Tick-borne haemoparasite occurrence and *Anaplasma bovis* strain diversity in eastern rock sengis (*Elephantulus myurus*)

MSc research dissertation by

Roanda Jacobs

(16338147)

Supervisor: Prof Marinda C. Oosthuizen

Co-supervisor: Prof Armanda D.S. Bastos

Submitted in partial fulfilment of the requirements of the MSc (Veterinary Science) degree in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

December 2018

DEDICATION

To my ever-loving parents who continually mention how proud they are of me. This work is dedicated to you and our Saviour Jesus Christ.

DECLARATION

I declare that the 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, to be my own work and has not been previously submitted by me for a degree at another tertiary institution.

Roanda Jacobs

11 December 2018

Date

This dissertation emanates from project V081-16 approved by the Research Committee of the Faculty of Veterinary Science and the Animal Ethics Committee of the University of the University of Pretoria

ACKNOWLEDGEMENTS

Firstly I would like to thank God for putting me on the path to meet two of the most amazingly inspirational women: My supervisor Prof Marinda Oosthuizen and Co-Supervisor Prof Armanda Bastos. You have been so patient with all my silly questions and dumb mistakes and helped me abundantly throughout this study. Your effortless demeanour is something to admire. I am blessed to have you both as my Supervisors and I'm truly grateful for the opportunity that you have given me to conduct this study.

Prof Marinda, you have created such a calm environment for me to work in and for that I am truly thankful. We, your students, cannot thank you enough for your endless support and attempts to help us gain funding from various agencies. You never hesitate to include us in any opportunity that could benefit us.

Prof Armanda, you have played such a vital role in my studies with welcoming me to your laboratory. Every time when I thought there was no solution, you effortlessly solved every problem that arose.

To my parents and the rest of my family, I have only come so far with your support, guidance and patience. At times when I wanted to shout out with rage or cry with unbearable sadness, you have raised me up and reminded me to keep my head held high. You have never doubted in me even though I had every reason to quit. I thank the Lord every day for blessing me with such an amazing family.

Thank you to the South African Agricultural Sector Education and Training Authority (AgriSETA awarded to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria), the National Research Foundation (NRF grant 92739 to Marinda Oosthuizen), Centers for Disease Control and Prevention CDC Co-Ag 5 NU2GGH001874-02-00 and Sanger sequencing through NRF facility grant No: UID78566 (to Armanda Bastos) for funding this study. Without your help, this dissertation would not have been possible.

To the Department of Veterinary Tropical Diseases' staff and students, you all have been so welcoming and helpful. My journey has been effortless with all of your help and support. I will never forget the amazing people I have met and the life-long friends I have made here.

CONFERENCE PRESENTATIONS

1. Jacobs, R., Bastos A.D.S, Troskie, M., Medger, K & Oosthuizen M.C. Tick-borne haemoparasite occurrence in eastern rock sengi (*Elephantulus myurus*) of South Africa presented at the 26th International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP) 4-8 September 2017, Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia (Poster presentation).

2. Jacobs, R., Bastos A.D.S, Troskie, M., Medger, K & Oosthuizen M.C. Tick-borne haemoparasite occurrence in eastern rock sengi (*Elephantulus myurus*) of South Africa presented at the 3rd International Congress on Parasites of Wildlife (PARSA) 24-27 September 2017, Kruger National Park, South Africa (Poster presentation).

3. Jacobs, R., Bastos A.D.S, Troskie, M., Medger, K & Oosthuizen M.C. Tick-borne haemoparasite occurrence in eastern rock sengi (*Elephantulus myurus*) from South Africa presented at the Onderstepoort Campus Faculty Day 23 August 2018, Onderstepoort Campus, University of Pretoria, Pretoria, South Africa (Poster presentation).

Tick-borne haemoparasite occurrence and *Anaplasma bovis* strain diversity in eastern rock sengis (*Elephantulus myurus*)

Candidate: Ms Roanda Jacobs
Supervisor: Prof Marinda Oosthuizen
Co-supervisors: Prof Armanda Bastos
Department: Veterinary Tropical Diseases
Degree: MSc (Veterinary Science)

SUMMARY

Studying the relationships of parasites with their vertebrate hosts is important as they improve our knowledge on the ecology of diseases of veterinary and zoonotic importance. Eastern rock sengis (*Elephantulus myurus*) are small insectivores endemic to Africa that are known to have higher tick burdens than other small mammal species. Studies have shown that *E. myurus* from South Africa harbour an *Anaplasma bovis*-like strain, a rickettsial pathogen of cattle. *Anaplasma bovis* infects host monocytes causing monocytic- and bovine anaplasmosis.

The role of rock sengi as reservoirs of tick-borne pathogens has not been fully investigated. In addition, the phylogenetic position of the described *A. bovis*-like strain is unclear as genetic data are currently limited to the highly conserved 16S rRNA gene and studies are constrained by difficulties with cultivation of tick-borne Anaplasmataceae. The aim of the current study was, therefore, to determine the tick-borne haemoparasite diversity in rock sengi and to expand molecular characterization of the *A. bovis*-like strain in rock sengi (henceforth referred to as *A. bovis*-like (sengi)), using a more phylogenetically informative gene region. The specific objectives were to (i) screen blood samples for the presence of *Theileria, Babesia, Ehrlichia* and *Anaplasma* spp. using the Reverse Line Blot (RLB) hybridization assay, and (ii) to determine the taxonomic position of the *A. bovis* strain present in rock sengis by PCR-screening of additional rock sengi samples using 16S rRNA and *GroEL* assays, optimised for specific detection of the *A. bovis*-like (sengi) variant, in combination with Sanger sequencing and phylogenetic analysis.

Genomic DNA extracted from 160 eastern rock sengi blood samples collected from the Goro Game Reserve, Limpopo Province (n=112) and Ezemvelo Nature Reserve, Gauteng (n=48), South Africa, as part of previous studies, was subjected to the RLB hybridization assay and further molecular characterization. The RLB hybridization assay results revealed that PCR products hybridized with the *Theileria/Babesia* genus-specific probe in 5% (n=8) of the

samples and with the *Anaplasma/Ehrlichia* genus-specific probe in 31.9% (n=51) of the samples. A total of 86 (53.8%) of the samples tested negative or below the level of detection of the assay; none of the PCR products hybridized with any species-specific probes. Alignment of the near full-length 16S rRNA gene sequences of the *A. bovis*-like strain previously identified in rock sengi (Harrison *et al*., 2013) revealed that the *A. bovis* RLB probe differed at three nucleotide sites under the probe area explaining why the species-specific *A. bovis* RLB probe failed to hybridise. A new RLB probe was designed on the basis of available 16S rDNA sequences, to allow for the specific detection of the sengi-associated *A. bovis*-like strains. This custom-designed *A. bovis*-like (sengi) probe was subsequently used to screen a subset (n=108) of the original eastern rock sengi samples that previously tested RLB positive for the *Anaplasma/Ehrlichia* genus-specific probe. Despite the custom-design of the probe, only 17 (15.7%) tested positive.

The parasite 16S rRNA and *GroEL* genes were subsequently amplified, purified and sequenced from the 17 *A. bovis*-like (sengi) RLB positive samples and two previously negative samples. A total of twelve 16S rDNA and eight *GroEL* sequences were generated. Gene trees were inferred using the Neighbour-joining algorithm, prior to more rigorous Maximum likelihood and Bayesian inferences, using appropriate models of sequence evolution and priors, respectively. BLASTn homology searches showed that the obtained 16S rDNA sequences had 99% sequence identity with *A. bovis* (Accession no: U03775) previously described in South Africa, while the *GroEL* sequences were 88% identical to an uncultured *Anaplasma* sp. identified in a raccoon (Accession no: JN588562). The phylogenetic analyses revealed that the sequences were most closely related to *A. bovis* type sequences, but were sufficiently genetically distinct to represent a novel species. In contrast, the 16S rDNA results revealed that the novel sengi-associated *Anaplasma* lineage falls within an unresolved, polyphyletic lineage that includes *A. bovis*. Little is known about this sengi-associated variant that is closely related to *A. bovis*. However, given the relatedness to a pathogen of animal health concern it is important to establish the pathogenicity of this species in order to determine its potential impact on animal and/or human health. It is also imperative that more phylogenetic studies should be performed to clearly place this *A. bovis*-like (sengi) species within the broader genus *Anaplasma* phylogeny.

CONTENTS

ABBREVIATIONS

16S rRNA	16S ribosomal Ribonucleic acid
18S rRNA	18S ribosomal Ribonucleic acid
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
°C	Degrees Celsius
CAT	Card agglutination test
C-ELISA	Competitive enzyme-linked immunosorbent assay
CGA	Canine granulocytic anaplasmosis
comb. nov.	Combinatio nova
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DEET	N,N-Diethyl-meta-toluamide
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
dUTP	Deoxyuridine triphosphate
ECF	East Coast Fever
EDAC	1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide
EDTA	Ethylene diamine tertaacetic acid
EGA	Equine granulocytic anaplasmosis
ELISA	Enzyme-linked immunosorbent assay
et al	et alia
FABI	Forestry and Agricultural Biotechnology Institute
GroEL	heat shock protein
gltA	Citrate synthase
HGA	Human granulocytic anaplasmosis
HGE	Human granulocytic ehrlichiosis
I-ELISA	Indirect ELISA
IFAT	Immuno fluorescent antibody test
IUCN	International Union for Conservation of Nature
kb	kilobase
KCl	Potassium chloride
µl	Microlitre
µM	Micromolar
ml	Mililitre
mM	Milimolar
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium chloride
MSP	Major Surface Protein
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
PCR	Polymerase chain reaction
pmol	Picomoles

rDNA	Ribosomal deoxyribonucleic acid
RLB	Reverse Line Blot
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SMS	Smart Model Selection
SSPE	Saline Sodium Phosphate
TBF	Tick-borne fever
Tris-HCl	Hydroxymethyl aminomethane hydrochloride
UDG	Uracil DNA glycosylase
USA	United States of America

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

General Introduction

Small mammals are important hosts for the immature life stages of ixodid ticks throughout the world (Sonenshine, 1991). Rodents play an important role in supporting these life stages (Norval, 1979, Talleklint & Jaenson, 1997, Clark *et al*., 1998); and an equally important role as reservoir hosts of tick-borne pathogens (Donahue *et al*., 1987, Randolph *et al*., 1999, Bown *et al*., 2003, Karbowiak, 2004). The importance of sympatric insectivore species in supporting tick populations and as reservoirs of tick-borne pathogens is poorly understood, however.

The eastern rock sengi (*Elephantulus myurus*) is a small mammal that hosts a wide variety of arthropod parasites and is known to have higher tick burdens than most other small mammal species. Fourie *et al*. (1995) recorded 27 immature ixodid tick species on a single rock sengi, three of which (*Ixodes rubicundus, Rhipicentor nuttalli, Rhipicephalus warburtoni*) are known to cause paralysis in domestic animals. Moreover, Harrison *et al*. (2011) collected 22 739 ticks from 57 rock sengi individuals, underscoring the previously-mentioned high tick burden. Subsequent studies have shown that eastern rock sengi from South Africa, harbour a rickettsial pathogen that is closely related to *Anaplasma bovis* in cattle (Harrison *et al*., 2013). In their study, Harrison *et al*. (2013) were able to amplify an *A. bovis*-like sequence from 30% of rock sengi blood samples. The sequence differed at six nucleotide sites (nt) from the *A. bovis* reference strain (U03775) across the 1413 nt region analysed (Harrison *et al*., 2013). The high level of variation in such a highly conserved gene provided the impetus for this study. Collectively the results suggest that in southern Africa, insectivores may play a more important role as hosts for immature ticks than sympatric rodents, and that they are important reservoirs of tick-borne pathogens.

Anaplasma bovis infects the monocytes of their vertebrate hosts and causes monocytic anaplasmosis as well as bovine anaplasmosis. *Anaplasma bovis* infections are usually sub-clinical in cattle but when symptoms do occur, case fatality rates of up to 50% have been recorded (Santos & Carvalho, 2006, Rymaszewska & Grenda, 2008, Harrison *et al.,* 2013). In addition to *A. bovis*, bovine anaplasmosis is also caused by *A. marginale* and *A. centrale* and is associated with major economic losses. Furthermore, if cattle survive the acute infection of the disease, persistent infections may become established allowing these cattle to serve as reservoir hosts of *A. marginale* (Kocan *et al*., 2003).

Although infection of humans with *A. bovis* has not been reported, it has been suggested that *Ixodes dammini* may be capable of transmitting this pathogen to humans (Goethert & Telford lll, 2003). Furthermore, this pathogen has been found in a wide range of geographical areas and in various host and tick species.

In 2011, DNA of *A. bovis* was found in the nymphs of an undescribed *Rhipicephalus* tick species, designated *Rhipicephalus* sp. near. *warburtoni* due to the similar morphology to *R. warburtoni*, in South Africa (Harrison *et al.,* 2011). This previously undescribed tick species was subsequently proposed to be the vector for *A. bovis* due to the high abundance of this tick species on rock sengi while other tick species were absent (Harrison *et al.,* 2013). This hypothesis is yet to be confirmed, although a subsequent study revealed *A. bovis* infection rates of 30% in sengi sampled from the same area (Harrison *et al*., 2013).

There is presently a lack of information on the pathogen status of ticks that infest eastern rock sengi. Despite the confirmed presence of *A. bovis* in ticks, information on vector competence is limited compared to other *Anaplasma*, *Ehrlichia*, *Theileria* and *Babesia* species (Doan *et al*., 2013, Atif, 2016). Likewise, the phylogenetic position of this *A. bovis*-like strain found in the nymphs of *Rhipicephalus* sp. near. *warburtoni* is unclear, as the results were limited to reporting PCR-positivity, without confirmatory nucleotide sequencing. Moreover, gene sequence data are currently limited, and these data constraints are unlikely to change in the short term due to difficulties with cultivating tick-borne Anaplasmataceae *in vitro* (Moustafa *et al*., 2015), which in turn negatively impacts whole-genome sequencing (Battilani *et al*., 2017). The aim of the study was, therefore, to assess tick-borne haemoparasite prevalence and diversity in rock sengi and to molecularly characterize the *A. bovis*-like strain present in sengi using gene sequence analysis. The specific objectives were to:

(i) Screen blood samples for the presence of *Theileria, Babesia, Ehrlichia* and *Anaplasma* spp. using the Reverse Line Blot (RLB) hybridization assay.
(ii) Determine the taxonomic position of the *Anaplasma* strain present in rock sengis by PCR amplification and sequencing of the parasite's 16S rRNA and *GroEL* genes and subsequent phylogenetic analysis.
(iii) Optimise RLB and PCR-sequencing approaches for specific detection of the *A. bovis*-like variant present in rock sengi.

CHAPTER 2

Literature review

Wildlife species act as reservoirs for many pathogens which are of veterinary and zoonotic importance. These pathogens can be transmitted by various vectors, ticks being among the most important of these. Tick-borne pathogens cause diseases which infect humans and animals (Jongejan & Uilenberg, 2004) and in the last 20 years, researchers and clinicians have become increasingly aware of these emerging tick-borne diseases and their impacts on public health (Parola & Raoult, 2001a). Harrison *et al.* (2011) stated that the control of these pathogens, together with identification of their risks to human and animal health, hinges on identifying novel reservoirs and vectors of these infectious diseases.

2.1 Eastern rock sengi (*Elephantulus myurus*) as a host species

Rock sengis (also called elephant shrews) within the genus *Elephantulus*, family Macroscelidae, order Afrotheria, are small mammal insectivores that are endemic to Africa (Harrison *et al*., 2013). On the IUCN Red List of mammals, they are considered to be of "Least Concern" (Rathbun, 2015), as there is no indication that this species or the habitats it uses face any major threats.

Rock sengis' natural habitats are subtropical or tropical dry lowland grassland and rocky outcrops (Fourie *et al*., 1992, Lancaster & Pillay, 2010). They are primarily diurnal with a good deal of activity at sunrise and sunset. They are solitary animals and display social monogamy (Rathbun, 1979).

Rock sengis host a variety of ectoparasites (i.e. ticks, lice, mites, fleas) particularly ticks, that vary by season. These parasites are vectors for a variety of human and zoonotic diseases (Fourie *et al.,* 1992). Of the ticks in greatest abundance, *Ixodes rubicundus* and *Rhipicephalus warburtoni* cause paralysis in domestic livestock, *Haemaphysalis elliptica* is a vector for babesiosis in dogs and Q-fever in humans, and toxic excretions of *Rhipicentor nuttalli* cause paralysis in dogs (Fourie *et al.,* 1992). In addition, rock sengis have been found to harbour an *Anaplasma bovis*-like strain (Harrison *et al*., 2013), making them particularly well-suited to studying the relationship between parasites and their hosts (Fourie *et al*., 1995). In their study, Harrison *et al*. (2013) were able to amplify an *A. bovis*-like sequence from nearly 30% of rock sengi blood samples using primers that targeted the central regions of the 16S rRNA gene

(Parola *et al*., 2000). The sequences generated were shown to differ at six nucleotide sites from the reference *A. bovis* strain (Accession no: U03775) (Harrison *et al*., 2013).

2.1.1 Parasitic host burdens

A vector is described as an organism that promotes the spread of pathogens within a host population (Rymaszewska & Grenda, 2008). Ticks are among the most important vectors for pathogen transmission in humans and animals (Balashov, 1972, Wei *et al*., 2016). It is known that tick-borne pathogens have a specific host-parasite interaction with their vectors (Goethert & Telford lll, 2003). Thus identification of the factors driving distribution of vectors among their hosts plays an important role in managing the diseases they carry (Fagir *et al*., 2015). According to Wilson *et al.* (2002) these distribution patterns can be influenced by biotic and abiotic factors, such as climate change and reproductive activity, which could act on the host and/or the parasite.

Ticks are ecto-parasite arachnids that survive by feeding on the blood of their hosts and are classified within three families. The two highly speciose families are the Ixodidae (hard ticks) and the Argasidae (soft ticks) (Sauer *et al*., 2000), and the third, the Nuttalliellidae, which has features intermediate to those of hard and soft ticks, is monotypic. Each life stage of the ixodid ticks feeds only once but for long periods of time, while Argasid ticks feed more often but for shorter periods (Sonenshine, 1991, Parola & Raoult, 2001a, Parola & Raoult, 2001b).

Most ixodid ticks are exophilic meaning that they live independently, away from domestic environments, but some ixodid species are endophilic, living within domestic environments (Parola & Raoult, 2001a). Exophilic ticks prefer meadows, forests, and open environments, whereas endophilic ticks hide within burrows and nesting areas of their preferred hosts (Parola & Raoult, 2001a).

Ticks mainly display two host-seeking behaviours called the “hunter” and “ambush” approaches. During the hunting approach a tick will directly attack the host (i.e. runs towards the host) by sensing chemical stimuli, amongst others, of the intended target (Sonenshine, 1991, Parola & Raoult, 2001a). The ambush approach is where a tick climbs onto vegetation, and takes on a questing stance in which outstretched front legs are used to latch onto passing hosts (Parola & Raoult, 2001a). Exophilic ixodid ticks have been the focus of most studies, due to the fact that unlike soft ticks, these ticks act as vectors for a wider range of pathogens (Sauer *et al*., 2000) and are readily sampled from the environment.

Globally, rodents are known to be important hosts for immature ixodid tick species, but it is suggested that rock sengi host substantially higher tick loads than sympatric murid rodent species (Sonenshine, 1991, Fourie *et al*., 1992). This was demonstrated in previous studies in South Africa, such as the study of Harrison *et al.* (2011) that found high tick burdens on rock sengi, with a combined total of 22 739 ticks being recovered from just 57 rock sengi. This is in stark contrast to sympatrically occurring Namaqua rock mice (*Micaelamys namaquensis*), for which 177 ticks were recovered from 172 individuals (Harrison *et al*., 2011). Similarly, Fourie *et al.* (1992) found a total of 15 991 ticks on 132 rock sengi while 321 Namaqua rock mice, sampled from the same locality, were parasitized by 1352 ticks.

Harrison *et al.* (2011) suggested that the reason for the lower tick loads in the Namaqua rock mouse compared to the eastern rock sengi could be due to disparate levels of sociality as well as behavioural differences. Increased levels of transmission would be expected within social animals, such as the Namaqua rock mouse (Smithers & Wilson, 1979), compared to solitary rock sengi (Ribble & Perrin, 2005). However, with higher levels of sociality, the Namaqua rock mouse could benefit from allo-grooming within their social group (Harrison *et al*., 2012). Moreover, Namaqua rock mice are nocturnal animals (Fleming & Nicolson, 2004) whereas rock sengi are active both during the day and night (Ribble & Perrin, 2005).

In 1995, 27 mostly immature ixodid tick species were reported to occur on rock sengi in Africa (Fourie *et al*., 1995). Three of these tick species, i.e. *Ixodes rubicundus, Rhipicentor nuttalli* and *Rhipicephalus warburtoni*, are known to transmit toxins that cause paralysis in domestic animals (Fourie *et al*., 1992, Harrison *et al*., 2011), which is considered to be the most important tick toxicosis transmitted by 60 known tick species (Gothe & Neitz, 1991). This paralysis is caused by the neurotoxins produced within the female ticks' salivary glands (Parola & Raoult, 2001a). According to Du Toit and Fourie (1994), rock sengi are not affected by this paralysis-inducing tick toxicosis, even though they carry large burdens of these tick species. This suggests a long association between this tick species and rock sengi. The current lack of information on the ticks that infest rock sengi and the diseases that they carry requires attention as the limited data available confirm that many of the tick species hosted by rock sengi are of medical and veterinary importance (Harrison *et al*., 2011).

2.2 Tick-borne haemoparasites

Tick-borne haemoparasites occur on every continent and affect all animals (domestic and wildlife species); some also represent a concern to human public health. Piroplasms, with the two main genera being *Theileria* and *Babesia*, are tick-borne intracellular apicomplexan

parasites which inhabit erythrocytes, and sometimes other cells, of vertebrates. Both genera contain an abundance of species of key veterinary, zoonotic and economic importance. *Ehrlichia* and *Anaplasma,* the two major genera in the family Anaplasmataceae, order Rickettsiales, are also increasingly implicated in both human and animal disease. They are small, obligate intracellular pathogens that multiply in both vertebrate and invertebrate hosts; generally replicating within erythrocytes, leucocytes or endothelial cells.

The most economically important haemoparasitic tick-borne diseases of ruminants on a global scale are bovine babesiosis caused by *B. bovis* and *B. bigemina*, bovine anaplasmosis (*A. marginale, A. centrale* and *A. bovis*), bovine theileriosis caused by *T. annulata* and *T. parva* (Norval *et al*., 1992), and heartwater of cattle and small ruminants caused by *Ehrlichia ruminantium*. In Africa, all four of these diseases pose a serious threat to livestock production (Uilenberg, 1995). Tick-borne haemoparasites of zoonotic importance include *Babesia* spp. that cause human babesiosis (*B. microti*, *B. duncani*, *B. divergens* and *B. venatorum*), *A. phagocytophilum* (causing human granulocytic anaplasmosis), *E. chaffeensis* (causing human monocytotropic ehrlichiosis), and less commonly *E. ewingii* (causing granulocytic ehrlichiosis).

2.2.1 The genus *Anaplasma*

Anaplasma species are tick-borne, Gram-negative, obligate, intracellular bacteria that infect the blood of animals and humans (Dumler *et al*., 2001, Rar & Golovljova, 2011). They reside within cytoplasmic vacuoles, either singly and more often in compact inclusions (morulae) present in mature or immature haematopoietic cells of mammalian hosts (Dumler *et al*., 2001).

Bacteria from the genus *Anaplasma* belong to the kingdom Prokaryota, and family Anaplasmataceae. The present classification has been valid since 2001 when significant reorganizational changes were carried out. Based on the molecular analysis of the 16S rRNA and *groESL* genes, and supported by biological data, the families Rickettsiaceae and Anaplasmataceae were placed within the order Rickettsiales. Some changes were also made in the classification of the bacteria within the genera; the genus *Anaplasma* was emended to include *A. phagocytophilum (E. phagocytophila, E. equi* and the agent of human granulocytic ehrlichiosis were united within the single species designation), with two species formerly classified within the genus *Ehrlichia*, being assigned to *Anaplasma, viz. A. bovis* (formerly *E. bovis*) and *A. platys* (formerly *E. platys*) (Dumler *et al*., 2001).

There are currently six recognized species that belong to the genus *Anaplasma:* the ruminant-infecting *A. marginale* (the type species), *A. bovis*, *A. centrale* and *A. ovis*; the canine pathogen *A. platys*; and finally *A. phagocytophilum* which infects a wide range of hosts

including humans, rodents, birds, dogs and ruminants. Recently, *A. capra* was identified and described by Li *et al*. (2015). This species is zoonotic, infecting humans, sheep and goats and is widespread in China (Li *et al*., 2015; Yang *et al*., 2017), but is not a formally recognized species and not on the List of Prokaryotic Names with Standing in Nomenclature (www.bacterio.net/anaplasma.html). In addition, there have recently been a number of studies demonstrating that the genus *Anaplasma* is far more species-rich than previously thought. *Anaplasma odocoilei* (Tate *et al*., 2013) has been described from white-tailed deer; several "*Candidatus* Anaplasma" species have also been described, including "*Candidatus* Anaplasma camelii" from camels (Bastos *et al*., 2015), *Candidatus* Anaplasma boleense and *Candidatus* Anaplasma rodmosense from mosquitoes (Guo *et al*., 2016), to name a few. Given the focus of this study, the remainder of this section will concentrate on *A. bovis*, and the other *Anaplasma* species will only be briefly discussed.

2.2.1.1 *Anaplasma* life cycle

The family Anaplasmataceae has an enzootic life cycle between the tick vector and the vertebrate hosts (Figure 2.1) (Parola *et al*., 2005, Kawahara *et al*., 2006, Nicholson *et al*., 2010). The species within the genus *Anaplasma* are generally transmitted by ixodid ticks (Dumler *et al*., 2001) and these vectors play an important role in their life cycle because they circulate these pathogens in the environment (Rymaszewska & Grenda, 2008). On establishing a persistent infection in their vertebrate hosts, the hosts become reservoirs for the infection (Rar & Golovljova, 2011). This is important because although *Anaplasma* species are transstadially transmitted in ticks (transmitted from one life stage to another, larvae to nymph to adult), they cannot be maintained within the tick cycle because these pathogens cannot be transmitted transovarially (transmitted from one generation to another through ovaries of the female ticks). Mammalian reservoir hosts are therefore important for pathogen reproduction and maintenance (Parola *et al*., 2005, Rar & Golovljova, 2011).

