 2001; Simões et al., 2011). Clinical signs of *B. microti* infection in dogs include fever, lethargy, anorexia and pale mucous membranes (Simões et al., 2011). In humans it usually causes mild illness (Gray et al., 2010) except in the elderly and immunocompromised where signs of fever, chills, myalgia, haemolytic anaemia and haemoglobinuria have been reported (White et al., 1998). The known vectors of *B. microti* are ticks of the *Ixodes* genus (Gary et al., 2010) so it is notable that it was detected from a *R. simus* pool in this study. To our knowledge, this is the first report of *B. microti* detection in ticks collected from domestic dogs in South Africa.

Blood samples (22%) and 30% of tick pools and 50% of the flea pools were positive for the genus-specific probes of *Babesia* 1 on the RLB hybridization assay while 1% of blood samples, 30% of tick pools and 50% of the flea pools were positive for the genus-specific probes of *Babesia* 2. The *Babesia* 1 genus-specific probe detects species like *Babesia* sp. *venatorum*, *B. capreoli*, *B. crassa* and *B. odocoilei* while the *Babesia* 2 genus-specific probe detects species like *Babesia cf. microti* isolates, *B. leo*, *B. rodhaini* and *Babesia* sp. “spanish dog” isolates. Unfortunately specific species could not be confirmed for samples that tested positive for the genus-specific probes of *Babesia* 1 and 2 as attempts to reamplify the variable region of the 18S rRNA gene was unsuccessful. Since some of

the samples had faint signals detected on the RLB assay, it could be the level of parasitaemia was too low to be detected by conventional PCR for sequencing. Twenty-one (14%) blood samples were positive for the *Theileria*/*Babesia* genus-specific probes. Three (2%) of blood samples and one tick pool (*Ixodes* sp.) were positive for the *Theileria* genus-specific probes. Previous studies in South Africa have detected *Theileria* species closely related to *Theileria* sp. (sable) in 15% of dogs sampled in the Pietermaritzburg area and at the Onderstepoort Veterinary Academic hospital (OVAH) (Matjila et al., 2008a). Clinical signs of dogs sampled in the study revealed an immune-mediated condition with thrombocytopenia (Matjila et al., 2008a). Although the specific species of the *Theileria* detected in this study was not confirmed; of recent in South Africa, a *Theileria* sp. was detected by molecular techniques in four dogs and *Theileria* equi in two dogs at the Onderstepoort Veterinary Academic Hospital (Rosa et al., 2014). Common clinical signs seen in the dogs on presentation included pale mucous membranes, bleeding tendencies and lethargy (Rosa et al., 2014). Tick vectors implicated in the transmission of *Theileria* in dogs include *Ixodes hexagonus*, *I. ricinus* and *R. sanguineus* (Camacho et al., 2003; Lledó et al., 2010; Iori et al., 2010). The detection of *Theileria* sp. in canine blood and in an *Ixodes* pool in this study supports the findings of Matjila *et al*., (2008a), Camacho *et al*., (2003), and Rosa *et al*., (2014).

5.5 Detection of co-infections

Co-infections were detected in 14 (9%) blood samples and 20% tick pools (*A. hebraeum* and *R. sanguineus*). The occurrence of the co- infections in the blood samples could be related to the distribution of different arthropod vectors such as *R. sanguineus* and *H. elliptica* being present on the same host (Horak, 1995) at a particular time or the presence of a tick vector implicated in the transmission of different pathogens like *R. sanguineus* which is a known vector of *E*. *canis* and *B. vogeli*, and *A. hebraeum* which transmits *E. ruminantium* and *R. africae* (Bekker et al., 2002; Jensenius et al., 2003).

5.6 Conclusions

The detection of rickettsial agents *Rickettsia massiliae and Orientia tsutsugamushi* in canine blood suggests that dogs may play an important role in the life cycle of rickettsiae. The detection of

Ehrlichia, *Babesia* and *Theileria* species is consistent with previous studies in South Africa that have detected these organisms in canine blood. There should be increased surveillance for rickettsiae by serological and molecular means of both human and canine populations to determine the distribution and epidemiology of these organisms. Further studies is needed to characterize and isolate *Anaplasma* sp. South Africa dog strain and *Candidatus* Rickettsia asemboensis via culture and determine if these organisms are pathogenic to dogs and humans.

Public education of the community in Bushbuckridge should be embarked upon explaining the potential infection risk of these vector-borne pathogens and protection measures that can be taken with a focus on ectoparasite control of domestic dogs and the environment. These vector-borne zoonotic infections should be taken into consideration in the diagnosis of febrile conditions of unknown aetiology in the human population. Finally, these data provide preliminary information about the occurrence of zoonotic rickettsiae in domestic dogs and their ectoparasites in a South African rural community and highlight the potential risk of human infection with these pathogens.