Ticks become infected in their larval or nymphal stages by feeding on a reservoir host (Nicholson *et al*., 2010). These pathogens enter the midgut epithelium of the ticks where replication takes place (Kocan *et al*., 2003). From there they move to the epithelial cells of the tick salivary gland where they undergo secondary replication (Rodriguez *et al*., 2009). The pathogens can then be transtadially transmitted when the ticks become nymphs and adults (Nicholson *et al*., 2010). Lastly the pathogens enter the tick salivary glands where they will be transmitted to a new vertebrate host (Rar & Golovljova, 2011).

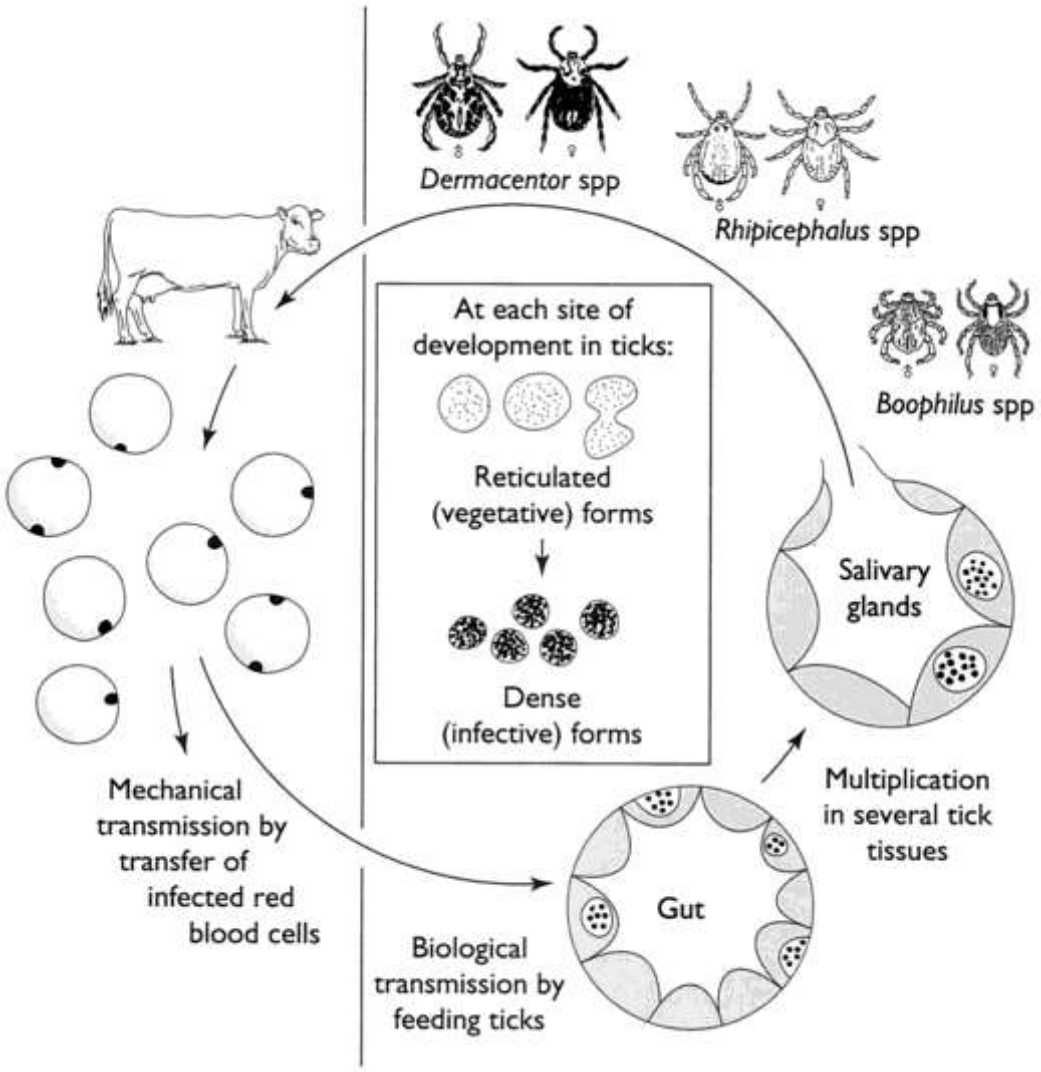

Figure 2.1: Representation of the life cycle of *Anaplasma marginale* in cattle and ticks. This life cycle is shown as a general life cycle representation of *Anaplasma* species. It should be noted that *A. bovis, A. phagocytophilum* and *A. platys* infect the leukocytes while *A. marginale, A. centrale* and *A. ovis* infect the erythrocytes of their vertebrate hosts. (Taken from Kocan *et al.* (2003) with permission from the American Society for Microbiology).

2.2.1.2 *Anaplasma bovis*

Anaplasma bovis is a bacterium detected mainly in cattle, but also observed in small mammals which are probably a reservoir of this bacterium (Goethert & Telford III, 2003). It is described as a pleomorphic, obligate, intravacuolar bacterium (Dumler *et al*., 2001). *Anaplasma bovis* infects the monocytes of their hosts and the disease it causes is called monocytic anaplasmosis which usually does not cause any clinical symptoms in cattle (Rar & Golovljova, 2011). When symptoms do occur, they include: fever, anorexia, debility, drowsiness, diarrhoea, weight loss, convulsions together with enlarged lymph nodes. Furthermore, when these symptoms occur the infection rates could be up to 50% within a herd, which could then have a case fatality rate of 40-50% (Santos & Carvalho, 2006, Rymaszewska & Grenda, 2008, Harrison *et al*., 2013). Goethert and Telford lll (2003) stated that there are no acknowledged

reports of *A. bovis* infections in humans but that *Ixodes dammini* (*scapularis*) could possibly transmit this pathogen to humans in their nymphal or adult life stages. *Anaplasma bovis* has been detected in Brazil, North America, Africa and Japan (Goethert & Telford III, 2003, Kawahara *et al*., 2006, Santos and Carvalho, 2006). African tick vectors include *H. excavatum*, *R. appendiculatus* and *A. variegatum* (Dumler *et al*., 2001).

History of *A. bovis* discovery: The first description of *A. bovis* was during transmission experiments done by Donatien and Lestoguard (1936) which involved the tick species *Hyalomma* feeding on French cattle breeds in Iran. It was subsequently identified by De Kock *et al.* (1937) in *R*. *appendiculatus* in domestic cattle from the Limpopo Province, South Africa. This pathogen, named *Rickettsia bovis* by Donatien and Lestoguard (1936), was transferred to the genus *Ehrlichia* by Scott (1994) and subsequently placed in the genus *Anaplasma* by Dumler *et al*. (2001).

Host range, geographical distribution and tick vectors: *Anaplasma bovis* has been found in a wide range of geographical areas and ticks from hosts that range from, among others, cottontail rabbits from Nantucket Island, United states (Goethert & Telford lll, 2003, Rar & Golovljova, 2011) to cattle, wild deer and feral racoons from Hokkaido, Japan (Kawahara *et al*., 2006, Jilintai *et al*., 2009, Sashika *et al*., 2011), cattle from Yonaguni Island, Japan (Ooshiro *et al*., 2008), sheep and goats from Tunisia (Said *et al*., 2015), Red and Sika deer from China (Li *et al*., 2016), ungulates from Africa, Asia and South America (Donatien & Lestoquard, 1936, Dumler *et al*., 2001), calves in Rio de Janeiro, Brazil (Santos & Carvalho, 2006) and birds in Spain (Palomar *et al*., 2015).

Anaplasma bovis has been previously detected from the tick species *Amblyomma variegatum*, *Hyalomma excavantum* and *R. appendiculatus* in Africa, and in Brazil they have been identified in *A. cajennense* (Dumler *et al*., 2001). Further molecular studies have shown that *A. bovis* can be transmitted by *Haemaphysalis* tick species in Far East Russia, Japan, China, South Korea and Thailand (Parola *et al*., 2003, Kawahara *et al*., 2006, Shpynov *et al*., 2006, Sashika *et al*., 2011, Doan *et al*., 2013, Wei *et al*., 2016), while in Saskatchewan, Canada, DNA of *A. bovis* was identified in *D. andersoni* (Dergousoff & Chilton, 2011). The main vector for *A. bovis* in rabbits on Nantucket Island was confirmed by Goethert and Telford lll (2003) as *H. leporispalustris* due to all the stages being infected and likewise, that *A. bovis* it is transmitted transstadially within this tick. They also found that *A. bovis* is transstadially transmitted within *I. dentatus*, but this was not as predominant as in *H. leporispalustris*. This could indicate that *I. dentatus* might be a secondary vector of *A. bovis* (Goethert & Telford lll, 2003). However, as *H. leporispalustris* usually only feed on small mammals it is unlikely that

this tick species will transmit *A. bovis* to cattle. Recently, *A. bovis* was discovered in *R. sanguineus* and interestingly in *R. turanicus* from ticks in Israel (Harrus *et al*., 2011). Although confirmed in a wide range of species and localities, information on vector-competence for *A. bovis* is limited compared to other *Anaplasma* species (Doan *et al*., 2013, Atif, 2016).

As previously mentioned, Harrison *et al.* (2011) found exceptionally high tick loads on rock sengi. These tick species were from an undescribed *Rhipicephalus* tick, which they denoted *R*. sp. near. *warburtoni* because of its similar morphology to *R. warburtoni* sensu stricto (Harrison *et al*., 2011). Within these nymphal ticks, they found DNA of *A. bovis* but this does not confirm that rock sengi are reservoir hosts of *A. bovis* nor that *R.* sp. near. *warburtoni* is the vector of this pathogen (Harrison *et al*., 2013). Harrison *et al.* (2013) suggested that the larvae of the ticks could have been infected while having a blood meal from another infected source, but also highlight that *R*. sp. near. *warburtoni* is the likely vector for *A. bovis* because they found an abundance of these tick species on rock sengi while other tick species were absent (Harrison *et al*., 2013). This suggests that further investigation of possible *A. bovis* involvement in an enzootic cycle with rock sengi and the tick *R.* sp. near. *warburtoni* (Harrison *et al*., 2013) is warranted. Harrison *et al*. (2013) further stated that, currently, information on the pathogenicity of the *A. bovis*-like strain found in these rock sengi is limited, which emphasises the importance of identifying the hosts of the adult life stages of *R*. sp. near. *warburtoni*, as well as to compare this *A. bovis*-like strain to other pathogenic strains found in cattle so that the impact of this bacterium can be determined.

Taxonomic position: Attempts at cultivating *A. bovis in vitro* having been unsuccessful (Moustafa *et al*., 2015), there is limited gene sequence information available for conserved and semi-conserved *A. bovis* genes. Based on initial 16S rRNA gene sequence analyses (Dumler *et al.,* 2001), it was found that *A. bovis* was closely related to *A. ovis* and *A. centrale*, but all subsequent studies placed *A. bovis* in a cluster with *A. phagocytophilum* (Figure 2.2). When Zobba *et al*. (2014) analysed the *GroEL* sequences of *A. bovis,* they found that it formed its own cluster distinct from *A. phagocytophilum.* This was also seen in the study of Ybañez *et al*. (2014) (Figure 2.3). According to Ybañez *et al*. (2014), when protein secondary structures are considered in multiple sequence alignment construction for the *gtlA* and G*roEL* genes, the phylogeny is more consistent than when nucleotides are used.

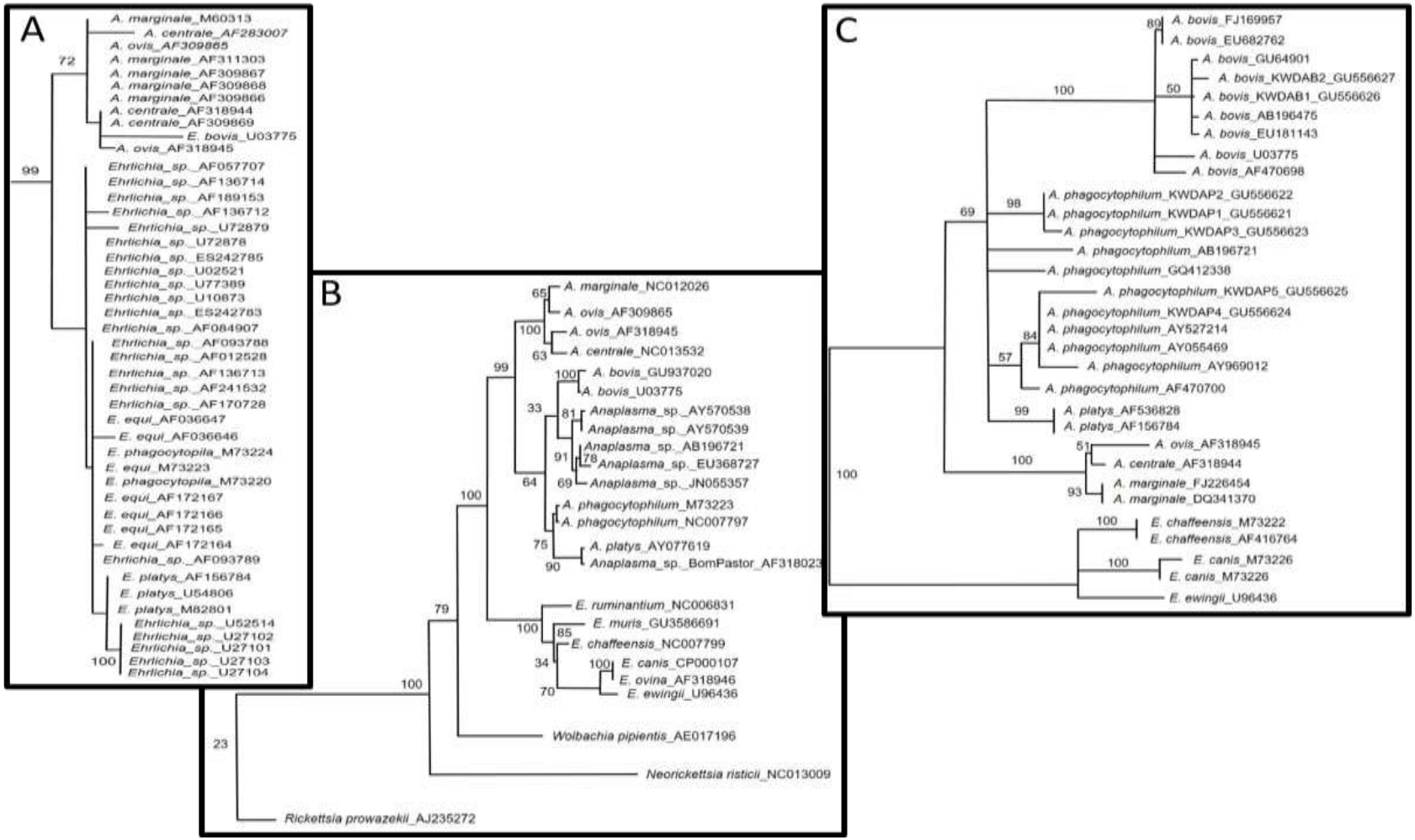

Figure 2.2: Based on the 16S rRNA phylogenetic tree from Dumler *et al.* (2001) it is clear in (A) that *Anaplasma bovis* (*E. bovis*) is closely related to *A. marginale* and *A. centrale* within a well-supported clade (72% bootstrap support), whereas the 16S rRNA phylogenetic trees of (B) Ybañez *et al*. (2014) and (C) Kang *et al.* (2011), indicate that *A. bovis* is related to the *A. phagocytophilum-platys* lineage (64% support) and that it forms its own distinct cluster, respectively. All figures were redrawn using data from the Dumler *et al*. (2001), Kang *et al*. (2011) and Ybañez *et al*. (2014) studies.

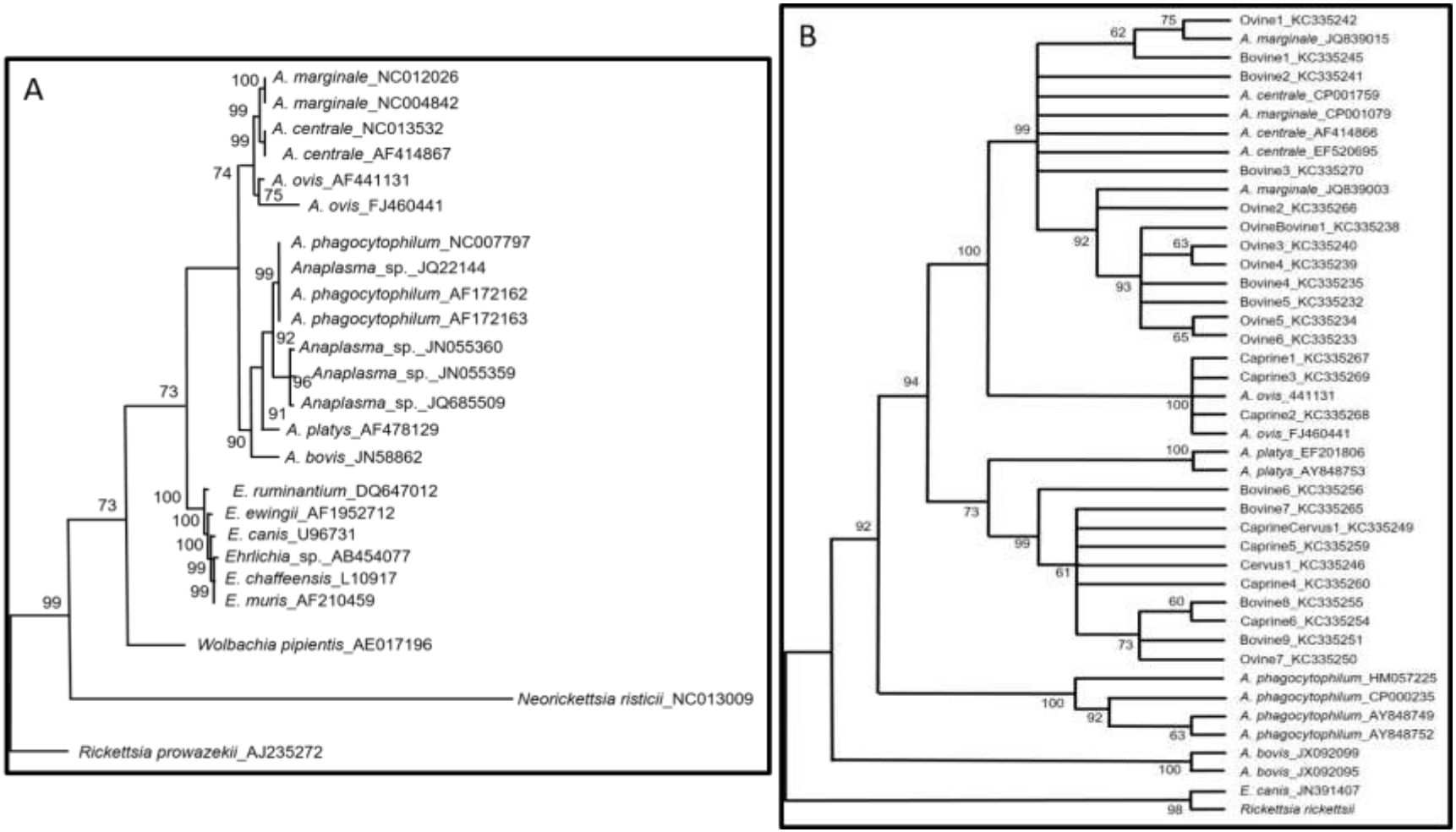

Figure 2.3: Based on the *GroEL* phylogenetic tree from (A) Ybañez *et al.* (2014) it is clear that *A. bovis* clusters with *A. platys* and *A. phagocytophilum* (90% bootstrap support), while the *GroEL* phylogenetic tree from (B) Zobba *et al.* (2014) indicates that *A. bovis* is genetically distinct from all the other *Anaplasma* species (Redrawn using data from Ybañez *et al*. (2014) and Zobba *et al.* (2014)).

2.2.1.3 Other *Anaplasma* species of importance

Anaplasma marginale* and *A. centrale infect erythrocytes of vertebrates and cause disease; specifically in ruminants in tropical and subtropical regions (Rymaszewska & Grenda, 2008, Rar & Golovljova, 2011). Symptoms include fever, abortion, anaemia, weight loss, icterus and lethargy (Rikihisa, 1991, Kocan *et al*., 2003). Bovine anaplasmosis is becoming more abundant which could be due to carrier cattle being transported more widely and due to higher ambient temperatures, which promote the expansion of tick ranges (Kocan *et al*., 2010). *Anaplasma centrale* is closely related to *A. marginale* (Rar & Golovljova, 2011). Their relative position within erythrocytes differs slightly where *A. marginale* is located marginally and *A. centrale* is located more centrally within the cell (Potgieter & Stoltsz, 2004). *Anaplasma centrale* is less pathogenic than *A. marginale* and when infection of *A. centrale* occurs, it provides a protective immunity against *A. marginale* (Rikihisa, 1991, Kocan *et al*., 2003). Moreover, cattle that become infected with *A. marginale* develop persistent infections, thus becoming reservoirs for this parasite (Kocan *et al*., 2003). Tick vectors for *A. marginale* include *Rhipicephalus microplus, R. simus, R. decoloratus, R. evertsi evertsi, Hyalomma marginatum rufipes* and *Dermacentor* species (Rikihisa, 1991, De Waal, 2000, Futse *et al*., 2003, Kocan *et al*., 2003). Whereas *A. centrale* is seemingly only transmitted by *R. simus; Haemaphysalis longicornis* is suggested as a potential vector for this pathogen in Japan (Potgieter & Van Rensburg, 1987, Kawahara *et al*., 2006). *Anaplasma marginale* and *A. centrale* can also be mechanically transmitted by blood-contaminated fomites such as nose tongs, ear tags and tattooing instruments (Kocan *et al*., 2003), as well as through biting flies from the order Diptera (Ewing, 1981).

Anaplasma ovis infects erythrocytes of their vertebrate hosts (Friedhoff, 1997). This pathogen is transmitted by ixodid tick species and causes persistent infections (Palmer *et al*., 1998) in small ruminants. This parasite has been found in various regions, including Italy (Torina *et al*., 2010), Iraq (Renneker *et al*., 2013), China (Liu *et al*., 2012, Li *et al*., 2016), Iran (Razmi *et al*., 2006) and the USA (de la Fuente *et al*., 2007), among others (Renneker *et al*., 2013). *Anaplasma ovis* DNA was described in sheep and deer keds (Diptera: Hippoboscidae) which suggests that these keds could potentially be vectors for *A. ovis* (Hornok *et al*., 2011). Some ticks found to harbour *A. ovis* include *D. hunteri* in northern Mexico and southwestern United Sates (Stiller *et al*., 1999), *R. sanguineus* in eastern Turkey (Aktas *et al*., 2009) and *D. marginatus* in Hungary (Hornok *et al*., 2007).

Anaplasma phagocytophilum is an emerging zoonotic pathogen which has gained a lot of attention in recent years (Wei *et al*., 2016). This species has not yet been reported to infect humans in South Africa. It is an intracytoplasmic bacterium that infects peripheral blood cells

from bone marrow (neutrophils and/or granulocyte endosomes) (Dumler *et al*., 2001) causing various diseases in humans and animals in Asia, Europe, South America, USA and Africa (Battilani *et al*., 2017): human granulocytic anaplasmosis (HGA), tick-borne fever (TBF) in ruminants, equine granulocytic anaplasmosis (EGA) and canine granulocytic anaplasmosis (CGA) (Rar & Golovljova, 2011, García-Pérez *et al*., 2016).

Chen *et al.* (1994) were the first to report HGA in humans caused by *A. phagocytophilum*. Patients with HGA could be asymptomatic or symptomatic, with some cases being fatal. These patients commonly suffer from fever, headaches, myalgia, lethargy, chills, reduced platelets and elevated liver-function enzymes (Bakken & Dumler, 2008, Nicholson *et al*., 2010). *Anaplasma phagocytophilum* is presumed to be mainly transmitted by Ixodid ticks (Dumler *et al*., 2007) having been detected in *D. reticulatus* (Paulauskas *et al*., 2012)*, D. variabilis* (Holden *et al*., 2003)*, D. silvarum, D. nuttalli* (Wei *et al*., 2016)*, Amblyomma americanum* (Clark, 2012), *H. longicornis* and *H. concinna* (Tomanovic *et al*., 2013, Wei *et al*., 2016) ticks across various regions around the world. In South Africa, in the absence of the *Ixodes* tick species that transmit *A. phagocytophilum* in other parts of the world, *R. sanguineus*, *R. e. evertsi, R. decoloratus, H. elliptica,* and *A. hebraeum* should be considered to be possible vectors/reservoirs of the pathogen.

Anaplasma platys infects blood platelets of canines and causes canine cyclic thrombocytopenia and canine monocytic ehrlichiosis (Rymaszewska & Grenda, 2008, Bastos *et al*., 2015). This pathogen has been identified in countries across the world, including Spain (Aguirre *et al*., 2006), the USA (Harvey *et al*., 1978), Chile (Abarca *et al*., 2007) and Italy (de la Fuente *et al*., 2006). These infections are mainly asymptomatic but fatal infections can arise with symptoms of anorexia, fever and depression (Baker *et al*., 1987, Rar & Golovljova, 2011). *Anaplasma platys* was initially reported as only infecting canines, but was found recently to infect platelets of cats (Lima *et al*., 2010, Qurollo *et al*., 2014) and cattle (Dahmani *et al*., 2015, Said *et al*., 2017). Moreover, *A. platys* has been found to infect humans which means that this pathogen could potentially be zoonotic (Maggi *et al*., 2013, Arraga-Alvarado *et al*., 2014). This pathogen is transmitted by *R. sanguineus* (Rar & Golovljova, 2011) and has also been reported in *D. auratus* ticks in dogs from Thailand (Parola *et al*., 2003), *R. turanicus* from Israel (Harrus *et al*., 2011) and in *H. longicornis* and *I. persulcatus* ticks from Korea (Kim *et al*., 2006). Interestingly, DNA of *A. platys* was found in *Heterodoxus spiniger*, more commonly known as the dog chewing louse, but the ability of the chewing louse to transmit *A. platys* still needs to be confirmed (Brown *et al*., 2005).

2.2.2 The genus *Ehrlichia*

The genus designation *Ehrlichia* was created in 1945 to honour Paul Ehrlich (Moshkovski, 1945, Silverstein, 1998). Members of the genus *Ehrlichia* are Gram-negative, small pleomorphic to ellipsoidal organisms that are found in cytoplasmic vacuoles. *Ehrlichia* are present in granulocytes, lymphocytes, monocytes, macrophages, neutrophils, in tissues and peripheral blood of their hosts and are transmitted by tick vectors, in which they mainly replicate (Rikihisa, 1991, Dumler *et al*., 2001).

The most economically important *Ehrlichia* species in South Africa is *E. ruminantium* (formerly known as *Cowdria ruminantium*), while *E. canis* has continental importance in Africa. *Ehrlichia chaffeensis* and *E. ewingii* are important in other parts of the world, due to the generally distinct geographical distributions of *Ehrlichia* species and their vectors. Furthermore, *Ehrlichia* species infect canines, horses, humans, rodents and ruminants (Rikihisa, 1991, Dumler *et al*., 2001).

Ehrlichia ruminantium mainly infects cattle, goats and sheep and is transmitted by *Amblyomma* tick species (more specifically *A. hebraeum* and *A. variegatum*) (Dumler *et al*., 2001, Peter *et al*., 2002). This species is important as it causes a sub-Sahara African endemic disease known as heartwater, which has a case fatality rate of 80% in sheep and cattle (Rikihisa, 1991, Rar & Golovljova, 2011, Mdladla *et al*., 2016). The first report of heartwater was in 1838, and it is mostly found in the hot, dry region of the Eastern Cape, Limpopo and KwaZulu-Natal provinces (Rikihisa, 1991, Mdladla *et al*., 2016). It is suggested that goats from non-endemic regions are more susceptible to heartwater than endemic goats (Mdladla *et al*., 2016). Peter *et al*. (2002) suggested that *E. ruminantium* and their tick vectors have co-evolved, where *E. ruminantium* has utilised the *Amblyomma* tick vector for their wide host ranges to increase their transmission possibilities.

According to Rikihisa (1991), when symptoms of acute disease occur, this disease can be fatal within a week but the possibility of recovery from milder forms of the disease is good. They further suggest that symptoms of anorexia, depression and fever occur with infection of *Ehrlichia* species and that each species has its own site-specific tissue tropism, i.e. *Ehrlichia ruminantium* infects the endothelial cells of the brain (Peter *et al*., 2002) whereas *E. canis* infects the microvasculature cells of kidneys, lungs and meninges (Simpson, 1974).

Ehrlichia canis is a pathogen that infects dogs and is transmitted largely by the tick vector *R. sanguineus* (Dumler *et al*., 2001). This pathogen was also detected in *H. logicornis* and *I. turdus* ticks from Korea (Kim *et al*., 2006) and in *R. turanicus* from Israel (Harrus *et al*., 2011).

Although Rikihisa (1991) indicate that *E. canis* infection only occurs in canines, subsequent studies have confirmed presence in Sika deer from China (Li *et al*., 2016). This pathogen is the only *Ehrlichia* species known to have a worldwide distribution, while other species are geographically restricted (Rikihisa, 1991). *Ehrlichia canis* causes canine ehrlichiosis, as well as tropical canine pancytopenia in domestic and wild canines (Rikihisa, 1991). The first report of canine monocytic ehrlichiosis was from Algeria in 1935 (Rar & Golovljova, 2011). Symptoms of this fatal disease include anaemia, depression, dyspnoea, fever, leukopenia and myalgia, together with a decrease in blood platelets and white blood cells which can lead to bleeding disorders (Nicholson *et al*., 1999, Rar & Golovljova, 2011).

Ehrlichia species are known to cause disease in humans and are more commonly reported from the USA. These human infections are mainly caused by *E. chaffeensis* and *E. ewingii* (Buller *et al*., 1999, Dumler *et al*., 2007). However, Pritt *et al*. (2011) reported an ehrlichial pathogen in humans which is closely related to *E. muris*. According to Nicholson *et al.* (2010), humans infected with *E. chaffeensis* could develop a severe *Ehrlichia* disease where acute infections display symptoms of myalgia, lethargy, fever and headaches, together with decrease in blood platelets and sodium levels and likewise an increase in liver enzymes.

2.2.2.1 *Ehrlichia* life cycle

The life cycle of *Ehrlichia* has not been fully elucidated, but is similar to *Anaplasma* in that *Ehrlichia* species are transmitted by ixodid ticks and infection with *Ehrlichia* results in their vertebrate hosts becoming reservoirs due to the establishment of persistent infections (Rar & Golovljova, 2011). The life cycle of *E. ruminantium* is better known than other *Ehrlichia* species and can be used as an example to explain their developmental cycle.

Briefly, *Ehrlichia ruminantium* has a similar growth cycle to that of *Chlamydia* within the vertebrate host (Jongejan *et al*., 1991). The infectious and metabolically inactive form, known as elementary bodies, is present at the start of the infection of a host cell. These elementary bodies develop into reticulate bodies, which are metabolically active. These bodies will then multiply *via* binary fission and revert to elemental bodies which will then infect new hosts cells (Jongejan *et al*., 1991). These elemental bodies are released into the blood stream where they are taken up by ticks during a blood meal. They are likewise transmitted transstadially by their tick vectors.

2.2.3 The genera *Theileria* and *Babesia*

Theileria and *Babesia* are piroplasms that cause major impacts on the economy and on human health and are therefore of veterinary and zoonotic relevance (Fuehrer *et al*., 2013, Zanet *et al*., 2014). These piroplasms are tick-transmitted, intracellular parasites which cause piroplasmosis in various vertebrate animals (Duh *et al*., 2008, Zhang *et al*., 2016). These infections are pathogenic for domestic animals and livestock and result in great economic losses due to high morbidity and case fatality rates (Duh *et al*., 2008). Some *Theileria* species are less pathogenic and this could be due to the fact that the parasites and hosts have had a long evolutionary relationship (Zanet *et al*., 2014).

The probability of clinical symptoms occurring increases when animals are put under stressful conditions such as translocation for conservation and commercial purposes (Hofle *et al*., 2004, Nijhof *et al*., 2005, Zanet *et al*., 2014). Immunity to disease caused by these piroplasms is possible with persistent tick exposure. This is common in young animals that are infected and which then develop immunity as they mature (Garcia-Sanmartin *et al*., 2007). Unfortunately, this means that the parasites can establish a persistent infection within the infected animal, causing them to become reservoir hosts (Garcia-Sanmartin *et al*., 2007).

One apparent difference between these two pathogens is that *Theileria* initially infect the leukocytes and later erythrocytes of their vertebrate hosts, while *Babesia* only infects erythrocytes (Duh *et al*., 2008). *Babesia* species, such as *B. microti*, can cause zoonotic infections such as human babesiosis, whereas *Theileria* is not known to cause any zoonotic infections (Yabsley and Shock, 2013).

It is very difficult to distinguish between these two piroplasms within the erythrocytes due to morphological similarities, however paired merozoites are only present in *Babesia* species (Garner *et al*., 2012), which could be used to distinguish between these two piroplasms in blood smears.

2.2.3.1 Economically important *Theileria* species

Theileria is a tick-borne haemoprotozoan parasite that is classified under the phylum Apicomplexa, order Piroplasmida (Rar *et al.,* 2014) and family Theileriidae (Kundave *et al*., 2015).

It causes theileriosis in small ruminants and cattle, which is a major problem in South Africa (Slodki *et al*., 2011). Theileriosis can be chronic or acute with some species being highly

pathogenic whereas other species are benign (Slodki *et al*., 2011, Fuehrer *et al*., 2013). Mild and asymptomatic theileriosis in cattle and bovine benign theileriosis is caused by *Theileria buffeli, T. orientalis* and *T. sergenti* within the Asia-Pacific region (Jenkins & Bogema, 2016, Maharana *et al*., 2016). Other *Theileria* species that are mildly pathogenic are *T. mutans, T. taurotragi* and *T. velifera* (Lorusso *et al*., 2016). These *Theileria* species are considered to have less economic importance in South Africa. Species that are of importance are *Theileria annulata* and *T. parva* as they cause severe disease in bovines called bovine theileriosis (Habibi, 2016) and *Theileria equi* (together with *Babesia caballi*) which causes equine piroplasmosis. *Theileria annulata* is an important pathogen of cattle as it causes bovine tropical theileriosis, which results in high mortality and morbidity rates. It is, however, not present in South Africa (Sibeko, 2010). Bovine theileriosis is economically important because it severely impacts the cattle farming industry due to high morbidity rates and major losses in productivity (Habibi, 2016), while equine piroplasmosis affects the health of horses, with major impact on the equine industry (Kappmeyer *et al*., 2012).

Theileria parva is economically important as it causes a range of fatal diseases, including Corridor disease (caused by buffalo-derived *T. parva*), East Coast fever and January disease (both caused by cattle-derived *T. parva*) and is transmitted by the tick vector *R. appendiculatus* (Koch *et al*., 1993, Mbizeni *et al*., 2013, Patel *et al*., 2016). In South Africa, theileriosis caused by *T. parva* infection exists as Corridor disease, and it is a controlled disease.

2.2.3.2 *Theileria* life cycle

Theileria species have a complex life cycle in which their intermediate hosts are mammals and their ultimate hosts are hard ticks (Slodki *et al*., 2011). *Theileria parva* will be used to represent the life cycle of *Theileria* species (Figure 2.4). The main tick vector for this species is *R. appendiculatus* which is a three-host tick and displays transstadial transmission within these tick vectors (Bishop *et al*., 2004).

Sporozoites are formed within the tick salivary gland where they are released into a vertebrate host during a blood meal (Bishop *et al*., 2004). These sporozoites then invade the lymphocytes of their buffalo or cattle host and disrupt the cells' defence mechanism by dissoluting the cell membrane of the host (Bishop *et al*., 2004). The sporozoites develop into schizonts in which many merozoites are formed. These are released into the blood stream of the host, invade erythrocytes and develop into piroplasms. Infected erythrocytes are ingested by engorging ticks. In the tick gut, the piroplasms are released due to cell lysis (Bishop *et al*., 2004). Piroplasms are transformed into gametes which in turn fuse to form zygotes. These zygotes invade the gut epithelium of the tick forming kinetes that invade the salivary glands.

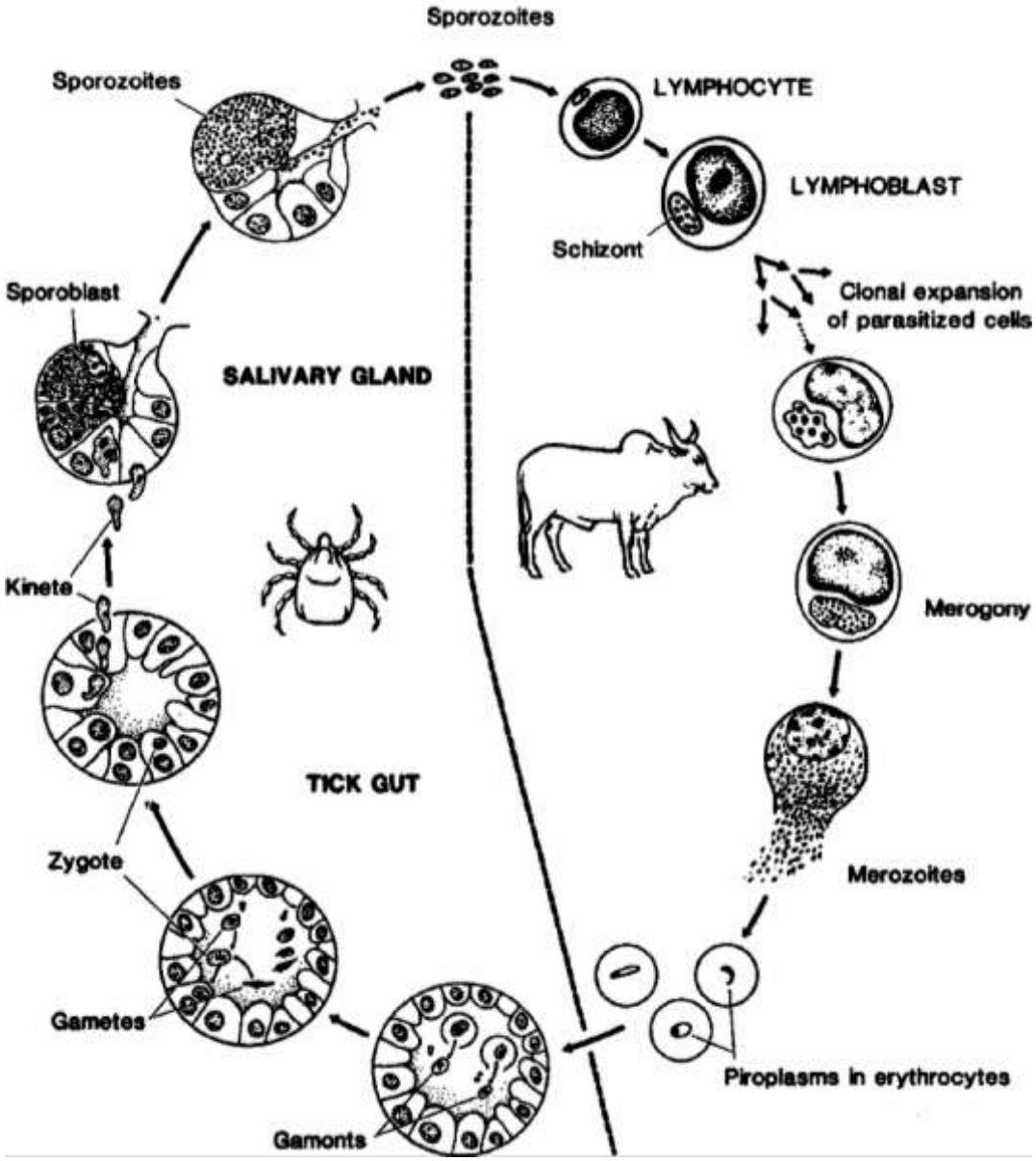

Figure 2.4: Representation of the life cycle of *Theileria parva* in cattle and ticks. This is a basic representation of the life cycle of *Theileria* species. (Taken from Bishop *et al.* (2004) with permission from Cambridge University Press).

2.2.3.3 Economically important *Babesia* species

Similar to *Theileria, Babesia* is a tick-borne haemoprotozoan parasite that infects the erythrocytes of its vertebrate host (Baviskar *et al.,* 2007, Bashir *et al.,* 2014). This genus belongs to the family Babesiidae, the order Piroplasmida (Maharana *et al*., 2016) and the phylum Apicomplexa (Rar *et al.,* 2014). *Babesia* species occur worldwide and more than 100 species of *Babesia* have been described to date (Slodki *et al*., 2011). This pathogen causes the zoonotic disease known as babesiosis (Kjemtrup & Conrad, 2000, Maharana *et al*., 2016).

Some of these diverse species are: *B. rossi, B. vogeli, B. canis, B. felis* (which infect cats and dogs); *B. microti* (which infects rodents); *B. bovis, B. bigemina, B. divergens, B. ovis* and *B. equi* which infect horses and cattle (Bajer *et al*., 2014). *Babesia*-derived zoonotic diseases (that infects humans) are caused by *B. divergens, B. venatorum* and *B. microti* (Lack *et al*., 2012, Wei *et al*., 2016).

Bovine babesiosis, also known as redwater, is an economically important disease affecting cattle and buffalo globally, which is caused by *B. bovis, B. bigemina*, *B. divergens* and *B. major* (Uilenberg, 1995, Potgieter & Stoltsz, 2004, Omar *et al*., 2016), with *B. bovis* and *B. bigemina* being the most economically important species (Bock *et al*., 2008). This disease largely occurs within tropical and subtropical regions, around the world, where their tick vectors (*Rhipicephalus* ticks) are present (Gubbels *et al*., 1999, Brown *et al*., 2006).

Babesia bovis is considered to be highly pathogenic in cattle, whereas *B. bigemina* is moderately pathogenic (Uilenberg, 1995) and after recovering from an infection with *B. bigemina*, cattle develop life-long immunity (Lorusso *et al*., 2016). *Rhipicephalus decoloratus* only transmits *B. bigemina* while *R. annulatus* and *R. microplus* transmit both *B. bovis* and *B. bigemina* (Molad *et al.,* 2015).

2.2.3.4 *Babesia* life cycle

The life cycles of *Theileria* and *Babesia* are very similar. But unlike *Theileria*, the sporozoites from *Babesia* species directly penetrate the erythrocytes of the vertebrate host, which is where their whole developmental cycle occurs (Figure 2.5) (Chauvin *et al*., 2009). When these erythrocytes are ingested during a blood meal, ray bodies are formed. They then transform into zygotes within the tick's digestive tract. The zygote penetrates a cell and transforms into an ookinete which then moves from the midgut epithelium to the tick's tissues (Chauvin *et al*., 2009). *Babesia* is then transovarially and transstadially transmitted within the ticks.

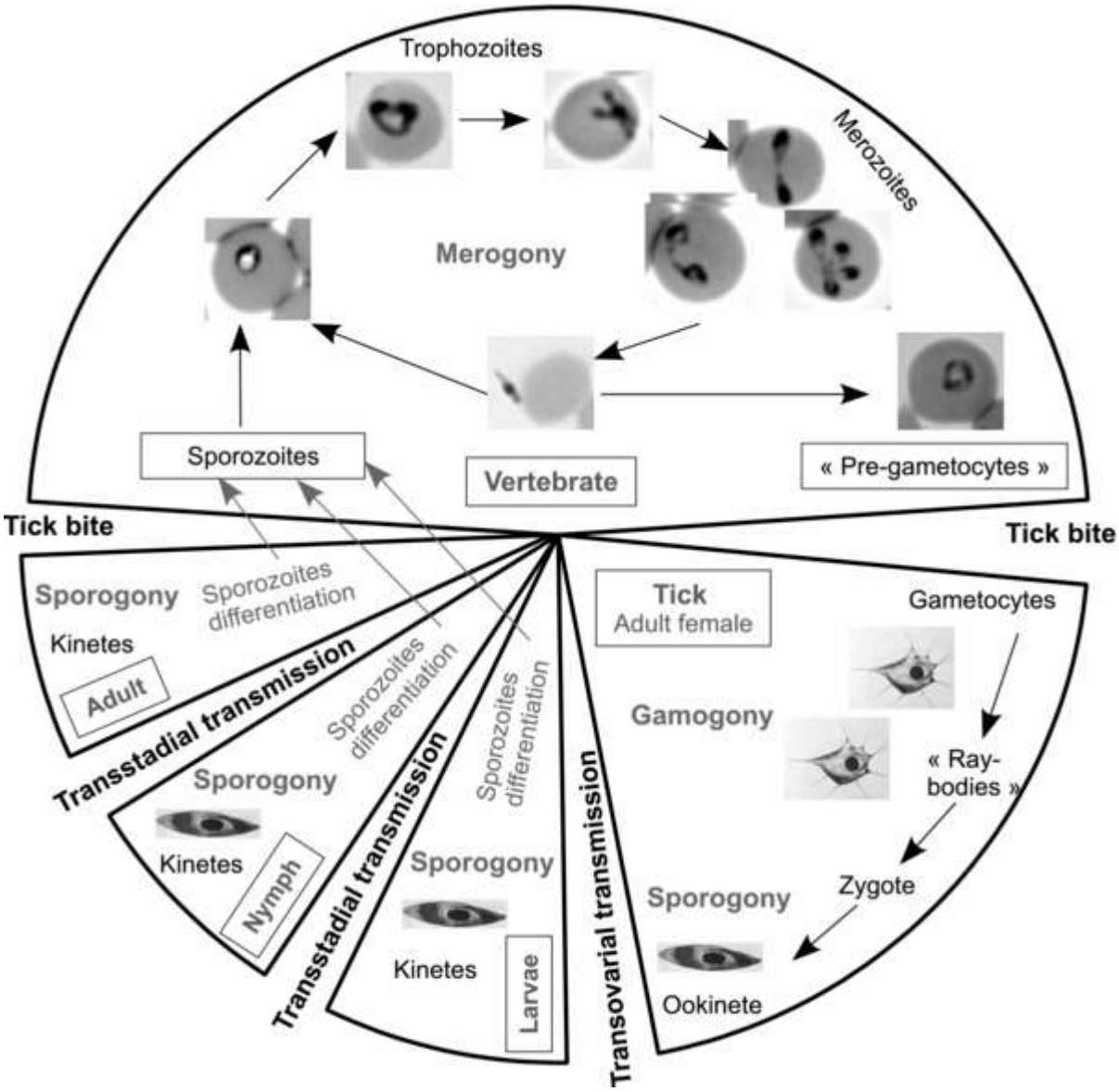

Figure 2.5: Representation of the life cycle of *Babesia* species. (Taken from Chauvin *et al.* (2009) with permission from BioMed Central under the terms of the Creative Commons Attribution-Noncommercial License).

2.3 Laboratory diagnosis

Diagnosis of *Anaplasma, Ehrlichia, Theileria* and *Babesia* includes a wide range of methods, each having their own advantages and disadvantages. It is important to consider the characteristics of the infectious agent and the persistence of the infection to ensure that the best method for diagnosis is chosen (Nicholson *et al*., 2010). Cell culture isolation, direct microscopic examination of stained blood smears, impression smears, or tissue sections, serology and PCR assays are the most effective methods used for the diagnosis of many tick-borne diseases (Nicholson *et al*., 2010, Maharana *et al*., 2016).

2.3.1 Conventional parasitological techniques

Conventional microscopic examination provides the basis for diagnosing *Anaplasma, Ehrlichia, Theileria* and *Babesia* as it is fast and cheap. However, this technique lacks specificity and sensitivity and a trained eye is needed when distinguishing these pathogens (Salih *et al*., 2015, Maharana *et al*., 2016). Furthermore, although this technique does not require expensive equipment that has to be maintained, it is labour intensive (Salih *et al*., 2015).

Anaplasma, as well as *Ehrlichia and Babesia*, can be identified by observing organisms within the erythrocyte and monocytes in Giemsa- or Wright-stained blood smears (Potgieter & Stoltsz, 2004). However, *Anaplasma* infection is usually only diagnosed through microscopy when the clinical signs are most pronounced, which poses a problem as most of the infected erythrocytes have been removed from the circulation by the time that samples are taken for diagnostic purposes (De Waal, 2000, Potgieter & Stoltsz, 2004). *Anaplasma bovis* was previously examined in Leishman's stained blood smears as intracytoplasmic inclusion bodies within the lymphocytes of an infected cow (Chirayath *et al.,* 2012), but as monocytes only account for approximately 1% of all leukocytes, *A. bovis* infected cells are rarely seen in blood smears (Goethert & Telford lll, 2003). Lastly, microscopic diagnosis of *Theileria* species is achieved through confirmation of the presence of schizonts in lymph nodes of biopsy smears (Potgieter & Stoltsz, 2004, Slodki *et al*., 2011, Kundave *et al*., 2015).

2.3.2 Serological techniques

Various indirect diagnostic tests exist that are used for the detection of antibodies and antigens of *Anaplasma, Ehrlichia, Theileria* and *Babesia* (Salih *et al*., 2015). No specific serological diagnosis tool has been reported for *A. bovis* but serological techniques can be used to diagnose other *Anaplasma* species. When an animal is infected with *Anaplasma*, it could be difficult to detect within blood smears (for the reasons mentioned above) and sometimes end-PCR might not be able to detect the presence of these pathogens within the blood of asymptomatic cattle (OIE, 2015). Therefore, various serological diagnostic tools have been developed in order to identify and detect infected cattle but some of these tests have variable sensitivity and specificity, raising questions regarding the reliability of these serological tests (OIE, 2015).

Some of the most important serological methods include enzyme-linked immunosorbent assay (ELISA), competitive enzyme-linked immunosorbent assay (C-ELISA), card agglutination test (CAT), indirect fluorescent antibody technique (IFAT) and indirect ELISA (I-ELISA) (Salih *et*

al., 2015, Maharana *et al*., 2016). The indirect fluorescent antibody test (IFAT) uses whole, cultured organisms as antigens. The competitive enzyme-linked immunosorbent assay uses the recombinant Major Surface Protein 5 (MSP5) as an antigen (Strik *et al*., 2007). The card agglutination test uses a suspension of pathogen particles which is retrieved by washing and lysing erythrocytes and then these pellets are sonicated, washed and resuspended in stain solution (OIE, 2015). ELISA techniques use the same antigen as for CAT (OIE, 2015). Furthermore, infected blood is used to produce antigen smears used for IFAT. However, this test is dependent on specific parasite antigens which are not identified yet. Another problem is the possibility of piroplasm presence after antibodies have disappeared, meaning that the serology test could be negative while the patient could still transmit the infection (Maharana *et al*., 2016). On the other hand, antibodies could also remain within the patient for many years after recovery which means that the patient could still be seropositive even though the parasite is no longer present (Salih *et al*., 2015, Maharana *et al*., 2016).

2.3.3 Molecular techniques

Diagnoses which are based upon nucleic acid, in particular DNA-based diagnostic techniques, are widely and more commonly used for the identification and characterization of *Anaplasma, Ehrlichia, Theileria* and *Babesia* (Salih *et al*., 2015). Achieving DNA amplification and obtaining DNA sequences is used throughout the world by diagnostic laboratories and is known as the "golden standard" of molecular diagnosis (Sobel & Akins, 2015). There are molecular diagnostic techniques that can be used, including: nested PCR, real-time PCR, PCR-ELISA, Reverse Line Blot (RLB) hybridization assay, microarrays etc. (Salih *et al*., 2015).

Polymerase chain reaction is a sensitive molecular technique used for the detection of parasites as it is capable of detecting parasitaemia as low as a few parasites per millilitre (Maharana *et al*., 2016). This method holds more advantages than microscopy, one being that PCR is more uniform and objective, which makes this method less error-prone (Meurs *et al*., 2015). It is one of the most commonly used methods for diagnosis of haemoparasites. With nested PCR, as few as 30 infected erythrocytes can be identified within a millilitre of blood (Torioni de Echaide *et al*., 1998). It is suggested that real-time PCR be used instead of nested PCR (OIE, 2015) but currently, there is no real-time PCR probe available for *A. bovis*. Real-time PCR gives a semi-quantitative assay result as well as reducing the possibility of contamination of the amplicons (OIE, 2015). Although real-time PCR is preferred over other PCR methods, this technique is expensive and requires preventative maintenance to prevent the equipment from failing (OIE, 2015).

The Reverse line blot (RLB) hybridization assay can simultaneously detect different parasites even if their parasitaemia levels are extremely low. Specific oligonucleotide probes are linked to a membrane and hybridized with PCR products (Gubbels *et al*., 1999). This enables different samples to be analysed against different probes for simultaneous detection (Gubbels *et al*., 1999). RLB presents many advantages, one being that only one PCR reaction is needed for the simultaneous detection of several haemoparasite species (Bekker *et al*., 2002). This method can also be used to detect any variant genes of specific species as well as to detect undescribed haemoparasite species (Bekker *et al*., 2002).