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APPENDIX

Nucleotide sequences used in the phylogenetic analyses

Genbank accession number	Taxonomic classification	Strain designation	Source
JN558811	*Anaplasma phagocytophilum*	YDH6 clone 6	Goat
AY570540	*Anaplasma* sp.	South Africa dog-1245	Dog
AY570539	*Anaplasma* sp.	South Africa dog-1076	Dog
AY570538	*Anaplasma* sp.	South Africa dog-1108	Dog
AB196721	*Anaplasma phagocytophilum*	SS33P-L	Deer
KC455366	*Anaplasma phagocytophilum*	GDR4	Camel
KC800963	*Anaplasma phagocytophilum*	ApGDr1	Camel
KC800964	*Anaplasma phagocytophilum*	ApGDr2	Camel
KF826284	*Anaplasma platys*	103	Dog
KC989957	*Anaplasma platys* isolate	CA07	Dog
KF055356	*Anaplasma bovis* isolate	PZHB7	Cattle
KC189853	*Anaplasma* sp.' *Omatjenne*'	HIP/A8/b	Buffalo
KJ459342	*Anaplasma ovis*	Ovis 6	Sheep
KF055364	*Anaplasma bovis* isolate	LY4	Cattle
AB588977	*Anaplasma centrale*	Ac22D	Deer
JQ839012	*Anaplasma marginale*	C6A	Cattle
HQ843851	*Wolbachia pipientis* isolate	wPas	*Phloeomyzus passerinii*
JN315973	*Candidatus* Rickettsia asemboensis isolate	F82	*Ctenocephalides felis*
KJ459344	*Rickettsia massiliae*	8	NA
AB781707	*Candidatus* Rickettsia kotlanii	HM-2	*Haemaphysalis megaspinosa*
NR_103923	*Rickettsia rickettsii*	Iowa	Guinea pig
NR_074497	*Rickettsia parkeri*	Portsmouth	Human
NR_074480	*Rickettsia conorii*	Malish 7	*Rhipicephalus sanguineus*
D38625	*Orientia tsutsugamushi*	Kawasaki	Human
NR_074485	*Rickettsia canadensis*	Mckiel	*Haemaphysalis leporispalustris*
L36103	*Rickettsia bellii*	369L42-1	*Dermacentor variabilis*
NR_074483	*Rickettsia felis*	URRWXCal2	*Ctenocephalides felis*
KJ740388	*Rickettsia helvetica*	IR-698.9-AF	*Ixodes ricinus*
NR044656	*Rickettsia prowazekii*	Brein 1	Human
NR_074394	*Rickettsia typhi*	Wilmington	Human
HM050271	*Rickettsia sibirica*	RH05	*Hyalomma truncatum*
JF949783	*Rickettsia africae*	Clone 95.2	*A. variegatum*, *R. decoloratus*, *R. sanguineus*
NR_118837	*Rickettsia peacockii*	Skalkaho	*Dermacentor*

			andersoni
NR_074469	*Rickettsia heilongjiangensis*	054	*Dysmicoccus sylvarum*
M83798	*Caulobacter crescentus*	CCRRNAB	Marine habitat
DQ111760	*Babesia canis rossi* isolate	Dog-44	Dog
HQ148664	*Babesia canis vogeli*	TWN2	Dog
JQ929916	*Babesia divergens*	RD54	Roe deer
EU152128	*Babesia canis canis*	18S ribosomal RNA gene complete sequence	*Dermacentor reticulatus*
JN982344	*Babesia rossi*	NGR clone 22	Dog
GQ304526	*Babesia capreoli* isolate	CVD5	Roe deer
HM585429	*Babesia canis rossi*	18S ribosomal RNA gene	Dog
JX110650	*Babesia gibsoni* isolate	11	Dog
AF152702	*Babesia conradae*	18S ribosomal RNA gene	Dog
KJ649311	*Babesia microti* isolate	224864NIRAA	*Ixodes ricinus*
FJ537055	*Babesia bovis*	Clone SS11C05	Cattle
JX648554	*Babesia bigemina*	Rap-1a	Cattle
KF429795	*Theileria annulata*	Vaccine S15	Cell culture
AF046927	*Theileria parva*	Muguga	Vaccine strain
KF773741	*Theileria annae*	407_08	Red fox
EU700057	*Theileria orientalis* isolate	ANU-RP	Cattle
AY488170	*Toxoplasma gondii* isolate	S05	Sea otter

NA *=Not available