Gene markers such as the 16S rRNA, the heat shock protein (*GroEL*), the housekeeping enzyme citrate synthase (*gltA*) and different major surface proteins (MSP) have been used to detect various *Anaplasma* species (Sisson *et al*., 2017) but only a few studies have been done on the amplification of different gene regions of *A. bovis*. Presently, 16S rRNA is the most commonly amplified gene region of most haemoparasites but this gene region is highly conserved and incapable of differentiating between closely related species (Harrison *et al*., 2013). The *GroEL* and *gtlA* gene regions, which are more variable are capable of higher levels of discrimination between closely related species (Lee *et al*., 2003).

2.4 Treatment

Treatment of *Anaplasma, Ehrlichia, Theileria* and *Babesia* is very broad. *Anaplasma* infections in cattle can be treated with amicarbalide and imidocarb, among other carbanilides in various countries (Potgieter & Stoltsz, 2004). Whereas oxytetracycline and doxycycline can be used to treat human ehrlichiosis (Rikihisa, 1991) and tetracycline can be used to treat *Ehrlichia* infections, *E. chaffeensis* could also be treated with rifampin (Parola & Raoult, 2001a). *Theileria* infections can be treated with theilericidal drugs like parvaquone and buparvaquone (Gharbi & Darghouth, 2015) and infection with *B. bovis* and *B. bigemina* can be treated with diminazene aceturate and imidocarb dipropionate (Bock *et al*., 2008). According to Atif (2016) no controlled studies for the treatment of *A. bovis* exist. However, one case study in which oxytetracycline was administered to an infected cow, with a supportive treatment of Livobex, resulted in an improvement of clinical symptoms after five days (Chirayath *et al.,* 2012).

Doxycycline's pharmacokinetic properties make this the most preferred agent for treatment of various tick-borne diseases in humans and animals (Nicholson *et al*., 2010, Bakken & Dumler, 2015). To prevent fatal disease, patients with suspected or documented HGA should be treated with doxycycline hyclate orally or intravenously (Bakken & Dumler, 2015). Patients who have been treated with this drug usually show clinical improvement within 48 hours,

therefore, if patients show no improvement after 48 hours, they should be re-examined for an alternative diagnosis (Bakken & Dumler, 2015). Bakken and Dumler (2015) further suggest that patients who have a history of drug allergy, pregnant patients or children under 8 years of age, should be treated with rifampin.

2.5 Prevention and control

Vectors and vertebrate species that are involved in *Anaplasma* and *Ehrlichia* maintenance may differ according to their specific region (Bastos *et al*., 2015). Some molecular studies show that bacterial variants exist that are distinct to specific geographical regions (Potgieter & Stoltsz, 2004) and evidence has been found of locality-specific ticks and vertebrate reservoir hosts, as in the study of Harrison *et al.* (2013) and Kawahara *et al.* (2006). Thus it is important to identify the mammalian host/reservoir of the infection, together with the enzootic ticks that are involved in the natural life cycle of the diseases when evaluating the prevalence and the diversity of the regional bacterial species so as to control the disease as well as to limit pathogen transmission to other hosts within a specific region (Bastos *et al*., 2015). Furthermore, to ensure the effective control of tick-borne diseases, knowledge on how different vector stages amplify and transmit these pathogens is essential (Ueti *et al*., 2008).

Nicholson *et al.* (2010) suggest that the main course of prevention for anaplasmosis and ehrlichiosis, is to educate the public about ticks, tick-bite prevention and tick control. However, these messages should include education about free-roaming pets and the environment to ensure that public education is successful (Nicholson *et al*., 2010). The public should avoid tick bites but if ticks cannot be avoided they should be removed as soon as possible because when ticks are allowed to feed for longer periods, infection is more likely to occur (Bakken & Dumler, 2015). By wearing protective clothing such as long-legged pants, long-sleeved shirts, closed shoes and socks wrapped outside of the pants it will be harder for ticks to reach bare skin when individuals enter tick habitats (Bakken & Dumler, 2015). Tick repellents (DEET and picaridin) can be used by individuals who will be exposed to tick habitats but caution should be given as some repellents should not be applied to the skin but rather be applied to clothes (Bakken & Dumler, 2015). Vector tick populations can furthermore be controlled through spraying barns and kennels and by dipping animals (Rikihisa, 1991). According to De Waal (2000), dairy farmers prefer dipping their animals whereas beef farmers prefer using a pour on application.

Arthropods can be controlled by prophylactic treatment of antibiotics, acaricides and vaccinations (Kocan *et al*., 2010). But rigorous treatment with antibiotics increases the risk of

the organisms' ability to become resistant to treatment (Kocan *et al*., 2010). Tetracycline is a popular antibiotic used to treat infections. This antibiotic is a bacteriostatic agent with various antimicrobial activities (Scholar and Pratt, 2000). According to Scholar and Pratt (2000), types of tetracycline are synthesised differently: minocycline is produced through chemical alteration of tetracycline, doxycycline is produced semi-synthetically from oxytetracycline whereas tetracycline, demeclocycline and oxytetracycline are naturally developed.

In the case of *A. marginale*, control of arthropods is not 100% successful due to this parasites' ability to be transmitted mechanically. Kocan *et al.* (2010) advocate vaccination as an economically effective method to control bovine anaplasmosis. However, vaccination has limitations. This is because new genetic strains evolve constantly which might not be controlled by established vaccines. Furthermore, the vaccine could potentially be incapable of protecting animals against field strain challenges (De Waal, 2000). De Waal (2000) suggests that this could be solved by exposing the vaccinated animal to field challenges right after they are vaccinated to ensure that the immunity is properly developed. Vaccines can also become ineffective when they are stored or handled incorrectly (De Waal, 2000). There are various types of vaccines available for most illnesses, but for anaplasmosis, live and killed vaccines are used which produce protective immunity towards *Anaplasma* (more specifically *A. marginale*) that inhibits clinical disease (Kocan *et al*., 2010).

When cattle are infected with *A. centrale* they develop a protective immunity towards *A. marginale* infection which is more pathogenic than *A. centrale*. Thus *A. centrale* is used as a live vaccine against *A. marginale* (Kocan *et al*., 2003, Kocan *et al*., 2010, Rar & Golovljova, 2011). Calves are less susceptible to *Anaplasma* infection and they react less severely than older cattle, thus it is suggested that young claves be vaccinated at about six months of age (Potgieter & Stoltsz, 2004).

Moreover, chemotherapy is often used in the United States and South Africa to prevent anaplasmosis transmitted by arthropods but this method is very expensive and is not applicable to range cattle (De Waal, 2000, Kocan *et al*., 2003, Kocan *et al*., 2010). Chemotherapy can be used for controlling reactions caused by live vaccines and to control primary infections (De Waal, 2000). Kocan *et al.* (2010) further suggest that new antimicrobials should be developed for the effective treatment or prevention of anaplasmosis and persistent infections in cattle.

According to Rikihisa (1999), the long-term use of tetracycline prophylaxis has successfully controlled epizootics of canine ehrlichiosis as well as heartwater, which is effective when tick

exposure cannot be prevented. Furthermore, there exists an infected-blood vaccine for heartwater (Rikihisa, 1991).

Control of East Coast Fever (ECF) could be done through an infection and treatment method (Nene *et al*., 2016). This consists of infecting the cattle with live *T. parva* sporozoites together with the associated drug treatment of oxytetracycline (Radley, 1981, Nene *et al*., 2016). Thus, this controls the parasite but does not kill it allowing for the cattle to develop the necessary levels of protective immunity toward future infection (Nene *et al*., 2016). Chemotherapy is also done to control ECF but this treatment method is very expensive and has low efficacy in late treatment cases (Mbwambo *et al*., 2006). Furthermore, theileriosis can be controlled through the application of acaricides (Habibi, 2016). But this control method is very expensive and is very hazardous towards the environment.

Controlling bovine babesiosis could be achieved by destroying infected erythrocytes through splenic macrophages and neutralizing antibodies which are directed at extracellular merozoites (Jaramillo Ortiz *et al*., 2016). Likewise, the use of live attenuated vaccines which consist of bovine erythrocytes infected with selected strains is increasingly being used in South Africa and other countries to control and prevent *B. bovis* and *B. bigemina* infections in cattle (Bock *et al*., 2008). Another, uncommon method for controlling these infections is to develop vaccines derived from *in vitro* cultures (Bock *et al*., 2008). Although there are various vaccines that decrease mortality rates, no vaccine fully prevents *Babesia* infections (Munkhjargal *et al*., 2016).

CHAPTER 3

Materials and methods

3.1 Study area and samples collected

A total of 160 rock sengi DNA extracts previously collected from two locations in South Africa, were made available for this project. During 2007-2008, 104 rock sengi were trapped and blood was collected at the Goro Game Reserve in the Limpopo Province, South Africa (22°58'S, 22°57'S; 29°25'E, 29°24'E) (Figure 3.1) (Medger *et al.,* 2012). These samples formed the basis of the Harrison *et al.* (2013) study. In 2015, a further eight samples were collected at the same locality and in 2016, a total of 48 rock sengi were trapped at the Ezemvelo Nature Reserve, Gauteng (25°42'30.2"S 28°55'46.9"E) and blood was collected on filter paper (Hatyoka 2018, Department of Entomology and Zoology, University of Pretoria).

Figure 3.1: Map of South Africa showing the sample collection sites. The red star indicates the location of the Goro Game Reserve and the blue star represents the location of the Ezemvelo Nature Reserve. Modified from Google Map Data ©2017 AfriGIS (Pty.) Ltd.

3.2. Reverse line blot (RLB) hybridization

3.2.1 Whole genome amplification

The illustra GenomiPhi™ V2 DNA Amplification Kit (GE Healthcare Life Science) was used to perform whole genome amplification on 104 (65%) of the samples for which DNA was limited (less than 20 µl of extracted DNA). The whole genome was amplified using isothermal strand displacement, where the DNA is denatured at 95°C for three minutes after adding 9 µl sample buffer which contains random hexamers that bind non-specifically to the 1 µl DNA template and then cooled. The illustra GenomiPhi™ V2 protocol suggests that the samples should be cooled to 4°C after denaturisation by placing them on ice. However, in this study, the samples were instantaneously frozen in liquid nitrogen, prior to being placed on ice, to ensure that the strands remain denatured. After this, a 10 µl master-mix was prepared from 9 µl reaction buffer and 1 µl enzyme mix which altogether contains buffers, nucleotides, salts, other hexamers, and DNA polymerase. The master-mix was added to the 10 µl denatured products, resulting in a final reaction volume of 20 µl. Samples were then incubated at 30°C for 2-4 hours, after which the enzyme was inactivated at 65°C for 10 minutes.

3.2.2 RLB-PCR

The V1 hypervariable region (492-498 bp fragment) of the 16S rRNA gene of *Anaplasma* and *Ehrlichia* was amplified with forward primer Ehr-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') (Schouls *et al*., 1999) and reverse primer Ehr-R (5'-biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3') (Bekker *et al*., 2002). The V4 hypervariable region (460-520 bp fragment) of the 18S rRNA of *Theileria* and *Babesia* was amplified with the forward primer RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and reverse primer, RLB-R2 (biotin-5'-CTA AGA ATT TCA CCT CTG ACA GT-3') (Nijhof *et al*., 2003).

The PCR reaction consisted of 12.5 µl Platinum® Quantitative PCR SuperMix-UDG (40 mM Tris-HCl (pH 8.4), 100 mM KCl, 6 mM $MgCl_2$, 400 µM dATP, 400 µM dCTP, 400 µM dGTP, 800 µM dUTP, 40 units/ml UDG, 60 units/ml Platinum® *Taq* DNA Polymerase and stabilizers) (ThermoFisher Scientific™), 5 µl of DNA template, 0.5 µM of each primer (forward and reverse) and distilled water was added for the total reaction volume of 25 µl. PCR master mix with no DNA template (negative control), and known *Anaplasma centrale* and *Babesia bovis* DNA samples (Onderstepoort Biological Products, South Africa) were included to monitor the occurrence of false positive or false negative results, respectively. Amplification was performed on the Gene Amp® PCR system 9700 (Applied Biosystems). The PCR program included a cycle of enzyme activation at 34°C for 3 minutes, an initial denaturation step at 94°C for 10 minutes, then 10 cycles of 20 seconds at 94°C, 30 seconds at 64°C and 30

seconds at 72°C. This was followed by 40 cycles of a denaturation step at 72°C for 30 seconds and an annealing step at 57°C for 30 seconds. The final extension was done at 72°C for 7 minutes, followed by a holding step at 4°C.

3.2.3 RLB hybridization

The PCR products were subjected to the RLB hybridization assay as described by Gubbels *et al.* (1999). A blotting membrane was activated by incubating it for 10 minutes in 10 ml of 16% 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide (EDAC) at room temperature. Washing of the membrane was done with distilled water for 2 minutes after which the membrane was placed in a MN45 miniblotter. *Anaplasma* and *Ehrlichia*, together with *Theileria* and *Babesia*-specific oligonucleotides probes, were selected for this study (Table 3.1). These specific probes were diluted to a 2 pmol/150 µl concentration by adding 500 mM $NaHCO_3$ (pH 8.4) which was then covalently linked to the membrane with an amino linker. The membrane was incubated for one minute at room temperature and afterwards the oligonucleotide solution was aspirated. Furthermore, the membrane was inactivated by incubation for 10 minutes in a 100 ml of 100 mM NaOH solution at room temperature. The membrane was washed under shaking in a 125 ml of 2x SSPE-0.1% sodium dodecyl sulfate solution for 5 minutes at 60°C in a shaker incubator.

Before using the membrane, it was washed under gentle shaking for 5 minutes at room temperature with a solution of ~50 ml of 2x SSPE-0.1% SDS and placed in a miniblotter with the slots positioned perpendicular to the specific oligonucleotide probes. The PCR products were diluted by adding 130 µl of 2x SSPE-0.5% SDS to 40 µl of PCR products and then denatured for 10 minutes at 99.9°C. The denatured PCR product was added to the slots and then incubated for 60 minutes at 42°C. The PCR products were aspirated and the blot was washed twice in a solution of 125 ml of 2x SSPE-0.5% SDS in an incubator under gentle shaking at 50°C for 10 minutes. The membrane was incubated in a solution of 10 ml of 1:4,000-diluted peroxidase-labelled streptavidin in 2x SSPE-0.5% SDS under gentle shaking for 30 minutes at 42°C. After this, the membrane was washed twice again in 125 ml solution of 2x SSPE-0.5% for 10 minutes at 42°C with gentle shaking, followed by another two washes in 125 ml of 2x SSPE for 5 minutes each at room temperature. The membrane was incubated for one minute at room temperature in 10 ml solution of ECL detection fluid after which the membrane was exposed to an ECL hyperfilm for a few seconds after which the film was developed and fixed.

The membrane was washed twice in 1% SDS for 30 minutes each at 80°C under gentle shaking for the PCR products to be stripped from the membrane. Lastly, the membrane was

washed in 20 mM EDTA (pH 8.0) for 15 minutes under gentle shaking at room temperature and stored in fresh EDTA solution at 4°C to be reused.

Table 3.1: Species-specific oligonucleotide probes used for RLB

Oligonucleotide probe	Sequence 5'-3'	Reference
Anaplasma/Erhlichia genus-specific	GGG GGA AAG ATT TAT CGC TA	Bekker *et al.* (2002)
Anaplasma bovis	GTA GCT TGC TAT GRG AAC A	Bekker *et al*. (2002)
Anaplasma bovis-like (sengi)	ATA GCT TGC TAC GAA AAC A	This study
Anaplasma centrale	TCG AAC GGA CCA TAC GC	Bekker *et al.* (2002)
Anaplasma marginale	GAC CGT ATA CGC AGC TTG	Bekker *et al.* (2002)
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG	Bekker *et al.* (2002)
Anaplasma sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC	Bekker *et al.* (2002)
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA	Schouls *et al.* (1999)
Ehrlichia chaffiensis	ACC TTT TGG TTA TAA ATA ATT GTT	Schouls *et al.* (1999)
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG	Bekker *et al.* (2002)
Theileria/Babesia genus-specific	TAA TGG TTA ATA GGA RCR GTT G	Gubbels *et al*. (1999)
Babesia 1 genus-specific	ATT AGA GTG TTT CAA GCA GAC	Nijhof (unpublished)
Babesia 2 genus-specific	ACT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
Babesia bicornis	TTG GTA AAT CGC CTT GGT	Nijhof *et al.* (2003)
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels *et al.* (1999)
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels *et al.* (1999)
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT	Butler *et al.* (2008)
Babesia canis	TGC GTT GAC GGT TTG AC	Matjila *et al.* (2004)
Babesia divergens	ACT RAT GTC GAG ATT GCA C	Nijhof *et al.* (2003)
Babesia felis	TTA TGC TTT TCC GAC TGG C	Bosman *et al*. (2007)
Babesia gibsoni	TAC TTG CCT TGT CTG GTT T	Yisaschar-Mekuzas *et al*. (2010)
Babesia leo	TTA TGC TTT TCC GAC TGG C	Bosman *et al*. (2007)
Babesia major	TCC GAC TTT GGT TGG TGT	Georges *et al*. (2001)
Babesia microti	GRC TTG GCA TCW TCT GGA	Nijhof *et al.* (2003)
Babesia rossi	CGG TTT GTT GCC TTT GTG	Matjila *et al.* (2004)
Babesia sp. (sable)	GCG TTG ACT TTG TGT CTT TAG C	Oosthuizen *et al*.(2008)
Babesia vogeli	AGC GTG TTC GAG TTT GCC	Matjila *et al*. (2004)
Theileria genus-specific	ATT AGA GTG CTC AAA GCA GGC	Nijhof (unpublished)
Theileria annae	CCG AAC GTA ATT TTA TTG ATT G	Yisaschar-Mekuzas *et al*. (2010)
Theileria annulata	CCT CTG GGG TCT GTG CA	Georges *et al*. (2001)
Theileria bicornis	GCG TTG TGG CTT TTT TCT G	Nijhof *et al.* (2003)
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT	Gubbels *et al.* (1999)
Theileria equi	TTC GTT GAC TGC GYT TGG	Butler *et al*. (2008)
Theileria lestoquardi	ATT GCT TGT GTC CCT CCG	Schnittger *et al.*(2004)
Theileria mutans	CTT GCG TCT CCG AAT GTT	Gubbels *et al.* (1999)
Theileria parva	GGA CGG AGT TCG CTT TG	Nijhof *et al.* (2003)
Theileria separata	GGT CGT GGT TTT CCT CGT	Schnittger *et al.*(2004)
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T	Oura *et al*. (2004)
Theileria sp.(kudu)	CTG CAT TGT TTC TTT CCT TTG	Nijhof *et al*. (2005)
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C	Nijhof *et al*. (2005)
Theileria taurotragi	TCT TGG CAC GTG GCT TTT	Gubbels *et al.* (1999)
Theileria velifera	CCT ATT CTC CTT TAC GAG T	Gubbels *et al.* (1999)

Symbols for degenerate positions: R=A/G and W=A/T.

3.3 RLB probe design

An *A. bovis*-like (sengi) RLB oligonucleotide probe was designed in the V1 hypervariable region of the 16S rRNA gene and synthesized containing an N terminal *N*-(trifluoracetamidohexylcyanoethyl, *N*,*N*-diisopropyl phosphoramidite)-C6 amino linker (Southern Cross Biotechologies, South Africa). The new probe, designated *A. bovis*-like (sengi) (5'-ATA GCT TGC TAC GAA AAC A-3') was incorporated into the RLB assay and used to screen a subset (n=108) of the original rock sengi blood sample extracts.

3.4 Genetic characterisation of *Anaplasma*-positive samples

3.4.1 PCR amplification of the 16S rRNA and *GroEL* genes of *Anaplasma* species

Samples that hybridized to the *A. bovis*-like (sengi) probe during the RLB hybridization assay (n=17), together with two samples that tested negative, were selected for the amplification of the 16S rRNA and *GroEL* genes. Conventional PCR was carried out using DreamTaq polymerase (Inqaba Biotec™). The final PCR reaction mixture contained 1X DreamTaq buffer, 0.2 mM dNTP's, 0.4 µM of each primer (Table 3.2), 1.5 U of DreamTaq polymerase. All PCR reactions contained 3 µl of template DNA and were performed in a final volume of 50 µl. Positive controls (previously identified to be positive for the *A. bovis*-like (sengi) by Harrison *et al*. (2013) and Bastos *et al*. (unpublished) through PCR amplification) and negative controls (no added DNA to mixture) were added to every PCR screening process. Touchdown PCR was performed in which an initial denaturation step at 96°C for 12 seconds, was followed by three stages consisting of a denaturation step at 96°C for 10 seconds, primer annealing at various temperatures and cycles (Table 3.3), elongation at 70°C for up to one minute and afterwards a final elongation step at 70°C for one minute. The PCR products were run on a 1.5% agarose gel, against a 100 bp molecular weight marker, to estimate the product sizes of the positive samples.

As amplification of the 16S rRNA and *GroEL* gene regions was poor with primer pairs taken from previous articles (Parola *et al*., 2000, Frank *et al*., 2008, Ybañez *et al*., 2012, Bastos *et al*., 2015), *A. bovis*-specific primers were designed based on available homologous congener data. For the 16S rRNA gene, this included the *A. bovis*-like sequences detected previously in rock sengi (Harrison *et al*., 2013), whereas for *GroEL* it was based on sequence spans, conserved across a 571 base pair (bp) region of two rock sengi *Anaplasma* sequences (Bastos, unpublished) for which data were generated from AnaGro712R (Ybañez *et al*., 2012) and EhrCanF3 (Bastos *et al*., 2015) primer amplicons, and closely related *Anaplasma*

sequences identified through BLASTn searches against the Genbank database (www.ncbi.nlm.nih.gov/blast).

Table 3.2: Forward and reverse primers for amplification of different gene regions

Primer pairs	Sequence (5'-3')	Expected amplicon size	Reference
16S rRNA			
Abov-16SF	CGG CAA GCT TAA CAC ATG CAA	~1,4 kb	This study
Abov-16SR	GCT ACC TTG TTA CGA CTT CAC		This study
Ehr-f (16S8FE)	GGA ATT CAG AGT TGG ATC MTG GYT CAG	~1,4 kb	Bergmans *et al*. (1995)
Abov-16SR	GCT ACC TTG TTA CGA CTT CAC		
EHR16SD	GGT ACC YAC AGA AGA AGT CC	~1,0 kb	Parola *et al*. (2000)
1492R	TAC CTT GTT ACG ACTT		Frank *et al.* (2008)
GroEL			
Abov-GroEF	GTG AGA AGT TGG ATA AAG CCA T	~495 bp	This study
Abov-GroER	ATC ACT CCG TCT CTT CCA ACT T		This study
EhrlCanF3	GAC ATG GCA AAT GTA GTT GTA AC	~595 bp	Bastos *et al*. (2015)
AnaGroEL712R	CCG CGA TCA AAC TGC ATA CC		Ybañez *et al.* (2012)

3.4.2 Purification of PCR amplicons

The PCR amplicons of the correct size were purified before further analysis by using the Roche High PCR Product Purification Kit by following the manufacturer's instructions. Briefly this entailed adding 50 µl double distilled water to the PCR products to make up 100 µl of solution before adding 500 µl Binding buffer and mixing the solution well, and loading the mixture onto a column. After centrifugation, and binding of the PCR amplicons to the column, the sample was purified by successive Wash steps, initially with 500 µl of Wash buffer and then with 200 µl. Elution of the PCR amplicons was achieved by adding 50 µl of a 2:1 ddH_2O: elution buffer, incubation at room temperature for 10 minutes, and a final centrifugation step.

Table 3.3: Primer annealing temperatures at different cycles with their elongation times.

Stage	Primer annealing and amount of cycles	Elongation Time
16S rRNA (Abov-16SF + Abov-16SR)		
1	57°C for 2 cycles	1 Minute; 10 sec
2	56°C for 3 cycles	1 Minute; 5 sec
3	55°C for 35 cycles	1 Minute
16S rRNA (Ehr-f + Abov-16SR)		
1	57°C for 2 cycles	1 Minute; 10 sec
2	56°C for 3 cycles	1 Minute; 5 sec
3	55°C for 35 cycles	1 Minute
16S rRNA (EHR-16SD + 1492R)		
1	58°C for 2 cycles	1 Minute; 20 sec
2	57°C for 3 cycles	1 Minute; 15 sec
3	56°C for 35 cycles	1 Minute; 10 sec
***GroEL* (Abov-GroEF + Abov-GroER)**		
1	56°C for 2 cycles	35 sec
2	55°C for 3 cycles	33 sec
3	54°C for 35 cycles	30 sec
***GroEL* (EhrlCanF3 + AnaGroEL712R)**		
1	53°C for 2 cycles	55 sec
2	52°C for 3 cycles	50 sec
3	51°C for 35 cycles	45 sec

3.4.3 DNA sequencing

Sequencing was performed using the BigDye Terminator Cycler Sequencing Kit (Applied Biosystems) following the manufacturer's instructions and then run on an ABI 3130 automated sequencer (Applied Biosystems) at the DNA Sequencing Facility situated within the Forestry and Agricultural Biotechnology Institute (FABI) on the Hatfield Campus of the University of Pretoria. Forward and reverse primers (3.2 pmol/µl) used for Sanger sequencing are included in Table 3.2.

3.4.4 Phylogenetic analysis

The sequences were assembled and edited using the MEGA7 software program (Tamura *et al.,* 2013). Homologous sequence searches of databases were performed using the BLASTn package (Altschul *et al*., 1990). A multiple sequence alignment was performed using ClustalW in the MEGA7 software program and included all related available homologous sequences from GenBank (Table 3.4). End-unaligned base pairs were trimmed, prior to analysis. This resulted in an aligned final dataset that was 675 nt in length for the 16S rRNA gene and a

dataset of 372 nt for *GroEL*. The most appropriate model of sequence evolution was identified by the Smart Model Selection (SMS) program (Lefort *et al.,* 2017).

For the 16S rRNA dataset, all positions containing gaps and missing data were removed in a pairwise manner when inferring the NJ tree. The MEGA7 software program was used to construct phylogenetic trees using the Neighbour-joining (NJ) and Maximum likelihood (ML). Bayesian inference (BI) was performed using the MrBayes software program (Ronquist *et al*., 2011). Nodal support for the NJ and ML analyses was determined through 5000 bootstrap replicates, using the most appropriate model selected in SMS. Bayesian inference in MrBayes was performed over 10 million generations, with sampling every 1000 generations. For each of the two independent runs, four chains, one cold and three with the default heated chain settings, were run. The first 10% of each run was discarded as burn-in. The selection of priors was guided by the best evolutionary model identified in SMS and from the results of the ML likelihood analysis.

Table 3.4: The 16S rRNA and *GroEL* gene sequences used in the phylogenetic analyses.

Accession Number	Taxonomic Classification	Location	Reference
16S rRNA			
KY007145	*Anaplasma* sp.	China	Zhuang *et al.* (2016)
GU937020	*Anaplasma bovis*	Japan	Sashika *et al.* (2011)
JX092094	*Anaplasma bovis*	Russia	Rar *et al*. (2012)
U03775	*Anaplasma bovis*	South Africa	Visser and Allsopp (1993)
KC811530	*Anaplasma bovis*	South Africa	Harrison *et al*. (2013)
CP000235	*Anaplasma phagocytophilum*	USA	Dunning *et al*. (2006)
CP006617	*Anaplasma phagocytophilum* str JM	USA	Barbet (2013)
NR 118489	*Anaplasma odocoilei*	Greece	Tate *et al.* (2013)
KF843825	*Candidatus* Anaplasma camelii	Saudi Arabia	Bastos *et al*. (2015)
AY077619	*Anaplasma platys*	Japan	Inokuma *et al.* (2002)
JQ396431	*Anaplasma platys*	Germany/Croatia (Dog)	Dyachenko *et al.* (2012)
KU686784	*Anaplasma centrale*	Uganda	Byaruhanga *et al*. (2016)
CP001759	*Anaplasma centrale* str. Israel	Israel	Herndon *et al.* (2010)
KJ639879	*Anaplasmaovis*	China	Li *et al*. (2014)
AF414870	*Anaplasma ovis*	South Africa	Lew *et al.* (2003)
KU686794	*Anaplasma marginale*	Uganda	Byaruhanga *et al.* (2016)
CP00030	*Anaplasma marginale* str. St. Maries	USA	Brayton *et al*. (2004)
CP001079	*Anaplasma marginale* str. Florida	USA	Visser *et al.* (1992)
AF414876	*Anaplasma marginale*	Israel	Lew *et al.* (2003)
CR925677	*Ehrlichia ruminantium*	France	Frutos *et al*. (2006)
CR767821	*Ehrlichia ruminantium* str Welgevonden	South Africa	Collins *et al*. (2005)
NR044747	*Ehrlichia ewingii*	USA	Anderson *et al.* (1992)
AB074459	*Candidatus* Ehrlichia shimanensis	USA	Kawahara *et al*. (2001)
CP007474	*Ehrlichia* sp. HF,	Japan	Lin *et al*. (2014)
CP006917	*Ehrlichia muris*	Japan	Thirumalapura *et al*. (2014)
CP000236	*Ehrlichia chaffeensis* str. Arkansas	USA	Hotopp *et al.* (2006)
KF843826	*Candidatus* Ehrlichia regneryi	Saudi Arabia	Bastos *et al.* (2015)
CP000107	*Ehrlichia canis* str. Jake	USA	Palenik *et al*. (2005) (direct submission)
GU810149	*Ehrlichia canis*	Taiwan	Huang *et al.* (2010)
JX629805	*Ehrlichia minasensis*	Brazil	Cruz *et al.* (2012)
AM999887	*Wolbachia* sp.	Sri Lanka	Klasson *et al*. (2008)

Accession Number	Taxonomic Classification	Location	Reference
GroEL			
KY523001	*Anaplasma centrale*	Uganda	Byaruhanga *et al*. (2017)
KY523038	*Anaplasma marginale*	Uganda	Byaruhanga *et al*. (2017)
AF414861	*Anaplasma marginale*	Israel	Lew *et al.* (2003)
CP00030	*Anaplasma marginale* str. St. Maries	USA	Brayton *et al.* (2004)
CP001079	*Anaplasma marginale* str. Florida	USA	Visser *et al.* (1992)
CP001759	*Anaplasma centrale* str. Israel	Israel	Herndon *et al.* (2010)
AF441131	*Anaplasma ovis isolate OVI*	South Africa	Lew *et al*. (2003)
MG778624	*Anaplasma ovis*	Sudan	Lee *et al.* (2018)
KJ410304	*Anaplasma sp.*	China	Kang *et al.* (2014)
KX987399	*Anaplasma bovis*	China	Lu *et al*. (2016)
JN588562	*Anaplasma sp.*	Japan	Ybañez *et al*. (2011)
JX092095	*Anaplasma bovis*	Russia	Rar *et al*. (2012)
CP000235	*Anaplasma phagocytophilum HZ*	USA	Dunning *et al.* (2006)
JF494841	*Anaplasma phagocytophilum*	USA	Rejmanek *et al.* (2012)
KF031393	*Anaplasma phagocytophilum*	Italy	Barakova *et al.* (2013)
JX876642	*Anaplasma odocoilei*	USA	Tate *et al.* (2012)
KJ814955	*Candidatus* Anaplasma camelli	Saudi Arabia	Bastos *et al*. (2015)
AY077621	*Anaplasma platys*	Japan	Inokuma *et al.* (2002)
EU516386	*Anaplasma platys*	Brazil	Cardozo *et al.* (2008)
CR767821	*Ehrlichia ruminantium* str. Welgevonden	South Africa	Collins *et al.* (2005)
CR925677	*Ehrlichia ruminantium* str. Gardel	France	Frutos *et al.* (2006)
AB074462	*Candidatus* Ehrlichia shimanensis	USA	Kawahara *et al*. (2001)
AF195273	*Ehrlichia ewingii*	Unknown	Sumner *et al. (*2000)
CP006917	*Ehrlichia muris*	Japan	Thirumalapura *et al*. (2014)
DQ672553	*Candidatus* Ehrlichia ovata	Japan	Ohashi *et al.* (2006)
CP000236	*Ehrlichia chaffeensis* str. Arkansas	USA	Hotopp *et al.* (2006)
KJ814961	*Candidatus* Ehrlichia regneryi	Saudi Arabia	Bastos *et al.* (2015)
JX629806	*Ehrlichia mineirensis*	Brazil	Cruz *et al.* (2012)
CP000107	*Ehrlichia canis* str. Jake	USA	Palenik *et al.* (2005) (Direct submission)
JN391407	*Ehrlichia canis*	Philippines	Ybañez *et al*. (2011)
AM999887	*Wolbachia* sp.	Sri Lanka	Klasson *et al.* (2008)

CHAPTER 4

Results

4.1 Reverse line blot hybridization assay

The RLB hybridization assay was used to screen rock sengis (n=160) for the presence of *Theileria, Babesia, Ehrlichia* and *Anaplasma* species. An example of a blot membrane is illustrated in Figure 4.1. The results of the RLB assay indicated that the majority of PCR products hybridized with the genus-specific probes only (Table 4.1); 51 (31.9%) with the *Anaplasma/Ehrlichia* genus-specific probe and eight (5%) with the *Theileria/Babesia* genus-specific probe. Only one PCR product obtained from a rock sengi captured at the Goro Game Reserve hybridized with the *B. bovis* probe. None of the other PCR products hybridized with the *A. bovis* probe. A total of 86 (53.8%) PCR products failed to hybridize with any probes; these samples are considered negative for infection or to be infected at a level lower than the detection level of the assay.

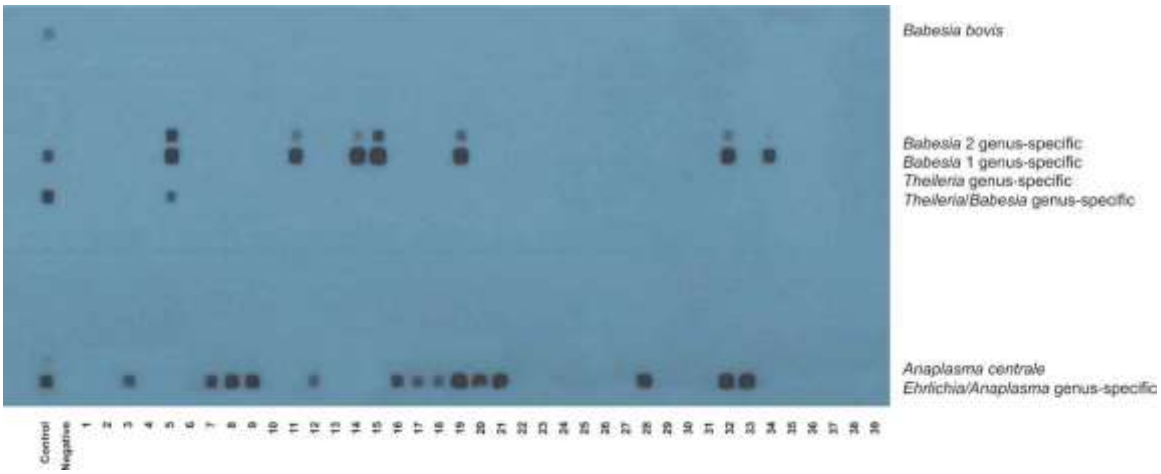

Figure 4.1 Representative reverse line blot (RLB) hybridization assay results for the simultaneous detection of *Theileria, Babesia, Ehrlichia* and *Anaplasma* species in the rock sengi samples. The genus and species-specific oligonucleotides probes were applied in horizontal lanes and the PCR products in vertical lanes. Positive and negative control, respectively were loaded in lanes labelled control and negative. Lanes 1 to 39 were loaded with sample DNA.

Table 4.1: Tick-borne haemoparasite infections in rock sengi investigated in this study as determined with the Reverse Line Blot hybridization assay.

	Goro Game Reserve (n=112)	Ezemvelo Nature Reserve (n=48)	Total (n=160)
Babesia bovis	1 (0.9%)	0	1 (0.6%)
Anaplasma/Ehrlichia genus-specific	35 (31.3%)	16 (33.3%)	51 (31.9%)
Theileria/Babesia genus-specific	6 (5.4%)	2 (4.2%)	8 (5%)
Babesia genus-specific 1	19 (17%)	9 (18.8%)	28 (17.5%)
Babesia genus-specific 2	17 (15.2%)	7 (14.6%)	24 (15%)
Theileria genus-specific	5 (4.5%)	11 (22.9%)	16 (10%)
Negative/Below detection limit	61 (54.5%)	25 (52.1%)	86 (53.8%)

*Number in column headings indicates the total samples from every locality and the ultimate total of samples screened. This does not indicate totals from the underlying columns as there were multiple infections present within some of the samples.

4.2 Development of *A. bovis*-like (sengi) RLB probe

The V1 hypervariable region of the 16S rRNA gene of a published *Anaplasma bovis* sequence from rock sengi (Harrison *et al*., 2013), was aligned with the currently used *A. bovis* RLB probe (Bekker *et al.,* 2002). This alignment revealed that the current *A. bovis* probe differed at three nucleotide sites (Figure 4.2) from the *A. bovis*-like sequences identified in rock sengi, which would explain why the *A. bovis* RLB probe could not detect *A. bovis*-like sequences within the rock sengi samples during our initial RLB screening. These sequences were then used to design an *A. bovis*-like (sengi) RLB probe for the specific detection of the *A. bovis*-like strains. The custom-designed probe was subsequently used to screen 108 rock sengi samples that previously tested negative for *A. bovis*.

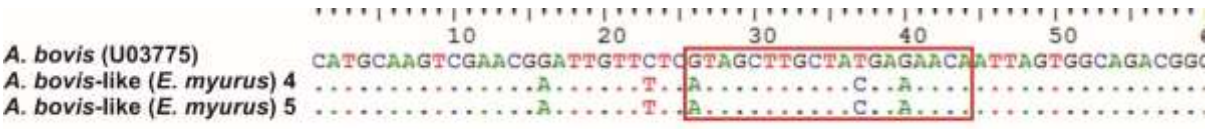

Figure 4.2: Nucleotide alignment of a 60 bp region each of the V1 hypervariable region of the published 16S rRNA gene of *A. bovis* and the *A. bovis*-like strain previously identified from eastern rock sengi. The region where the *A. bovis*-like (sengi) RLB probe was designed is indicated in a red block.

The new *A. bovis*-like (sengi) probe was able to detect *A. bovis*-like sequences within the rock sengi DNA samples (Figure 4.3). Of the 108 samples tested, 17 (15.7%) of the PCR products hybridized with the *A. bovis*-like (sengi) probe; 12 (10.5%) from the Goro Game Reserve, and five (10.4%) from the Ezemvelo Nature Reserve.

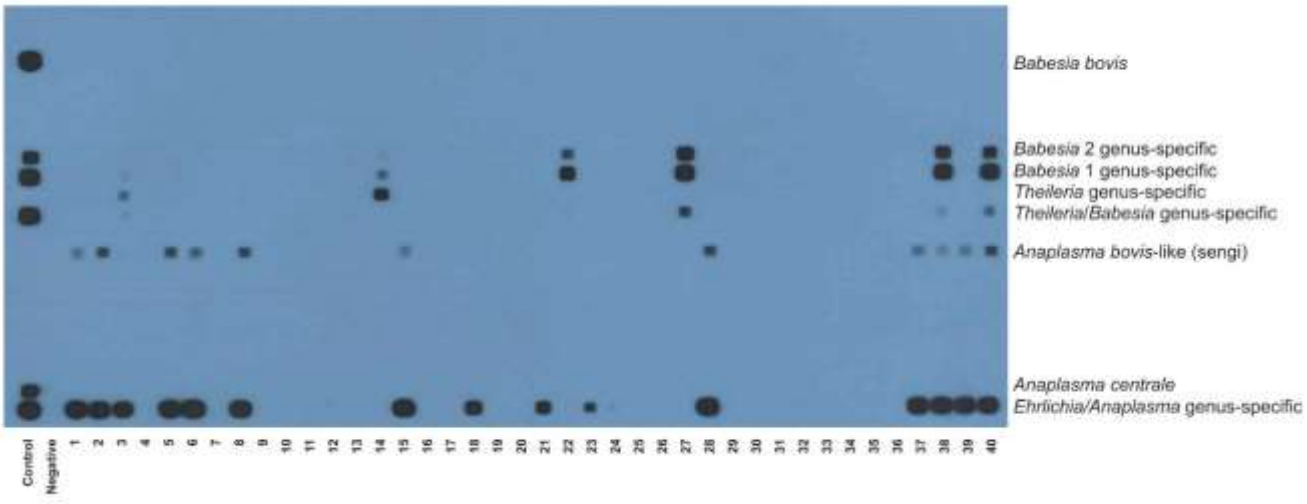

Figure 4.3: Representative RLB hybridization assay results illustrating the use of the newly designed *A. bovis*-like (sengi) RLB probe that was used to screen a subset of rock sengi blood samples. Positive and negative control, respectively were loaded in lanes numbered Control and Negative. Lanes 1 to 40 were loaded with sample DNA.

4.3 Genetic characterization of *Anaplasma*-positive samples

The samples that previously tested positive for *A. bovis*-like (sengi) during RLB hybridization (n=17), together with two samples that were negative, were selected for further PCR screening and molecular characterization, using primers targeting the parasite 16S rRNA and *GroEL* genes.

Primers previously described for the amplification of the 16S rRNA (Parola *et al*., 2000, Frank *et al*., 2008, Ybañez *et al*., 2012, Bastos *et al*., 2015) failed to amplify the parasite 16S rRNA and *GroEL* genes from the rock sengi samples, with no amplification showing on the agarose gel. The new *A. bovis*-like (sengi) specific primers that were subsequently designed (Table 3.2), successfully amplified both gene regions.

4.3.1 16S rRNA gene sequence analysis

After amplification of the 16S rRNA gene region, a single band the size of 1.0-1.4 kb was observed on 1.5% agarose gel. After purification and sequencing, 12 samples were identified to be closely related to *A. bovis* (Accession no: KC811530 and U03775). The 12 partial- to near full-length 16S rDNA sequences (700-1300 bp) generated in this study, when complemented with homologous reference sequences in the Genbank database (www.ncbi.nlm.nih.gov), and trimmed of end-aligned sequences, resulted in a final dataset 675 nt in length.

BLASTn homology searches showed that the obtained sequences were most closely related (99-100% sequence identity) to an uncultured *Anaplasma* sp. (Accession no: KC811530) previously identified in eastern rock sengis (Harrison *et al*., 2013). The obtained sequences further showed 99% sequence identity with *A. bovis* (Accession no: U03775) previously described from bovines in South Africa by Visser & Allsopp (1993) (unpublished). The geographical origin of the rock sengi, the RLB assay results and phylogenetic classification for the obtained sequences are summarised in Table 4.2.

Pairwise comparisons of gene sequences generated in this study and those of closely related *Anaplasma* sequences in the Genbank database allowed for determination of the number of base differences in the final dataset (675 nt). The two genotypes identified from the NJ p-distance analysis were designated *A. bovis*-like (sengi) genotype A (represented by two sequences) and *A. bovis*- like (sengi) genotype B (represented by 10 sequences). The two genotypes differed at one nucleotide position from each other with genotype A differing at one nucleotide position from the *A. bovis*-like sequence previously detected in rock sengi (Accession no: KC811530) by Harrison *et al*. (2013), and genotype B perfectly matching this sequence.

The observed sequence similarities were confirmed by phylogenetic analyses (Figure 4.4). Of interest is that both the Maximum likelihood and Bayesian analyses recovered near-significant levels of support (68% and 92%, respectively) for a sister taxon relationship between the *A. bovis* clade, within which the sengi genotypes clustered and the well-supported clade (83% and 100%, respectively) containing the *Anaplasma phagocytophilum-odocoilei-platys* species. The sister taxon relationship between the afore-mentioned clades and a clade containing the *Anaplasma central-ovis-marginale* species complex was 97% and 99%, respectively (Figure 4.4). The phylogeny further revealed that the previously identified *A. bovis*-like sequences described by Harrison *et al*. (2013) formed a well-supported monophyletic group (99% and 100%, respectively) within which published *A. bovis* sequences, including sequences described from South Africa (U03775), Japan (GU937020) and China (KY007145) also clustered.

Table 4.2: Origin and results of the rock sengi samples selected for 16S rRNA gene characterization.

SAMPLE NR	LOCALITY	RLB RESULTS	SEQUENCE LENGTH (BP)		*A. BOVIS*-LIKE (SENGI) GENOTYPE	
			16S rRNA	*GroEL*	16S rRNA	*GroEL*
5	Goro	A/E catch-all, *A. bovis*-like (sengi)	1104	-	B	-
28	Goro	T & B1 catch-all	939	568	B	D
42	Goro	A/E catch-all, *A. bovis*-like (sengi)	943	568	B	D
43	Goro	A/E catch-all, *A. bovis*-like (sengi)	1199	380	B	D
55	Goro	A/E catch-all, *A. bovis*-like (sengi)	1236	-	B	-
95	Goro	A/E catch-all, *A. bovis*-like (sengi), T, B1 & B2 catch-all	1199	473	B	D
105	Goro	A/E catch-all, *A. bovis*-like (sengi)	1064	474	B	D
109	Goro	A/E catch-all, *A. bovis*-like (sengi), T, B1 & B2 catch-all	885	473	B	D
L10	Ezemvelo	A/E catch-all, *A. bovis*-like (sengi)	821	475	A	C
L11	Ezemvelo	A/E catch-all, *A. bovis*-like (sengi)	733	-	A	-
L65	Ezemvelo	A/E catch-all, T catch-all	1030	497	B	C
L77	Ezemvelo	A/E catch-all, *A. bovis*-like (sengi)	733	-	B	-

A/E catch-all = *Anaplasma/Ehrlichia* genus-specific probe
B1 catch-all = *Babesia* genus-specific probe 1
B2 catch-all = *Babesia* genus-specific probe 2
T catch-all = *Theileria* genus-specific probe
Spaces marked with "-" indicates that no sequence data was obtained

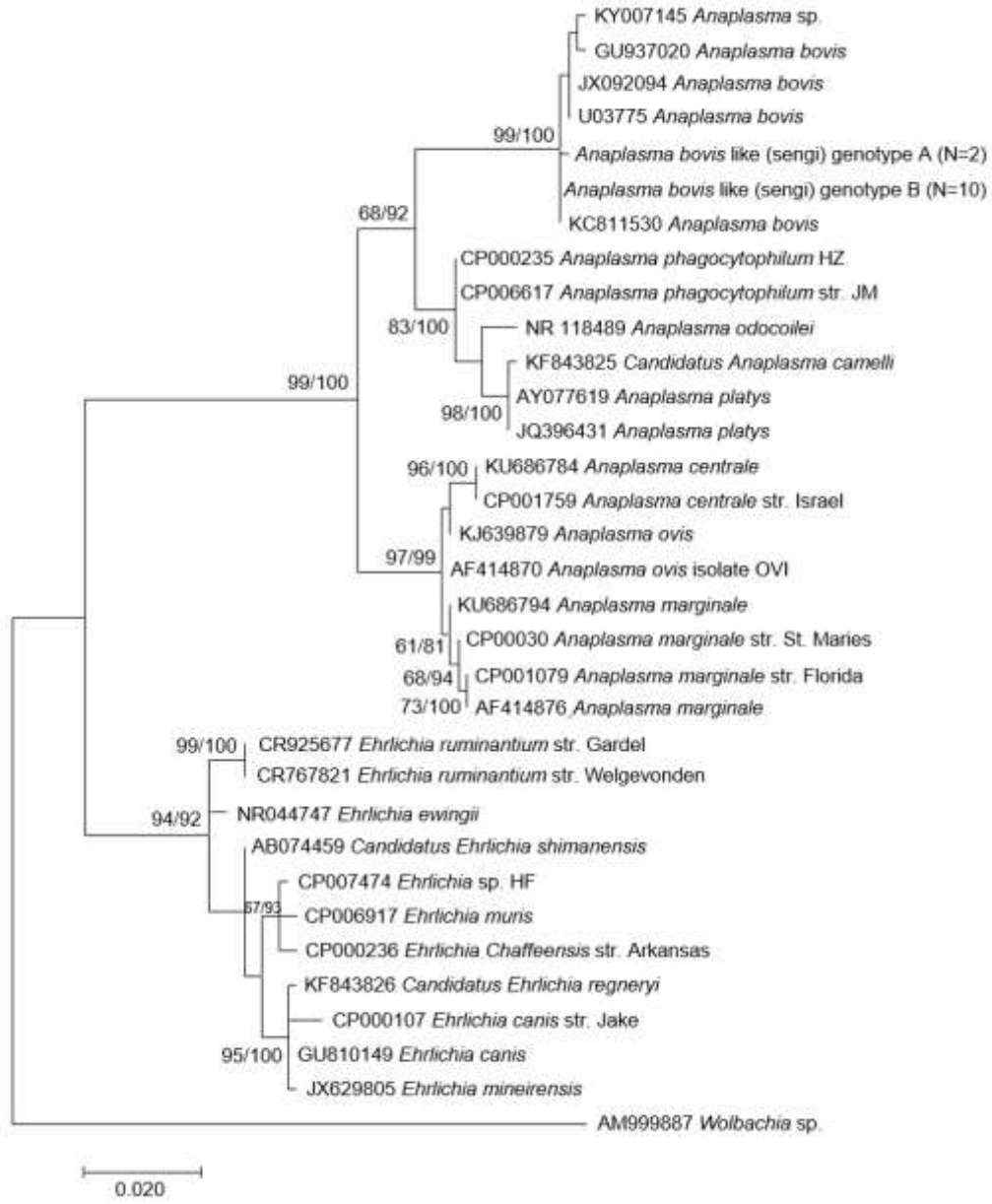

Figure 4.4: Maximum likelihood tree of the 16S rRNA gene found in rock sengi, inferred using the Kimura 2-parameter (K2P) model of sequence evolution with a gamma distribution shape parameter (G) of 0,19. This analysis involved 32 sequences (excluding the outgroup *Wolbachia*) from the genus *Anaplasma* and *Ehrlichia* and was based on a 675 nucleotide region, corresponding to the 5' end of the gene. Bootstrap support values from maximum likelihood (5000 replicates) and nodal support values from Bayesian Inference (10 million generations, four chains with one cold and default heated chain settings, and a 10% burn-in) are indicated as ML/BI next to each node. Only those values were higher than 60% and 90%, respectively are indicated.

4.3.2 *GroEL* gene sequence analysis

Purification and sequencing of bands of the expected band size of 500-600 bp observed on 1.5% agarose gel after amplification of the *GroEL* gene region with the *A. bovis*-like (sengi) primers designed specifically for this study, resulted in eight *GroEL* sequences (400-600 nt in length). BLASTn homology searches showed that the obtained sequences were 88% identical (99% query cover) to an uncultured *Anaplasma* sp. in a raccoon identified by Ybañez *et al*. (2011) (Accession no: JN588562) and 87% identical (99% query cover) to an *A. bovis* sequence in a *Haemaphysalis concinna* tick from Russia (Accession no: JX092095) (Figure 4.5).

Pairwise comparisons of gene sequences generated in this study and those of closely related *Anaplasma* sequences in the Genbank database allowed for determination of the number of base differences in the final dataset (372 nt). From the eight *GroEL* sequences obtained from rock sengi, two variants could be identified, which were designated *GroEL A. bovis*-like (sengi) genotype C (represented by two sequences) and *A. bovis*-like (sengi) genotype D (represented by six sequences). The two genotypes differed at two nucleotide sites from each other, and differed substantially (>11%) from other *A. bovis* sequences within the analysis. It is evident from the *GroEL* analysis that the *A. bovis*-like (sengi) form a discrete lineage. Although this lineage clusters with other *A. bovis* strains (88% and 99% support from ML and BI, respectively), the relationships between the three major lineages within this clade are unresolved. There is also no support for the deeper/internal nodes of the *Anaplasma* clade, rendering them polyphyletic (Figure 4.5). This contrasts markedly with the 16S rRNA phylogeny, for which internal node support was high.

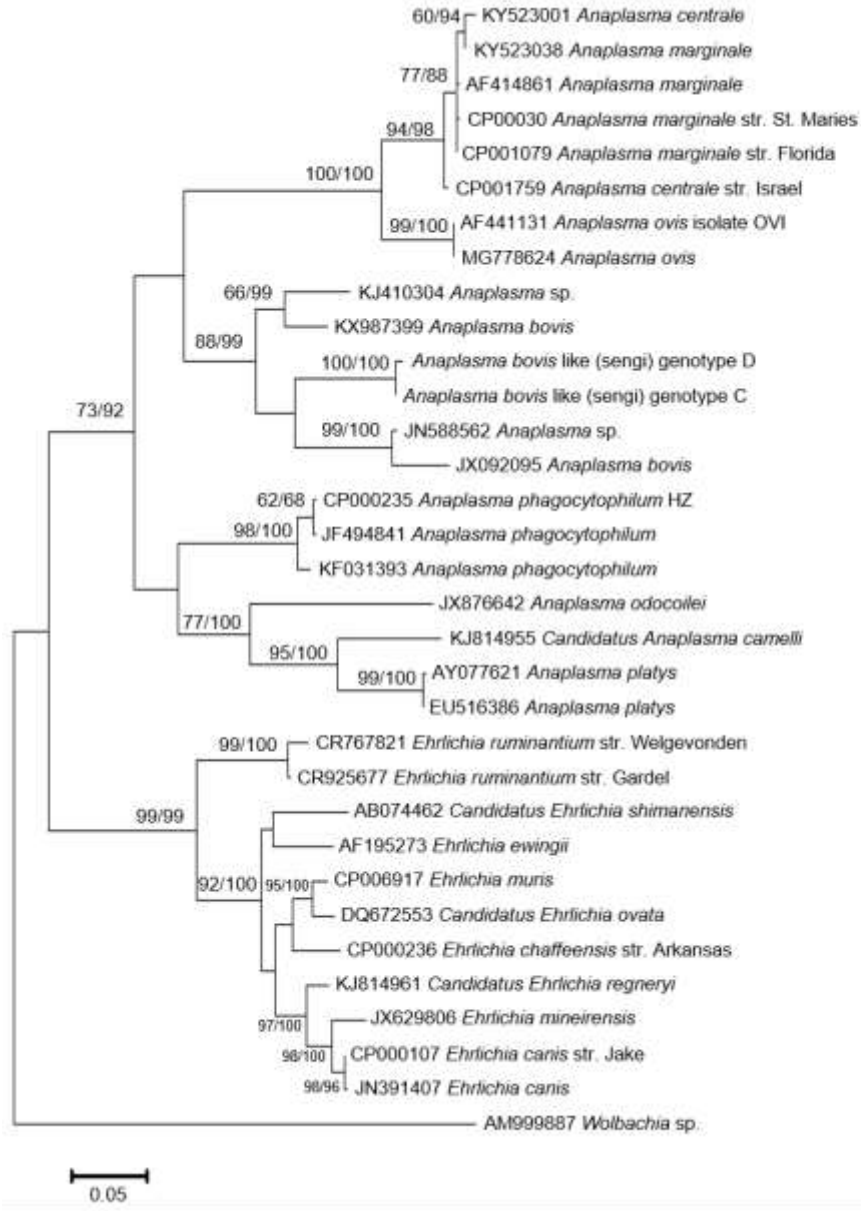

Figure 4.5: Maximum likelihood tree depicting *GroEL* gene relationships of in the *Anaplasma* species detecting in rock sengi and other members of the tick-borne Anaplasmataceae. This analysis involved 32 sequences (excluding the outgroup *Wolbachia*) from the genus *Anaplasma* and *Ehrlichia* and was based on a 372 base pair region. The HKY+G (G=0.26) model of nucleotide sequence evolution was used to infer the tree. Bootstrap support values from maximum likelihood (5000 bootstraps) and posterior probability estimates expressed as percentage, from Bayesian Inference (10 million generations, four chains with one cold and default heated chain settings, and a 10% burn-in) are indicated as ML/BI next to each node.

CHAPTER 5
Discussion

It is commonly known that small mammals like rodents and shrews play important roles in the ecology of a broad range of zoonotic and veterinary diseases, and in parasite-host relationships between these small mammals and the parasites that infect them. But, even though rock sengi are particularly well-suited to studying these relationships, due to high levels of infestation with a range of arthropod parasites (Fourie *et al.,* 1995), there is a gap in our knowledge about the potential tick-borne haemoparasites that these small mammals carry and/or transmit.

In our study, the Reverse Line Blot (RLB) hybridization assay results revealed that all samples failed to hybridize with the species-specific probes and only hybridized with the genus-specific probes; suggestive of novel species or variants within the positive genera. Only one PCR product obtained from a rock sengi captured at the Goro Game Reserve tested positive for *B. bovis* with the original species-specific RLB probe. The RLB hybridization assay has been developed and used to simultaneously detect and differentiate tick-borne haemoparasite infections (Gubbels *et al*., 1999, Bekker *et al*., 2002, Decaro *et al*., 2008, Sibeko *et al*., 2008, Muhanguzi *et al*., 2010a, Muhanguzi *et al*., 2010b, Njiiri *et al*., 2015), and has proven to be a valuable tool in the identification of novel parasites (Nijhof *et al*., 2005, Oosthuizen *et al*., 2008). The sensitivity of the RLB assay was determined at 0.000001% parasitaemia, enabling detection of the carrier state of most parasites (Gubbels *et al*., 1999).

The detection of *B. bovis* DNA in one sengi sample was of interest as *B. bovis* is a pathogen of cattle that causes babesiosis (redwater) and is responsible for major economic losses (Uilenberg, 1995, Omar *et al*., 2016). *Babesia bovis* is highly host-specific, occurring in cattle and in the one-host tick *Rhipicephalus microplus* in South Africa. This tick is not known to feed on rodents (L Neves 2018, Personal communication, 14 November 2018). The reason for detection of *B. bovis* within the blood of a rock sengi is thus unclear. No sequence data have been obtained to support this finding and we can therefore only speculate whether this was a true or an incidental finding. An incidental infection could occur when a non-host is accidentally infected with a parasite through for example a tick bite (Tembo, 2012). The parasite is usually eliminated by the incidental host, but can sometimes remain in the host for a short while, but it does not cause disease. The presence of previously unrecognized species or variants of existing species (with slightly different 18S rDNA genotypes) that might have cross-reacted with the RLB probes could also explain some of these results (Tembo, 2012). Alternatively

these results could be due to contamination of one sample with target DNA from another sample, or contamination with PCR products from a previous experiment. To elucidate this in a further study, the full-length 18S rRNA genes should be amplified and sequenced.

Despite contention surrounding the basis of the sole species-specific result, it was clear from the RLB genus-specific results that co-infections were abundant, and that these likely involved novel genotypes or variants as the majority failed to bind to available species-specific probes. Previous studies reporting significantly higher tick loads on eastern rock sengis compared to other sympatric small mammals (Fourie *et al*., 1992, Harrison *et al*., 2011) found a wide variety of tick species infesting eastern rock sengi, most of which belonged to the ixodid tick family. Extensive studies have been done on tick infestation in cattle and other livestock and it is commonly known that ixodid tick species transmit a wide variety of haemoparasites, yet the information regarding the three most common ticks found on eastern rock sengi (*Ixodes rubicundus, Rhipicentor nuttalli, Rhipicephalus warburtoni*) is minimal. It is clear that more studies are needed in order to clarify which haemoparasite infections are present within these rock sengi, and their associated ectoparasites.

We expected to identify *Anaplasma bovis* in the rock sengi sample set since previous studies revealed that eastern rock sengi from South Africa harbour a rickettsial pathogen that is closely related to *A. bovis* in cattle (Harrison *et al*., 2013). *Anaplasma bovis* is mostly studied within cattle, where it causes monocytic anaplasmosis (Rar & Golovljova, 2011). However, recent reports reveal that this pathogen occurs in a broad range of geographical areas and hosts, but has not been studied as extensively as other *Anaplasma* species. This contributes to the urgency for undertaking additional scientific studies on this species, especially in small mammals such as rock sengi, which are likely to play an important, but largely overlooked role in perpetuation of these bacteria.

Sequence alignment in the RLB probe region for *A. bovis* showed three nucleotide site differences between the *A. bovis*-like sequences previously described from rock sengi (Harrison *et al*., 2013) and the RLB probe sequence. The nucleotide differences most likely prevented hybridisation of the PCR amplicons to the RLB probe, resulting in failure of the RLB assay to identify *A. bovis*-like DNA in the samples. A new *A. bovis*-like (sengi) probe was subsequently developed and was able to detect the sengi-associated *A. bovis*-like strain. The occurrence of the *A. bovis*-like (sengi) in only 15.7% of the samples would suggest that the probe did not optimally detect *A. bovis*-like (sengi). Full optimisation of the probe was not done during this study and future steps should be taken to ensure that the probe and/or PCR-RLB conditions are fully optimised to detect *A. bovis*-like (sengi). Another explanation for the low

A. bovis-like (sengi) detection rate could be the presence of other genetic variants not detected by the *A. bovis*-like (sengi) probe. It is also possible that reservoirs can be infected with multiple strains from the same genus, one popular co-infection is that of *A. marginale* and *A. centrale* (Belkahia *et al.,* 2015, Khumalo *et al*., 2016, Chaisi *et al*., 2017, Hove *et al*., 2018). As such, we were only able to amplify and obtain 16S rDNA and *GroEL* gene sequence data for 12 and eight animals, respectively.

There is limited gene sequence information available for conserved and semi-conserved *A. bovis* genes due to the unsuccessful attempts to cultivate *A. bovis in vitro* (Moustafa *et al*., 2015). This difficulty in cultivating *Anaplasma* spp. makes it difficult to generate enough target DNA for whole-genome sequencing (Battilani *et al*., 2017). With most gene sequence information being available for *A. marginale* and *A. phagocytophilum* (Battilani *et al*., 2017), the phylogenetic position of the *A. bovis*-like strain is uncertain (Ooshiro *et al*., 2008). According to Kang *et al*. (2011), 16S rRNA gene sequence analysis has shown that *A. bovis* has a closer relationship with *A. phagocytophilum* than with *A. centrale*. This was also been demonstrated by Zobba *et al*. (2014), where phylogenetic analysis of two 16S rRNA *A. platys*-like sequences demonstrated that the *A. platys*-like sequences grouped into the same cluster as *A*. *phagocytophilum* and *A. bovis*. Our results support the closer relationship between *A. bovis* and its closely related strains, including those in sengi, and the monophyletic lineage containing *A. phagocytophilum, A. odocoilei, A. platys* and "*Candidatus* A. camelii". However, the levels of support for this relationship were below the 70% and 95% nodal support values corresponding to statistical significance for ML and BI, respectively (Hillis and Bull, 1993, Alfaro *et al.,* 2003).

The *Anaplasma* sp. sequence obtained in this study resembles that of *A. bovis* but is sufficiently genetically distinct to tentatively suggest that it likely represents a novel species within an *A. bovis* species complex, which in addition to containing the sengi strain, comprises of two additional discrete lineages, *viz.* KJ410304 + KX987399 and JN588562 + JX092095 in the *GroEL* gene phylogeny. However, the higher levels of variation within the *GroEL* gene region characterised did not assist with improved phylogenetic placement of the sengi strain, nor of *A. bovis* within the broader *Anaplasma* phylogeny as only the terminal nodes had high levels of support. In addition, the number of genotypes recovered for the *GroEL* gene region matched that identified with the more conserved 16S rRNA gene. However, the combination of the two datasets revealed the presence of at least three genetically discernible genotype combinations, viz. A-C, B-C and B-D. Together these results suggest that concatenation of the two gene regions prior to phylogenetic analysis may be warranted, and that characterisation of additional gene regions is needed to resolve the phylogenetic position of

A. bovis and its closely related genotypes/species within the tick-borne Anaplasmataceae phylogeny with confidence. Sequence data on its own is not sufficient to describe new species; thus we suggest that a type specimen be obtained by attempting to culture the strain. The full description of the novel species would highly benefit from additional morphological studies as well.

During 16S rRNA sequence analysis of this *Anaplasma* species found within rock sengi, it was clear that this species shares recent common ancestry with *A. bovis* (99% bootstrap support) but that these are polyphyletic, which means that the common ancestor cannot be identified with confidence. These sequences might also stem from the same ancestor as *A. phagocytophilum, A. platys* and *A. odocoilei* but this is only supported by a 68% bootstrap support. Further studies on this novel species are very important so that we could clearly place this unknown species within the genus *Anaplasma*. When *A. bovis* is present within cattle and symptoms occur, case fatality rates can reach 50%. It is unclear how pathogenic the *A. bovis*-like (sengi) strain is. Harrison *et al*. (2013) clearly stated how important it is to compare this novel species to other pathogenic strains found in cattle to determine its potential impact.

The fact that twelve 16S rRNA *A. bovis*-like sequences were obtained from rock sengi from two localities eliminates the possibility of 'incidental infection'. It is widely known that pathogens can infect vertebrates that are not their natural hosts but this is more common in humans (Baxby *et al*., 1994, Hartskeerl & Terpstra, 1996, Barnett *et al*., 1999, Chantrey *et al*., 1999).

In their study, Harrison *et al*. (2013) amplified *A. bovis*-like sequences from 30 samples using Parola (EHR 16SD and EHR 16SR) primers. These primers were less successful within our study where no *A. bovis* DNA could be amplified with these primers. However, the *A. bovis* found within their study was identical to the *A. bovis*-like (sengi) genotype B across a 675 bp region of the 16S rRNA gene. The 16S rRNA *Anaplasma* sequences identified within their study were closely related to *A. platys* and *A. phagocytophilum*, which is consistent with the results obtained in our study. Obtaining *A. bovis*-like sequences from two different studies done on rock sengi, indicates that a novel *A. bovis* species occurs within rock sengi.

Ybañez *et al*. (2014) stated that previous studies on the phylogeny of *Anaplasma* spp. through the use of the *GroEL* gene, did not include *A. bovis* because no sequence data were available at that time for this bacterial species. Consequently, the phylogeny on *A. bovis* was only done with the 16S rRNA gene (Ybañez *et al*., 2014). After the introduction of the analysis of the *GroEL* gene of *A. bovis*, a number of studies were published which helped to place *A. bovis*

more accurately in the phylogeny of the genus *Anaplasma* (Kang *et al*., 2011, Ybañez *et al*., 2014, Zobba *et al*., 2014). The study of Ybañez *et al*. (2014) showed that *A. bovis* clusters with *A. platys* and *A. phagocytophilum* (90% bootstrap support), whereas that of Zobba *et al*. (2014) indicated that *A. bovis* strains are genetically distinct from all other species within the genus *Anaplasma.* The results in our study correspond to the results found in Zobba *et al*. (2014), again showing the need of further studies to clarify the position of *A. bovis*-like (sengi) within the phylogeny of *Anaplasma.*

CHAPTER 6

Conclusion

Detecting an *Anaplasma bovis*-like strain in rock sengi, which usually only infects bovines, makes it clear that more studies needs to be done on the genus *Anaplasma*. It has been proven how extensively rock sengi are infected with ticks, yet this is the first study in which the simultaneous detection of haemoparasites infecting rock sengi has been investigated. This underscores the current lack of knowledge regarding the role that rock sengi play as reservoir hosts for a range of infectious pathogens, vector-borne agents which could pose a potential threat to animal and human health. Thus, further research needs to be done on the pathogenicity and the potential threat to animal and human health of this *A. bovis*-like (sengi) strain, as well as the other haemoparasites infecting rock sengi.

REFERENCES

ABARCA, K. LOPEZ, J. PERRET, C. GUERRERO, J. GODOY, P. VELOZ, A. VALIENTE-ECHEVERRIA, F. LEON, U. GUTJAHR, C. & AZOCAR, T. (2007). *Anaplasma platys* in dogs, Chile. ***Emerging Infectious Diseases,*** 13: 1392-1395.

AGUIRRE, E. TESOURO, M. RUIZ, L. AMUSATEGUI, I. & SAINZ, A. (2006). Genetic characterization of *Anaplasma* (*Ehrlichia*) *platys* in dogs in Spain. ***Zoonoses and Public Health,*** 53: 197-200.

AKTAS, M. ALTAY, K. DUMANLI, N. & KALKAN, A. (2009). Molecular detection and identification of *Ehrlichia* and *Anaplasma* species in ixodid ticks. ***Parasitology Research,*** 104: 1243.

ALFARO, M.E. ZOLLER, S. & LUTZONI, F. (2003). Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. ***Molecular Biology and Evolution,*** 20: 255-266.

ALTSCHUL, S.F. GISH, W. MILLER, W. MYERS, E.W. & LIPMAN, D.J. (1990). Basic Local Alignment Search Tool. ***Journal of Molecular Biology***, 215: 403-410.

ANDERSON, B.E. DAWSON, J.E. JONES, D.C. & WILSON, K.H. (1991). *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. ***Journal of Clinical Microbiology,*** 29: 2838-2842.

ARRAGA-ALVARADO, C.M. QUROLLO, B.A. PARRA, O.C. BERRUETA, M.A. HEGARTY, B.C. & BREITSCHWERDT, E.B. (2014). Molecular evidence of *Anaplasma platys* infection in two women from Venezuela. ***The American Journal of Tropical Medicine and Hygiene,*** 91: 1161-1165.

ATIF, F.A. (2016). Alpha proteobacteria of genus Anaplasma (Rickettsiales: *Anaplasmataceae*): Epidemiology and characteristics of *Anaplasma* species related to veterinary and public health importance. ***Parasitology,*** 143: 659-685.

BABES, V. (1888). Sur l'hemoglobinurie bacterienne du boeuf [on the bacterian hemoglobinuria of cattle] (in French). ***Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences,*** 107: 692-694.

BAJER, A. ALSARRAF, M. BEDNARSKA, M. MOHALLAL, E.M.E. MIERZEJEWSKA, E.J. BEHNKE-BOROWCZYK, J. ZALAT, S. GILBERT, F. & WELC-FALECIAK, R. (2014). *Babesia behnkei* sp nov., a novel *Babesia* species infecting isolated populations of Wagner's gerbil, *Dipodillus dasyurus*, from the Sinai Mountains, Egypt. ***Parasites & Vectors,*** 7: 572.

BAKER, D.C. SIMPSON, M. GAUNT, S.D. & CORSTVET, R.E. (1987). Acute *Ehrlichia platys* Infection in the Dog. ***Veterinary Pathology,*** 24: 449-453.

BAKKEN, J.S. & DUMLER, S. (2008). Human Granulocytic Anaplasmosis. ***Infectious Disease Clinics of North America,*** 22: 433.

BAKKEN, J.S. & DUMLER, J.S. (2015). Human Granulocytic Anaplasmosis. ***Infectious Disease Clinics of North America,*** 29: 341.

BAKKEN, J.S. KRUETH, J.K. LUND, T. MALKOVITCH, D. ASANOVICH, K. & DUMLER, J.S. (1996). Exposure to deer blood may be a cause of human granulocytic ehrlichiosis. ***Clinical Infectious Diseases,*** 23: 198.

BALASHOV, Y.S. (1972). Bloodsucking ticks (*Ixodoidea*)-vectors of disease in man and animals. ***Miscellaneous Publications of the Entomological Society of America,*** 8: 376-376.

BARNETT, J.K. BARNETT, D. BOLIN, C.A. SUMMERS, T.A. WAGAR, E.A. CHEVILLE, N.F. HARTSKEERL, R.A. & HAAKE, D.A. (1999). Expression and distribution of leptospiral outer membrane components during renal infection of hamsters. ***Infection and Immunity,*** 67: 853-861.

BASHIR, T. RABBANI, I. KHAN, M. ZAMAN, M. YOUSAF, M. KHAN, M. & SHARIF, F. (2014). Effect of Anti-Piroplasm Drug on Various Physiological Indices in Healthy Mongrel Dogs. ***Journal of Veterinary and Animal Science***, 4: 5-10.

BASTOS, A.D.S. MOHAMMED, O.B. BENNETT, N.C. PETEVINOS, C. & ALAGAILI, A.N. (2015). Molecular detection of novel *Anaplasmataceae* closely related to *Anaplasma platys* and *Ehrlichia canis* in the dromedary camel (*Camelus dromedarius*). ***Veterinary Microbiology,*** 179: 310-314.

BATTILANI, M. DE ARCANGELI, S. BALBONI, A. & DONDI, F. (2017). Genetic diversity and molecular epidemiology of *Anaplasma*. ***Infection, Genetics and Evolution,*** 49: 195-211.

BAVISKAR, B.S. GAWANDE, P.J. MASKE, D.K. JAYRAW, A.K. BAWASKAR, S.S. & BHANDARKAR, A.G. (2007). Occurrence of *Babesia* infection in Leopard *Panthera pardus* at Nagpur. ***Zoos' Print Journal,*** 22: 2736-2737.

BAXBY, D. BENNETT, M. & GETTY, B. (1994). Human cowpox 1969–93: a review based on 54 cases. ***British Journal of Dermatology,*** 131: 598-607.

BEKKER, C.P.J. DE VOS, S. TAOUFIK, A. SPARAGANO, O.A.E. & JONGEJAN, F. (2002). Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. ***Veterinary Microbiology,*** 89: 223-238.

BELKAHIA, H. SAID, M.B. ALBERTI, A. ABD, K. ISSAOUI, Z. HATTAB, D. GHARBI, M. & MESSADI, L. (2015). First molecular survey and novel genetic variants' identification of *Anaplasma marginale*, *A. centrale* and *A. bovis* in cattle from Tunisia. ***Infection, Genetics and Evolution***, 34:361-371.

BERGMANS, A.M. GROOTHEDDE, J. SCHELLEKENS, J.F. VAN EMBDEN, J.D. OSSEWAARDE, J.M. & SCHOULS, A.L.M. (1995). Etiology of cat scratch disease: comparison of polymerase chain reaction detection of Bartonella (formerly *Rochalimaea*) and *Afipia felis* DNA with serology and skin tests. ***Journal of Infectious Diseases,*** 171: 916-923.

BILGIÇ, H.B. KARAGENÇ, T. SIMUUNZA, M. SHIELS, B. TAIT, A. EREN, H. & WEIR, W. (2013). Development of a multiplex PCR assay for simultaneous detection of *Theileria annulata*, *Babesia bovis* and *Anaplasma marginale* in cattle. ***Experimental Parasitology***, 133: 222-229.

BISHOP, R. MUSOKE, A. MORZARIA, S. GARDNER, M. & NENE, V. (2004). *Theileria*: intracellular protozoan parasites of wild and domestic ruminants transmitted by ixodid ticks. ***Parasitology,*** 129: 271-283.

BOCK, R.E. JACKSON, L.A. DE VOS, A.J. & JORGENSEN, W.K. (2008). Babesiosis of cattle. ***Ticks: Biology, Disease and Control,*** 281-307.

BOWN, K.J. BEGON, M. BENNETT, M. WOLDEHIWET, Z. & OGDEN, N.H. (2003). Seasonal dynamics of *Anaplasma phagocytophila* in a rodent-tick (*Ixodes trianguliceps*) system, United Kingdom. ***Emerging Infectious Diseases,*** 9: 63-70.

BRAIG, K. OTWINOWSKI, Z. HEGDE, R. BOISVERT, D.C. JOACHIMIAK, A. HORWICH, A.L. & SIGLER, P.B. (1994). The crystal-structure of the bacterial chaperonin *GroEL* at 2.8-Angstrom. ***Nature,*** 371: 578-586.

BROWN, G. MARTIN, A. ROBERTS, T. & DUNSTAN, R. (2005). Molecular detection of *Anaplasma platys* in lice collected from dogs in Australia. ***Australian Veterinary Journal,*** 83: 101-102.

BROWN, W.C. NORIMINE, J. KNOWLES, D.P. & GOFF, W.L. (2006). Immune control of *Babesia bovis* infection. ***Veterinary Parasitology,*** 138: 75-87.

BULLER, R.S. ARENS, M. HMIEL, S.P. PADDOCK, C.D. SUMNER, J.W. RIKIHISA, Y. UNVER, A. GAUDREAULT-KEENER, M. MANIAN, F.A. & LIDDELL, A.M. (1999). *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. ***New England Journal of Medicine,*** 341: 148-155.

CHANTREY, J. MEYER, H. BAXBY, D. BEGON, M. BOWN, K. HAZEL, S. JONES, T. MONTGOMERY, W. & BENNETT, M. (1999). Cowpox: reservoir hosts and geographic range. ***Epidemiology & Infection,*** 122: 455-460.

CHAISI, M.E. BAXTER, J.R. HOVE, P. CHOOPA, C.N. OOSTHUIZEN, M.C. BRAYTON, K.A. KHUMALO, Z.T. MUTSHEMBELE, A.M. MTSHALI, M.S. & COLLINS, N.E. (2017). Comparison of three nucleic acid-based tests for detecting *Anaplasma marginale* and *Anaplasma centrale* in cattle. ***Onderstepoort Journal of Veterinary Research***, 84(1):1-9.

CHAUVIN, A. MOREAU, E. BONNET, S. PLANTARD, O. & MALANDRIN, L. (2009). *Babesia* and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. ***Veterinary Research,*** 40: 37.

CHEN, S.M. DUMLER, J.S. BAKKEN, J.S. & WALKER, D.H. (1994). Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. ***Journal of Clinical Microbiology,*** 32: 589-595.

CHIRAYATH, D. LAKSHMANAN, B. PILLAI, U.N. ALEX, P.C. & REJITHA, T.S. (2012). *Anaplasma bovis* infection in a cow - a case report. ***Journal of Veterinary and Animal Sciences,*** 43: 83-84.

CLARK, K.L. (2012). *Anaplasma phagocytophilum* in small mammals and ticks in northeast Florida. ***Journal of Vector Ecology,*** 37: 262-268.

CLARK, K.L. OLIVER, J.H., JR. MCKECHNIE, D.B. & WILLIAMS, D.C. (1998). Distribution, abundance, and seasonal activities of ticks collected from rodents and vegetation in South Carolina. ***Journal of Vector Ecology,*** 23: 89-105.

CRIADO-FORNELIO, A. MARTINEZ-MARCOS, A. BULING-SARANA, A. & BARBA-CARRETERO, J.C. (2003). Molecular studies on *Babesia, Theileria* and *Hepatozoon* in southern Europe - Part II. Phylogenetic analysis and evolutionary history. ***Veterinary Parasitology,*** 114: 173-194.

DAHMANI, M. DAVOUST, B. BENTERKI, M.S. FENOLLAR, F. RAOULT, D. & MEDIANNIKOV, O. (2015). Development of a new PCR-based assay to detect *Anaplasmataceae* and the first report of *Anaplasma phagocytophilum* and *Anaplasma platys* in cattle from Algeria. ***Comparative Immunology, Microbiology and Infectious Diseases,*** 39: 39-45.

DECARO, N. CARELLI, G. LORUSSO, E. LUCENTE, M.S. GRECO, G. LORUSSO, A. RADOGNA, A. CECI, L. & BUONAVOGLIA, C. (2008). Duplex real-time polymerase chain reaction for simultaneous detection and quantification of *Anaplasma marginale* and *Anaplasma centrale*. ***Journal of Veterinary Diagnostic Investigation***, 20: 606-611.

DE KOCK, G. VAN HEERDEN, C.J. DU TOIT, R. & NEITZ, W.O. (1937). Bovine Theileriasis in South Africa with special reference to *Theileria mutans*. ***Onderstepoort Journal of Veterinary Science,*** 8: 9-125.

DE LA FUENTE, J. ATKINSON, M.W. NARANJO, V. DE MERA, ISABEL G FERNÁNDEZ MANGOLD, A.J. KEATING, K.A. & KOCAN, K.M. (2007). Sequence analysis of the msp4 gene of *Anaplasma ovis* strains. ***Veterinary Microbiology,*** 119: 375-381.

DE LA FUENTE, J. TORINA, A. NARANJO, V. NICOSIA, S. ALONGI, A. LA MANTIA, F. & KOCAN, K.M. (2006). Molecular characterization of *Anaplasma platys* strains from dogs in Sicily, Italy. ***BMC Veterinary Research,*** 2: 24.

DE WAAL, D.T. (2000). Anaplasmosis control and diagnosis in South Africa. ***Annals of the New York Academy of Science,*** 916: 474-483.

DERGOUSOFF, S.J. & CHILTON, N.B. (2011). Novel genotypes of *Anaplasma bovis*, "*Candidatus Midichloria*" sp. and *Ignatzschineria* sp. in the Rocky Mountain wood tick, *Dermacentor andersoni*. ***Veterinary Microbiology,*** 150: 100-106.

DOAN, H.T.T. NOH, J.H. CHOE, S.E. YOO, M.S. KIM, Y.H. REDDY, K.E. QUYEN, D.V. NGUYEN, L.T.K. NGUYEN, T.T.D. KWEON, C.H. JUNG, S.C. CHANG, K.Y. KANG, S.W. (2013). Molecular detection and phylogenetic analysis of *Anaplasma bovis* from *Haemaphysalis longicornis* feeding on grazing cattle in Korea. ***Veterinary Parasitology,*** 196: 478-481.

DONAHUE, J.G. PIESMAN, J. & SPIELMAN, A. (1987). Reservoir competence of white-footed mice for Lyme disease spirochetes. ***American Journal of Tropical Medicine and Hygiene***, 36: 92-96.

DONATIEN, A. & LESTOQUARD, F. (1936). *R. bovis*. New pathogenic species for cattle. ***Bulletin de la Societe de Pathologie Exotique,*** 29: 1057-1061.

DU TOIT, J.S. & FOURIE, L.J. (1994). The effect of feeding immature Karoo paralysis ticks *Ixodes rubicundus* (Acari: Ixodidae) on the metabolic rate of the rock elephant shrew. ***Experimental & Applied Acarology***, 18: 167-175.

DUH, D. PUNDA-POLIC, V. TRILAR, T. & AVSIC-ZUPANC, T. (2008). Molecular detection of *Theileria* sp in ticks and naturally infected sheep. ***Veterinary Parasitology,*** 151: 327-331.

DUMLER, J.S. & BAKKEN, J.S. (1995). Ehrlichial diseases of humans: emerging tick-borne infections. ***Clinical Infectious Diseases,*** 20: 1102-1110.

DUMLER, J.S. BARBET, A.F. BEKKER, C.P.J. DASCH, G.A. PALMER, G.H. RAY, S.C. RIKIHISA, Y. & RURANGIRWA, F.R. (2001). Reorganization of genera in the families *Rickettsiaceae* and

Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. ***International Journal of Systematic and Evolutionary Microbiology,*** 51: 2145-2165.

DUMLER, J.S. MADIGAN, J.E. PUSTERLA, N. & BAKKEN, J.S. (2007). Ehrlichioses in humans: Epidemiology, clinical presentation, diagnosis, and treatment. ***Clinical Infectious Diseases,*** 45: 45-51.

ELLIS, J. HEFFORD, C. BAVERSTOCK, P.R. DALRYMPLE, B.P. & JOHNSON, A.M. (1992). Ribosomal DNA Sequence Comparison of *Babesia* and *Theileria*. ***Molecular and Biochemical Parasitology,*** 54: 87-95.

EWING, S.A. (1981). Transmission of *Anaplasma marginale* by arthropods. ***Proceedings of the 7th National Anaplasmosis Conference.*** Mississippi State University, Mississippi State, 395-423.

FAGIR, D.M. HORAK, I.G. UECKERMANN, E.A. BENNETT, N.C. & LUTERMANN, H. (2015). Ectoparasite diversity in the eastern rock sengis (*Elephantulus myurus*): the effect of seasonality and host sex. ***African Zoology,*** 50: 109-117.

FLEMING, P.A. & NICOLSON, S.W. (2004). Sex differences in space use, body condition and survivorship during the breeding season in the Namaqua rock mouse, *Aethomys namaquensis*. ***African Zoology,*** 39: 123-132.

FOURIE, L.J. DU TOIT, J.S. KOK, D.J. & HORAK, I.G. (1995). Arthropod parasites of elephant shrews, with particular reference to ticks. ***Mammal Review,*** 25: 31-37.

FOURIE, L.J. HORAK, I.G. & VAN DEN HEEVER, J.J. (1992). The Relative Host Status of Rock Elephant shrews *Elephantulus myurus* and Namaqua rock mice *Aethomys namaquensis* for economically important ticks. ***South African Journal of Zoology,*** 27: 108-114.

FRANK, J.A. REICH, C.I. SHARMA, S. WEISBAUM, J.S. WILSON, B.A. & OLSEN, G.J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. ***Applied and Environmental Microbiology,*** 74: 2461-2470.

FRIEDHOFF, K.T. (1997). Tick-borne diseases of sheep and goats caused by *Babesia*, *Theileria* or *Anaplasma* spp. ***Parassitologia,*** 39: 99-109.

FUEHRER, H. BIRO, N. HARL, J. WORLICZEK, H.L. BEIGLBÖCK, C. FARKAS, R. JOACHIM, A. & DUSCHER, G.G. (2013). Molecular detection of *Theileria* sp. ZS TO4 in red deer (*Cervus elaphus*) and questing *Haemaphysalis concinna* ticks in Eastern Austria. ***Veterinary Parasitology,*** 197: 653-657.

FUTSE, J.E. UETI, M.W. KNOWLES, D.P. & PALMER, G.H. (2003). Transmission of *Anaplasma marginale* by *Boophilus microplus*: Retention of vector competence in the absence of vector-pathogen interaction. ***Journal of Clinical Microbiology,*** 41: 3829-3834.

GARCÍA-PÉREZ, A. OPORTO, B. ESPÍ, A. DEL CERRO, A. BARRAL, M. POVEDANO, I. BARANDIKA, J. & HURTADO, A. (2016). *Anaplasmataceae* in wild ungulates and carnivores in northern Spain. ***Ticks and Tick-Borne Diseases,*** 7: 264-269.

GARCIA-SANMARTIN, J. AURTENETXE, O. BARRAL, M. MARCO, I. LAVIN, S. GARCIA-PEREZ, A.L. & HURTADO, A. (2007). Molecular detection and characterization of piroplasms infecting cervids and chamois in Northern Spain. ***Parasitology,*** 134: 391-398.

GARNER, B.C. HOLMAN, P. & BERENT, L.M. (2012). Theileriosis in a reindeer (*Rangifer tarandus tarandus*) associated with a potentially novel *Theileria* sp. ***Veterinary Clinical Pathology,*** 41: 497-501.

GHARBI, M. & DARGHOUTH, M.A. (2015). Control of tropical theileriosis (*Theileria annulata* infection in cattle) in North Africa. ***Asian Pacific Journal of Tropical Disease,*** 5: 505-510.

GOETHERT, H.K. & TELFORD III, S.R., III (2003). Enzootic transmission of *Anaplasma bovis* in Nantucket cottontail rabbits. ***Journal of Clinical Microbiology,*** 41: 3744-3747.

GOTHE, R. & NEITZ, A.W.H. (1991). Tick paralyses: pathogenesis and etiology. ***Advances in Disease Vector Research,*** 8: 177-204.

GUBBELS, J.M. DE VOS, A.P. VAN DER WEIDE, M. VISERAS, J. SCHOULS, L.M. DE VRIES, E. & JONGEJAN, F. (1999). Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. ***Journal of Clinical Microbiology,*** 37: 1782-1789.

GUINDON, S. DUFAYARD, J. LEFORT, V. ANISIMOVA, M. HORDIJK, W. & GASCUEL, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. ***Systematic Biology,*** 59: 307-321.

GUO, W. TIAN, J. LIN, X. NI, X. CHEN, X. LIAO, Y. YANG, S. DUMLER, J.S. HOLMES, E.C. & ZHANG, Y. (2016). Extensive genetic diversity of *Rickettsiales* bacteria in multiple mosquito species. ***Scientific Reports***, 6: 38770.

HABIBI, G. (2016). In silico analysis of Ta9 gene polymorphism in an Iranian *Theileria annulata* schizont-infected cell line S15 vaccine strain and native isolates. ***Archives of Razi Institute,*** 71: 71-79.

HARRISON, A. BASTOS, A.D.S. MEDGER, K. & BENNETT, N.C. (2013). Eastern rock sengis as reservoir hosts of *Anaplasma bovis* in South Africa. ***Ticks and Tick-Borne Diseases,*** 4: 503-505.

HARRISON, A. BOWN, K.J. & HORAK, I.G. (2011). Detection of *Anaplasma bovis* in an Undescribed Tick Species Collected from the Eastern Rock Sengi *Elephantulus myurus*. ***Journal of Parasitology,*** 97: 1012-1016.

HARRISON, A. ROBB, G.N. BENNETT, N.C. & HORAK, I.G. (2012). Differential feeding success of two paralysis-inducing ticks, *Rhipicephalus warburtoni* and *Ixodes rubicundus* on sympatric small mammal species, *Elephantulus myurus* and *Micaelamys namaquensis*. ***Veterinary Parasitology,*** 188: 346-354.

HARRUS, S. PERLMAN-AVRAHAMI, A. MUMCUOGLU, K. MORICK, D. EYAL, O. & BANETH, G. (2011). Molecular detection of *Ehrlichia canis*, *Anaplasma bovis*, *Anaplasma platys*, *Candidatus Midichloria mitochondrii* and *Babesia canis vogeli* in ticks from Israel. ***Clinical Microbiology and Infection,*** 17: 459-463.

HARTSKEERL, P. & TERPSTRA, W. (1996). Leptospirosis in wild animals. ***Veterinary Quarterly,*** 18: 149-150.

HARVEY, J.W. SIMPSON, C.F. & GASKIN, J.M. (1978). Cyclic thrombocytopenia induced by a *Rickettsia*-like agent in dogs. ***Journal of Infectious Diseases,*** 137: 182-188.

HENNINGSSON, A.J. HVIDSTEN, D. KRISTIANSEN, B. MATUSSEK, A. STUEN, S. & JENKINS, A. (2015). Detection of *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks from Norway using a realtime PCR assay targeting the *Anaplasma* citrate synthase gene *gltA*. ***BioMed Central Microbiology,*** 15: 153.

HILLIS, D.M. & BULL, J.J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. ***Systematic Biology***, 42: 182-192.

HOFLE, U. VICENTE, J. NAGORE, D. HURTADO, A. PENA, A. DE LA FUENTE, J. & GORTAZAR, C. (2004). The risks of translocating wildlife Pathogenic infection with *Theileria* sp and *Elaeophora elaphi* in an imported red deer. ***Veterinary Parasitology,*** 126: 387-395.

HOLDEN, K. BOOTHBY, J.T. ANAND, S. & MASSUNG, R.F. (2003). Detection of *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* in ticks (Acari: Ixodidae) from a coastal region of California. ***Journal of Medical Entomology,*** 40: 534-539.

HORNOK, S. DE LA FUENTE, J. BIRÓ, N. FERNÁNDEZ DE MERA, ISABEL G MELI, M.L. ELEK, V. GÖNCZI, E. MEILI, T. TÁNCZOS, B. & FARKAS, R. (2011). First molecular evidence of *Anaplasma ovis* and *Rickettsia* spp. in keds (Diptera: Hippoboscidae) of sheep and wild ruminants. ***Vector-Borne and Zoonotic Diseases,*** 11: 1319-1321.

HORNOK, S. ELEK, V. DE LA FUENTE, J. NARANJO, V. FARKAS, R. MAJOROS, G. & FÖLDVÁRI, G. (2007). First serological and molecular evidence on the endemicity of *Anaplasma ovis* and *A. marginale* in Hungary. ***Veterinary Microbiology,*** 122: 316-322.

HOTOPP, J.C.D. LIN, M. MADUPU, R. CRABTREE, J. ANGIUOLI, S.V. EISEN, J. SESHADRI, R. REN, Q. WU, M. & UTTERBACK, T.R. (2006). Comparative genomics of emerging human ehrlichiosis agents. ***PLoS Genetics***, 2: 21.

HOVE, P. CHAISI, M.E. BRAYTON, K.A. GANESAN, H. CATANESE, H.N. MTSHALI, M.S. MUTSHEMBELE, A.M. OOSTHUIZEN, M.C. & COLLINS, N.E. (2018). Co-infections with multiple genotypes of *Anaplasma marginale* in cattle indicate pathogen diversity**. *Parasites & Vectors***, 11(5): 1-13.

JARAMILLO ORTIZ, J.M. MOLINARI, M.P. GRAVISACO, M.J. PAOLETTA, M.S. MONTENEGRO, V.N. & WILKOWSKY, S.E. (2016). Evaluation of different heterologous prime-boost immunization strategies against *Babesia bovis* using viral vectored and protein-adjuvant vaccines based on a chimeric multi-antigen. ***Vaccine,*** 34: 3913-39139.

JENKINS, A. KRISTIANSEN, B.E. ALLUM, A.G. AAKRE, R.K. STRAND, L. KLEVELAND, E.J. VAN DE POL, I. & SCHOULS, L. (2001). *Borrelia burgdorferi* sensu lato and *Ehrlichia* spp. in *Ixodes* ticks from southern Norway. ***Journal of Clinical Microbiology,*** 39: 3666-3671.

JENKINS, C. & BOGEMA, D.R. (2016). Factors associated with seroconversion to the major piroplasm surface protein of the bovine haemoparasite *Theileria orientalis*. ***Parasites & Vectors,*** 9: 106.

JILINTAI, N.S. HAYAKAWA, D. SUZUKI, M. HATA, H. KONDO, S. MATSUMOTO, K. YOKOYAMA, N. & INOKUMA, H. (2009). Molecular survey for *Anaplasma bovis* and *Anaplasma phagocytophilum*

infection in cattle in a pastureland where sika deer appear in Hokkaido, Japan. ***Japanese Journal of Infectious Diseases,*** 62: 73-75.

JONGEJAN, F. & UILENBERG, G. (2004). The global importance of ticks. ***Parasitology,*** 129: 3-14.

JONGEJAN, F. ZANDBERGEN, T.A. VAN DE WIEL, PAUL A DE GROOT, M. & UILENBERG, G. (1991). The tick-borne rickettsia *Cowdria ruminantium* has a *Chlamydia*-like developmental cycle. ***Onderstepoort Journal of Veterinary Research*****,** 58: 227-237.

KANG, Y. DIAO, X. ZHAO, G. CHEN, M. XIONG, Y. SHI, M. FU, W. GUO, Y. PAN, B. & CHEN, X. (2014). Extensive diversity of Rickettsiales bacteria in two species of ticks from China and the evolution of the Rickettsiales. ***BioMed Central Evolutionary Biology,*** 14: 167.

KANG, J. KO, S. KIM, Y. YANG, H. LEE, H. SHIN, N. CHOI, K. & CHAE, J. (2011). New genetic variants of *Anaplasma phagocytophilum* and *Anaplasma bovis* from Korean water deer (*Hydropotes inermis argyropus*). ***Vector-Borne and Zoonotic Diseases***, 11: 929-938.

KAPPMEYER, L.S. THIAGARAJAN, M. HERNDON, D.R. RAMSAY, J.D. CALER, E. DJIKENG, A. GILLESPIE, J.J. LAU, A.O.T. ROALSON, E.H. SILVA, J.C. SILVA, M.G. SUAREZ, C.E. UETI, M.W. NENE, V.M. MEALEY, R.H. KNOWLES, D.P. & BRAYTON, K.A. (2012). Comparative genomic analysis and phylogenetic position of *Theileria equi*. ***BioMed Central Genomics,*** 13: 603.

KARBOWIAK, G. (2004). Zoonotic reservoir of *Babesia microti* in Poland. ***Polish Journal of Microbiology,*** 53: 61-65.

KAWAHARA, M. RIKIHISA, Y. LIN QUAN ISOGAI, E. TAHARA, K. ITAGAKI, A. HIRAMITSU, Y. & TAJIMA, T. (2006). Novel genetic variants of *Anaplasma phagocytophilum, Anaplasma bovis, Anaplasma centrale,* and a novel *Ehrlichia* sp. in wild deer and ticks on two major islands in Japan. ***Applied and Environmental Microbiology,*** 72: 1102-1109.

KHUMALO, Z.T. CATANESE, H.N. LIESCHING, N. HOVE, P. COLLINS, N.E. CHAISI, M.E. GEBREMEDHIN, A.H. OOSTHUIZEN, M.C. & BRAYTON, K.A. (2016). Characterization of *Anaplasma marginale* subspecies centrale strains using msp1aS genotyping reveals a wildlife reservoir. ***Journal of Clinical Microbiology,*** 54:2503-2512.

KIM, C.M. YI, Y.H. YU, D.H. LEE, M.J. CHO, M.R. DESAI, A.R. SHRINGI, S. KLEIN, T.A. KIM, H.C. SONG, J.W. BAEK, L.J. CHONG, S.T. O'GUINN, M.L. LEE, J.S. LEE, I.Y. PARK, J.H. FOLEY, J. & CHAE, J.S. (2006). Tick-borne rickettsial pathogens in ticks and small mammals in Korea. ***Applied and Environmental Microbiology,*** 72: 5766-5776.

KJEMTRUP, A.M. & CONRAD, P.A. (2000). Human babesiosis: an emerging tick-borne disease. ***International Journal for Parasitology,*** 30: 1323-1337.

KOCAN, K.M. DE LA FUENTE, J. BLOUIN, E.F. COETZEE, J.F. & EWING, S. (2010). The natural history of *Anaplasma marginale.* ***Veterinary Parasitology,*** 167: 95-107.

KOCAN, K.M. DE LA FUENTE, J. GUGLIELMONE, A.A. & MELENDEZ, R.D. (2003). Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. ***Clinical Microbiology Reviews,*** 16: 698.

KOCH, H.T. KAMBEVA, L. NORVAL, R.A.I. OCAMA, J.G.R. & MUNATSWA, F.C. (1993). Transmission of *Theileria parva bovis* by Nymphs of *Rhipicephalus appendiculatus*. ***Preventive Veterinary Medicine,*** 17: 89-93.

KOCK, R. (2005). What is this infamous "wildlife/livestock disease interface?" A review of current knowledge for the African continent. ***In: Osofsky SA (Ed.), IUCN Species Survival Commission report onConservation and Development Interventions at the Wildlife/Livestock Interface: Implications for Wildlife, Livestock and Human Health,*** 30: 1-13.

KUNDAVE, V.R. PATEL, A.K. PATEL, P.V. HASNANI, J.J. & JOSHI, C.G. (2015). Detection of theileriosis in cattle and buffaloes by polymerase chain reaction. ***Journal of Parasitic Diseases: Official Organ of the Indian Society for Parasitology,*** 39: 508-13.

LACK, J.B. REICHARD, M.V. & VAN DEN BUSSCHE, R.A. (2012). Phylogeny and evolution of the Piroplasmida as inferred from 18S rRNA sequences. ***International Journal for Parasitology,*** 42: 353-363.

LANCASTER, J. & PILLAY, N. (2010). Behavioral interactions between a coexisting rodent *Micaelamys namaquensis* and macroscelid *Elephantulus myurus*. ***Current Zoology,*** 56: 395-400.

LEE, J.H. PARK, H.S. JANG, W.J. KOH, S.E. KIM, J.M. SHIM, S.K. PARK, M.Y. KIM, Y.W. KIM, B.J. KOOK, Y.H. PARK, K.H. & LEE, S.H. (2003). Differentiation of *Rickettsiae* by *GroEL* gene analysis. ***Journal of Clinical Microbiology,*** 41: 2952-2960.

LEEMANS, I. FOSSUM, C. JOHANNISSON, A. & HOOSHMAND-RAD, P. (2001). Comparative studies on surface phenotypes of *Theileria lestoquardi* and *T*. *annulata* schizont-infected cells. ***Parasitology Research,*** 87: 768-777.

LEFORT, V. LONGUEVILLE, J-E. GASCUEL, O. (2017). SMS: Smart Model Selection in PhyML. ***Molecular Biology and Evolution***, 34: 2422-2424.

LI, Y. CHEN, Z. LIU, Z. LIU, J. YANG, J. LI, Q. LUO, J. & YIN, H. (2016). Molecular survey of *Anaplasma* and *Ehrlichia* of red deer and sika deer in Gansu, China in 2013. ***Transboundary and Emerging Diseases,*** 63: 228-236.

LI, Y. LIU, Z. YANG, J. CHEN, Z. GUAN, G. NIU, Q. ZHANG, X. LUO, J. & YIN, H. (2014). Infection of small ruminants and their red blood cells with *Theileria annulata* schizonts. ***Experimental Parasitology,*** 137: 21-24.

LI, H. ZHENG, Y. MA, L. JIA, N. JIANG, B. JIANG, R. HUO, Q. WANG, Y. LIU, H. & CHU, Y. (2015). Human infection with a novel tick-borne *Anaplasma* species in China: a surveillance study. ***The Lancet Infectious Diseases,*** 15: 663-670.

LIMA, M. SOARES, P. RAMOS, C. ARAÚJO, F. RAMOS, R. SOUZA, I. FAUSTINO, M. & ALVES, L. (2010). Molecular detection of *Anaplasma platys* in a naturally-infected cat in Brazil. ***Brazilian Journal of Microbiology,*** 41: 381-385.

LIU, Z. MA, M. WANG, Z. WANG, J. PENG, Y. LI, Y. GUAN, G. LUO, J. & YIN, H. (2012). Molecular survey and genetic identification of *Anaplasma* species in goats from central and southern China. ***Applied and Environmental Microbiology,*** 78: 464-470.

LORUSSO, V. WIJNVELD, M. MAJEKODUNMI, A.O. DONGKUM, C. FAJINMI, A. DOGO, A.G. THRUSFIELD, M. MUGENYI, A. VAUMOURIN, E. IGWEH, A.C. JONGEJAN, F. WELBURN, S.C. & PICOZZI, K. (2016). Tick-borne pathogens of zoonotic and veterinary importance in Nigerian cattle. ***Parasites and Vectors,*** 9.

LU, M. TIAN, J.H. YU, B. GUO, W.P. HOLMES, E.C. & ZHANG Y.Z. (2017). Extensive diversity of Rickettsiales bacteria in ticks from Wuhan, China. ***Ticks and Tick-Borne Diseases***, 8: 574-580.

MAGGI, R.G. MASCARELLI, P.E. HAVENGA, L.N. NAIDOO, V. & BREITSCHWERDT, E.B. (2013). Co-infection with *Anaplasma platys, Bartonella henselae* and *Candidatus Mycoplasma haematoparvum* in a veterinarian. ***Parasites & Vectors,*** 6: 103.

MAHARANA, B.R. TEWARI, A.K. SARAVANAN, B.C. & SUDHAKAR, N.R. (2016). Important hemoprotozoan diseases of livestock: Challenges in current diagnostics and therapeutics: An update. ***Veterinary World,*** 9: 487-95.

MANUAL, O.T. (2015). Bovine anaplasmosis, Chapter 2.4. 1. ***OIE Terrestrial Manual,*** 1-15.

MANUAL, O.T. (2015). Bovine babesiosis, Chapter 2.4. 2. ***OIE Terrestrial Manual,*** 1-16.

MBIZENI, S. POTGIETER, F.T. TROSKIE, C. MANS, B.J. PENZHORN, B.L. & LATIF, A.A. (2013). Field and laboratory studies on Corridor disease (*Theileria parva* infection) in cattle population at the livestock/game interface of uPhongolo-Mkuze area, South Africa. ***Ticks and Tick-Borne Diseases,*** 4: 227-234.

MBWAMBO, H.A. MAGWISHA, H.B. & MFINANGA, J.M. (2006). Evaluation of buparvaquone (BUTA-Kel™ KELA, Belgium) as a treatment of East Coast fever in cattle, in the peri-urban of Dar Es Salaam city, Tanzania. ***Veterinary Parasitology,*** 139: 67-73.

MDLADLA, K. DZOMBA, E.F. & MUCHADEYI, F.C. (2016). Seroprevalence of *Ehrlichia ruminantium* antibodies and its associated risk factors in indigenous goats of South Africa. ***Preventive Veterinary Medicine,*** 125: 99-105.

MEDGER, K. CHIMIMBA, C.T. & BENNETT, N.C. (2012). Seasonal reproduction in the eastern rock elephant-shrew: influenced by rainfall and ambient temperature? ***Journal of Zoology,*** 288: 283-293.

MEURS, L. BRIENEN, E. MBOW, M. OCHOLA, E.A. MBOUP, S. KARANJA, D.M.S. SECOR, W.E. POLMAN, K. & VAN LIESHOUT, L. (2015). Is PCR the next reference standard for the diagnosis of *Schistosoma* in stool? A comparison with microscopy in Senegal and Kenya. ***Plos Neglected Tropical Diseases,*** 9(7), p.e0003959.

MOLAD, T. ERSTER, O. FLEIDEROVITZ, L. ROTH, A. LEIBOVITZ, B. WOLKOMIRSKY, R. MAZUZ, M.L. BEHAR, A. & MARKOVICS, A. (2015). Molecular characterization of the Israeli *B. bigemina* vaccine strain and field isolates. ***Veterinary Parasitology,*** 212: 147-155.

MOSHKOVSKI, S. (1945). Cytotropic inducers of infection and the classification of the *Rickettsiae* with *Chlamydozoa*. ***Advances in Modern Biology (Moscow),*** 19: 1-44.

MOUSTAFA, M.A.M. LEE, K. TAYLOR, K. NAKAO, R. SASHIKA, M. SHIMOZURU, M. & TSUBOTA, T. (2015). Molecular characterization and specific detection of *Anaplasma* species (AP-sd) in sika deer and its first detection in wild brown bears and rodents in Hokkaido, Japan. ***Infection Genetics and Evolution,*** 36: 268-274.

MUHANGUZI, D. IKWAP, K. PICOZZI, K. & WAISWA, C. (2010a). Molecular characterization of *Anaplasma* and *Ehrlichia* species in different cattle breeds and age groups in Mbarara district (Western Uganda). ***International Journal of Animal and Veterinary Advances***, 2: 76-88.

MUHANGUZI, D. MATOVU, E. & WAISWA, C. (2010b). Prevalence and characterization of *Theileria* and *Babesia* species in cattle under different husbandry systems in western Uganda***. International Journal of Animal and Veterinary Advances***, 2: 51-58.

MUNKHJARGAL, T. ABOGE, G.O. UENO, A. ABOULAILA, M. YOKOYAMA, N. & IGARASHI, I. (2016). Identification and characterization of profilin antigen among *Babesia* species as a common vaccine candidate against babesiosis. ***Experimental Parasitology,*** 166: 29-36.

NEITZ, W.O. & THOMAS, A.D. (1948). *Cytauxzoon sylvicaprae* gen. nov., spec. nov., a protozoon responsible for a hitherto undescribed disease in the duiker, *Sylvicapra grimmia* (Linne). ***The Onderstepoort Journal of Veterinary Science and Animal Industry,*** 23: 63-76.

NENE, V. KIARA, H. LACASTA, A. PELLE, R. SVITEK, N. & STEINAA, L. (2016). The biology of *Theileria parva* and control of East Coast fever – Current status and future trends. ***Ticks and Tick-Borne Diseases,*** 7: 549-564.

NICHOLSON, W.L. ALLEN, K.E. MCQUISTON, J.H. BREITSCHWERDT, E.B. & LITTLE, S.E. (2010). The increasing recognition of rickettsial pathogens in dogs and people. ***Trends in Parasitology,*** 26: 205-212.

NICHOLSON, W.L. CASTRO, M.B. KRAMER, V.L. SUMNER, J.W. & CHILDS, J.E. (1999). Dusky-footed wood rats (*Neotoma fuscipes*) as reservoirs of granulocytic ehrlichiae (*Rickettsiales*: *Ehrlichieae*) in northern California. ***Journal of Clinical Microbiology,*** 37: 3323-3327.

NIJHOF, A.M. PENZHORN, B.L. LYNEN, G. MOLLEL, J.O. MORKEL, P. BEKKER, C.P.J. & JONGEJAN, F. (2003). *Babesia bicornis* sp nov and *Theileria bicornis* sp nov.: Tick-borne parasites associated with mortality in the black rhinoceros (*Diceros bicornis*). ***Journal of Clinical Microbiology,*** 41: 2249-2254.

NIJHOF, A.M. PILLAY, V. STEYL, J. PROZESKY, L. STOLTSZ, W.H. LAWRENCE, J.A. PENZHORN, B.L. & JONGEJAN, F. (2005). Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. ***Journal of Clinical Microbiology,*** 43: 5907-5911.

NJIIRI, N.E. COLLINS, N.E. STEYN, H.C. TROSKIE, M. VORSTER, I. THUMBI, S. SIBEKO, K.P. JENNINGS, A. VAN WYK, I.C. & MBOLE-KARIUKI, M. (2015). The epidemiology of tick-borne haemoparasites as determined by the reverse line blot hybridization assay in an intensively studied cohort of calves in western Kenya. **Veterinary Parasitology**, 210: 69-76.

NORVAL, R.A.I. (1979). The limiting effect of host availability for the immature stages on population growth in economically important ixodid ticks. ***Journal of Parasitology,*** 65: 285-287.

NORVAL, R.A.I. PERRY, B.D. & YOUNG, A.S. (1992). The epidemiology of theileriosis in Africa***. Academic Press (London)***, 481.

OMAR, M.A. SALAMA, A. ELSIFY, A. RIZK, M.A. AL-ABOODY, M.S. ABOULAILA, M. EL-SAYED, S.A.E. & IGARASHI, I. (2016). Evaluation of in vitro inhibitory effect of enoxacin on *Babesia* and *Theileria* parasites. ***Experimental Parasitology,*** 161: 62-67.

OOSHIRO, M. ZAKIMI, S. MATSUKAWA, Y. KATAGIRI, Y. & INOKUMA, H. (2008). Detection of *Anaplasma bovis* and *Anaplasma phagocytophilum* from cattle on Yonaguni Island, Okinawa, Japan. ***Veterinary Parasitology,*** 154: 360-364.

OOSTHUIZEN, M.C. ZWEYGARTH, E. COLLINS, N.E. TROSKIE, M. & PENZHORN, B.L. (2008). Identification of a novel *Babesia* sp. from a sable antelope (*Hippotragus niger* Harris, 1838). ***Journal of Clinical Microbiology***, 46: 2247-2251.

PALMER, G.H. ABBOTT, J.R. FRENCH, D.M. & MCELWAIN, T.F. (1998). Persistence of *Anaplasma ovis* infection and conservation of the msp-2 and msp-3 multigene families within the genus *Anaplasma*. ***Infection and Immunity,*** 66: 6035-6039.

PALOMAR, A.M. PORTILLO, A. SANTIBANEZ, P. MAZUELAS, D. RONCERO, L. GARCIA-ALVAREZ, L. SANTIBANEZ, S. GUTIERREZ, O. & OTEO, J.A. (2015). Detection of tick-borne *Anaplasma bovis*, *Anaplasma phagocytophilum* and *Anaplasma centrale* in Spain. ***Medical and Veterinary Entomology,*** 29: 349-353.

PAROLA, P. & RAOULT, D. (2001a). Tick-borne bacterial diseases emerging in Europe. ***Clinical Microbiology and Infection,*** 7: 80-83.

PAROLA, P. & RAOULT, D. (2001b). Ticks and tick-borne bacterial diseases in humans: an emerging infectious threat. ***Clinical Infectious Diseases,*** 32: 897-928.

PAROLA, P. CORNET, J.P. SANOGO, Y.O. MILLER, R.S. THIEN, H.V. GONZALEZ, J.P. RAOULT, D. TELFORD LLL, S.R. & WONGSRICHANALAI, C. (2003). Detection of *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and other eubacteria in ticks from the Thai-Myanmar border and Vietnam. ***Journal of Clinical Microbiology,*** 41: 1600-1608.

PAROLA, P. DAVOUST, B. & RAOULT, D. (2005). Tick- and flea-borne rickettsial emerging zoonoses. ***Veterinary Research,*** 36: 469-492.

PAROLA, P. ROUX, V. CAMICAS, J. BARADJI, I. BROUQUI, P. & RAOULT, D. (2000). Detection of *Ehrlichiae* in African ticks by polymerase chain reaction. ***Transactions of the Royal Society of Tropical Medicine and Hygiene,*** 94: 707-708.

PATEL, E. MWAURA, S. KIARA, H. MORZARIA, S. PETERS, A. & TOYE, P. (2016). Production and dose determination of the Infection and Treatment Method (ITM) Muguga cocktail vaccine used to control East Coast fever in cattle. ***Ticks and Tick-Borne Diseases,*** 7: 306-314.

PAULAUSKAS, A. RADZIJEVSKAJA, J. & ROSEF, O. (2012). Molecular detection and characterization of *Anaplasma phagocytophilum* strains. ***Comparative Immunology Microbiology and Infectious Diseases,*** 35: 187-195.

PENZHORN, B.L. KJEMTRUP, A.M. LOPEZ-REBOLLAR, L.M. & CONRAD, P.A. (2001). *Babesia leo* n. sp from lions in the Kruger National Park, South Africa, and its relation to other small piroplasms. ***Journal of Parasitology,*** 87: 681-685.

PETER, T.F. BURRIDGE, M.J. & MAHAN, S.M. (2002). *Ehrlichia ruminantium* infection (heartwater) in wild animals. ***Trends in Parasitology,*** 18: 214-218.

POTGIETER, F.T. & STOLTSZ, W.H. (2004). Bovine anaplasmosis. ***In: Coetzer, J. A. W. and Tustin R.C. (Eds), Infectious Diseases of Livestock, Oxford University Press: South Africa***: 594-616.

POTGIETER, F.T. & VAN RENSBURG, L. (1987). Tick transmission of *Anaplasma centrale*. ***Onderstepoort Journal of Veterinary Research,*** 54: 5-7.

PRITT, B.S. SLOAN, L.M. JOHNSON, D.K.H. MUNDERLOH, U.G. PASKEWITZ, S.M. MCELROY, K.M. MCFADDEN, J.D. BINNICKER, M.J. NEITZEL, D.F. & LIU, G. (2011). Emergence of a new pathogenic *Ehrlichia* species, Wisconsin and Minnesota, 2009. ***New England Journal of Medicine,*** 365: 422-429.

QUROLLO, B.A. BALAKRISHNAN, N. CANNON, C.Z. MAGGI, R.G. & BREITSCHWERDT, E.B. (2014). Co-infection with *Anaplasma platys, Bartonella henselae, Bartonella koehlerae* and '*Candidatus Mycoplasma haemominutum*' in a cat diagnosed with splenic plasmacytosis and multiple myeloma. ***Journal of Feline Medicine and Surgery,*** 16: 713-720.

RADLEY, D.E. (1981). Infection and treatment method of immunization against theileriosis. ***Advances in the Control of Theileriosis Proceedings of International Conference 9-13 Feb.1981, Nairobi.*** 227-237.

RANDOLPH, S.E. MIKLISOVA, D. LYSY, J. ROGERS, D.J. & LABUDA, M. (1999). Incidence from coincidence: patterns of tick infestations on rodents facilitate transmission of tick-borne encephalitis virus. ***Parasitology***, 118: 177-186.

RAR, V. & GOLOVLJOVA, I. (2011). *Anaplasma*, *Ehrlichia*, and "*Candidatus Neoehrlichia*" bacteria: Pathogenicity, biodiversity, and molecular genetic characteristics, a review. ***Infection Genetics and Evolution,*** 11: 1842-1861.

RAR, V.A. EPIKHINA, T.I. SUNTSOVA, O.V. KOZLOVA, I.V. LISAK, O.V. PUKHOVSKAYA, N.M. VYSOCHINA, N.P. IVANOV, L.I. & TIKUNOVA, N.V. (2014). Genetic variability of *Babesia* parasites in *Haemaphysalis* spp. and *Ixodes persulcatus* ticks in the Baikal region and Far East of Russia. ***Infection Genetics and Evolution***, 28: 270-275.

RATHBUN, G.B. 1979. The social structure and ecology of elephant-shrews. ***Zeitschrift für Tierpsychologie***, 20: 3–76.

RATHBUN, G.B. 2015. *Elephantulus myurus*. The IUCN Red List of Threatened Species 2015:e.T42662A21289491. http://dx.doi.org/10.2305/IUCN.UK.2015-RLTS.T42662A21289491.en. viewed on 2018/09/11.

RAZMI, G.R. DASTJERDI, K. HOSSIENI, H. NAGHIBI, A. BARATI, F. & ASLANI, M. (2006). An epidemiological study on *Anaplasma* infection in cattle, sheep, and goats in Mashhad suburb, Khorasan Province, Iran. ***Annals of the New York Academy of Sciences,*** 1078: 479-481.

RENNEKER, S. ABDO, J. SALIH, D. KARAGENÇ, T. BILGIÇ, H. TORINA, A. OLIVA, A. CAMPOS, J. KULLMANN, B. & AHMED, J. (2013). Can *Anaplasma ovis* in small ruminants be neglected any longer?. ***Transboundary and Emerging Diseases,*** 60: 105-112.

RIBBLE, D.O. & PERRIN, M.R. (2005). Social organization of the Eastern Rock Elephant-shrew (*Elephantulus myurus*): the evidence for mate guarding. ***Belgian Journal of Zoology,*** 135: 167-173.

RIKIHISA, Y. (1991). The tribe *Ehrlichieae* and *Ehrlichial* diseases. ***Clinical Microbiology Reviews,*** 4: 286-308.

RODRIGUEZ, S.D. GARCIA ORTIZ, M.A. JIMENEZ OCAMPO, R. & VEGA Y MURGUIA, C.A. (2009). Molecular epidemiology of bovine anaplasmosis with a particular focus in Mexico. ***Infection Genetics and Evolution,*** 9: 1092-1101.

RONQUIST, F. HUELSENBECK, J. & TESLENKO, M. (2011). Draft MrBayes version 3.2 manual: tutorials and model summaries. ***Distributed with the software from mrbayes.sourceforge.net/mb3.2_manual.pdf.***

RYMASZEWSKA, A. & GRENDA, S. (2008). Bacteria of the genus *Anaplasma* - characteristics of *Anaplasma* and their vectors: a review. ***Veterinarni Medicina,*** 53: 573-584.

SAID, M.B. BELKAHIA, H. EL MABROUK, N. SAIDANI, M. ALBERTI, A. ZOBBA, R. CHERIF, A. MAHJOUB, T. BOUATTOUR, A. & MESSADI, L. (2017). *Anaplasma platys*-like strains in ruminants from Tunisia. ***Infection, Genetics and Evolution,*** 49: 226-233.

SAID, M.B. BELKAHIA, H. KARAOUD, M. BOUSRIH, M. YAHIAOUI, M. DAALOUL-JEDIDI, M. & MESSADI, L. (2015). First molecular survey of *Anaplasma bovis* in small ruminants from Tunisia. ***Veterinary Microbiology,*** 179: 322-326.

SALIH, D. EL HUSSEIN, A. & SINGLA, L. (2015). Diagnostic approaches for tick-borne haemoparasitic diseases in livestock. ***Journal of Veterinary Medicine and Animal Health,*** 7: 45-56.

SALMON, D. & SMITH, T. (1896). Infectious diseases of cattle: southern cattle fever (Texas fever). ***Special Report on Diseases of Cattle and on Cattle Feeding. USDA Bureau of Animal Industry. Government Printing Office, Washington,*** 428-438.

SANTOS, C.F. & CARVALHO, C.B.D. (2006). [First report of *Anaplasma bovis* (Donatien and Lestoquard, 1936) at micro region of Campos dos Goytacazes, State of Rio de Janeiro, Brazil]. ***Brazilian Journal of Veterinary Parasitology,*** 15: 126-7.

SASHIKA, M. ABE, G. MATSUMOTO, K. & INOKUMA, H. (2011). Molecular survey of *Anaplasma* and *Ehrlichia* infections of feral raccoons (*Procyon lotor*) in Hokkaido, Japan. ***Vector-Borne and Zoonotic Diseases,*** 11: 349-354.

SAUER, J.R. ESSENBERG, R.C. & BOWMAN, A.S. (2000). Salivary glands in ixodid ticks: control and mechanism of secretion. ***Journal of Insect Physiology,*** 46: 1069-1078.

SCHOLAR, E.M. & PRATT, W.B. (2000). Bacteriostatic inhibitors of protein synthesis: tetracyclines. ***The Antimicrobial Drugs Second Edition. Oxford University Press, New York,*** 184-199.

SCHOULS, L.M. VAN DE POL, I. RIJPKEMA, S.G.T. & SCHOT, C.S. (1999). Detection and identification of *Ehrlichia, Borrelia burgdorferi s*ensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. ***Journal of Clinical Microbiology,*** 37: 2215-2222.

SCOTT, G. (1994). ***Lesser-known rickettsias infecting livestock. In: Coetzer, J. A. W., Thomson, G. R. & Tustin, R. C. Oxford University Press: Oxford, Infectious Diseases of Livestock with Special Reference to Southern Africa,*** 371-377.

SHPYNOV, S. FOURNIER, P. RUDAKOV, N. TARASEVICH, I. & RAOULT, D. (2006). Detection of members of the Genera *Rickettsia*, *Anaplasma*, and *Ehrlichia* in ticks collected in the Asiatic part of Russia. ***Annals of the New York Academy of Sciences,*** 1078(1): 378-383.

SIBEKO, K.P. (2010). Improved molecular diagnostics and characterization of *Theileria parva* isolates from cattle and buffalo in South Africa, PhD thesis, University of Pretoria, Pretoria, viewed 2018/11/13 < http://hdl.handle.net/2263/24872 >

SIBEKO, K.P. OOSTHUIZEN, M.C. COLLINS, N.E. GEYSEN, D. RAMBRITCH, N.E. LATIF, A.A. GROENEVELD, H.T. POTGIETER, F.T. & COETZER, J.A.W. (2008). Development and evaluation of a real-time polymerase chain reaction test for the detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle. ***Veterinary Parasitology***, 155: 37-48.

SILAGHI, C. HAMEL, D. PFISTER, K. & REHBEIN, S. (2011). *Babesia* species and co-infection with *Anaplasma phagocytophilum* in free-ranging ungulates from Tyrol (Austria). ***Tierärztliche Monatsschrift Veterinary Medicine Austria***, 98: 268-274.

SILVERSTEIN, A.M. (1998). On the naming of *rickettsiae* after Paul Ehrlich. ***Bulletin of the History of Medicine***, 72: 731-733.

SIMPSON, C.F. (1974). Relationship of *Ehrlichia canis* infected mononuclear-cells to blood-vessels of lungs. ***Infection and Immunity,*** 10: 590-596.

SISSON, D. HUFSCHMID, J. JOLLES, A. BEECHLER, B. & JABBAR, A. (2017). Molecular characterisation of *Anaplasma* species from African buffalo (*Syncerus caffer*) in Kruger National Park, South Africa. ***Ticks and Tick-Borne Diseases,*** 8: 400-406.

SLODKI, J. JASIK, K.P. KEPA, M. IDZIK, D. & WOJTYCZKA, R.D. (2011). Tick-transmitted diseases caused by Apicomplexa. ***Acta Protozoologica,*** 50: 155-161.

SMITH, T. & KILBORNE, F.L. (1893). Investigations into the nature, causation, and prevention of Texas or southern cattle fever. ***US Government Printing Office,*** 1.

SMITHERS, R.H.N. & WILSON, V.J. (1979). Check list and atlas of the mammals of Zimbabwe Rhodesia. ***Museum Memoir (Salisbury),*** i-vi, 1-193.

SOBEL, J.D. & AKINS, R.A. (2015). The role of PCR in the diagnosis of *Candida vulvovaginitis* - a new gold standard?. ***Current Infectious Disease Reports,*** 17: 488-488.

SONENSHINE, D.E. (1991). Biology of ticks. ***Oxford University Press: Oxford,*** **Volume 1.**

STARCOVICI, C. (1893). Bemerkungen über den durch Babès entdeckten Blutparasiten und die durch denselben hervorgebrachten Krankheiten, die seuchenhafte Hämoglobinurie des Rindes (Babès), das Texasfieber (Th. Smith) und der Carceag der Schafe (Babès). ***Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, Abteilung*** 14: 1-8.

STILLER, D. CROSBIE, P.R. BOYCE, W.M. & GOFF, W.L. (1999). *Dermacentor hunteri* (Acari: *Ixodidae*): an experimental vector of *Anaplasma marginale* and *A. ovis* (*Rickettsiales*: *Anaplasmataceae*) to calves and sheep. ***Journal of Medical Entomology,*** 36: 321-324.

STRIK, N.I. ALLEMAN, A.R. BARBET, A.F. SORENSON, H.L. WAMSLEY, H.L. GASCHEN, F.P. LUCKSCHANDER, N. WONG, S. CHU, F. FOLEY, J.E. BJOERSDORFF, A. STUEN, S. & KNOWLES, D.P. (2007). Characterization of *Anaplasma phagocytophilum* major surface protein 5 and the extent of its cross-reactivity with *A. marginale*. ***Clinical and Vaccine Immunology,*** 14: 262-268.

TALLEKLINT, L. & JAENSON, T.G.T. (1997). Infestation of mammals by *Ixodes ricinus* ticks (Acari: Ixodidae) in south-central Sweden. ***Experimental & Applied Acarology,*** 21: 755-771.

TAMURA, K. STECHER, G. PETERSON, D. FILIPSKI, A. & KUMAR, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. ***Molecular Biology and Evolution,*** 30: 2725-2729.

TATE, C.M. HOWERTH, E.W. MEAD, D.G. DUGAN, V.G. LUTTRELL, M.P. SAHORA, A.I. MUNDERLOH, U.G. DAVIDSON, W.R. & YABSLEY, M.J. (2013). *Anaplasma odocoilei* sp nov (family *Anaplasmataceae*) from white-tailed deer (*Odocoileus virginianus*). ***Ticks and Tick-Borne Diseases,*** 4: 110-119.

TEMBO, S. (2012). Occurrence of tick-borne haemoparasites in cattle in the Mungwi District, Northern Province, Zambia, MSc dissertation, University of Pretoria, Pretoria, viewed 2018/11/13< http://hdl.handle.net/2263/26218 >.

TOMANOVIC, S. CHOCHLAKIS, D. RADULOVIC, Z. MILUTINOVIC, M. CAKIC, S. MIHALJICA, D. TSELENTIS, Y. & PSAROULAKI, A. (2013). Analysis of pathogen co-occurrence in host-seeking adult hard ticks from Serbia. ***Experimental and Applied Acarology,*** 59: 367-376.

TORINA, A. GALINDO, R.C. VICENTE, J. DI MARCO, V. RUSSO, M. ARONICA, V. FIASCONARO, M. SCIMECA, S. ALONGI, A. CARACAPPA, S. KOCAN, K.M. GORTAZAR, C. & DE LA FUENTE, J. (2010). Characterization of *Anaplasma phagocytophilum* and *A. ovis* infection in a naturally infected sheep flock with poor health condition. ***Tropical Animal Health and Production,*** 42: 1327-1331.

TORIONI DE ECHAIDE, S. KNOWLES, D.P. MCGUIRE, T.C. PALMER, G.H. SUAREZ, C.E. & MCELWAIN, T.F. (1998). Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. ***Journal of Clinical Microbiology,*** 36: 777-82.

UETI, M.W. PALMER, G.H. SCOLES, G.A. KAPPMEYER, L.S. & KNOWLES, D.P. (2008). Persistently infected horses are reservoirs for intrastadial tick-borne transmission of the apicomplexan parasite *Babesia equi*. ***Infection and Immunity,*** 76: 3525-3529.

UILENBERG, G. (1995). International collaborative research - Significance of tick-borne hemoparasitic diseases to world animal health. ***Veterinary Parasitology,*** 57: 19-41.

WEI, F. SONG, M. LIU, H. WANG, B. WANG, S. WANG, Z. MA, H. LI, Z. ZENG, Z. & QIAN, J. (2016). Molecular detection and characterization of zoonotic and veterinary pathogens in ticks from Northeastern China. ***Frontiers in Microbiology,*** 7:1913.

WILSON, K. BJØRNSTAD, O. DOBSON, A. MERLER, S. POGLAYEN, G. RANDOLPH, S. READ, A. & SKORPING, A. (2002). Heterogeneities in macroparasite infections: patterns and processes. ***In: Hudson, P.J., Rizzoli, A., Grenfell, B.T., Heesterbeek, H. & Dobson, A.P. (Eds). The Ecology of Wildlife Diseases. Oxford University Press: Oxford***, 44: 6-44.

YABSLEY, M.J. & SHOCK, B.C. (2013). Natural history of zoonotic *Babesia*: Role of wildlife reservoirs. ***International Journal for Parasitology: Parasites and Wildlife***, 2: 18-31.

YANG, J. LIU, Z. NIU, Q. LIU, J. HAN, R. GUAN, G. HASSAN, M.A. LIU, G. LUO, J. & YIN, H. (2017). A novel zoonotic *Anaplasma* species is prevalent in small ruminants: potential public health implications. ***Parasites & Vectors***, 10: 264.

YBAÑEZ, A.P. MATSUMOTO, K. KISHIMOTO, T. & INOKUMA, H. (2012). Molecular analyses of a potentially novel *Anaplasma* species closely related to *Anaplasma phagocytophilum* detected in sika deer (*Cervus nippon yesoensis*) in Japan. ***Veterinary Microbiology,*** 157: 232-236.

YBAÑEZ, A.P. SASHIKA, M. & INOKUMA, H. (2014). The phylogenetic position of *Anaplasma bovis* and inferences on the phylogeny of the genus *Anaplasma*. ***Journal of Veterinary Medical Science,*** 76: 307-312.

ZANET, S. TRISCIUOGLIO, A. BOTTERO, E. FERNANDEZ DE MERA, I.G. GORTAZAR, C. CARPIGNANO, M.G. & FERROGLIO, E. (2014). Piroplasmosis in wildlife: *Babesia* and *Theileria* affecting free-ranging ungulates and carnivores in the Italian Alps. ***Parasites & Vectors,*** 7: 70.

ZHANG, Y. LI, T. CUI, Y. WANG, J. LV, Y. WANG, R. JIAN, F. ZHANG, L. WANG, J. YANG, G. & NING, C. (2016). The first report of *Anaplasma phagocytophilum* and a novel *Theileria* spp. co-infection in a South African giraffe. ***Parasitology International,*** 65: 347-351.

ZOBBA, R. ANFOSSI, A.G. PINNA PARPAGLIA, M.L. DORE, G.M. CHESSA, B. SPEZZIGU, A. ROCCA, S. VISCO, S. PITTAU, M. & ALBERTI, A. (2014). Molecular investigation and phylogeny of *Anaplasma* spp. in Mediterranean ruminants reveal the presence of neutrophil-tropic strains closely related to *A. platys*. ***Applied and Environmental Microbiology,*** 80: 271-280.

APPENDICES

Appendix I: Letter approval for the MSc study "Tick-borne haemoparasite occurrence and *Anaplasma bovis* strain diversity in eastern rock sengis (*Elephantulus myurus*)" issued by the Animal Ethics Committee, University of Pretoria, South Africa.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Haemoparasite occurrence and diversity in rock sengis *(Elepantulus myurus)* and their associated tick species**
PROJECT NUMBER	**V081-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**R Jacobs**

STUDENT NUMBER (where applicable)	**UP_16338147**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Rock sengis *(Elepantulus myurus)***	
NUMBER OF ANIMALS	**94**	
Approval period to use animals for research/testing purposes		**January 2017-January 2018**
SUPERVISOR	**Prof. M Oosthuizen**	

KINDLY NOTE:

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

APPROVED	Date 24 January 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

Appendix II: Permission to do research in terms of Section 20 of the Animal Diseases ACT, 1984 (ACT No. 35 of 1984) for the research project "Tick-borne haemoparasite occurrence and *Anaplasma bovis* strain diversity in eastern rock sengis (*Elephantulus myurus*)", issued by the Department of Agriculture, Forestry and Fisheries, Pretoria, South Africa.

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za

Prof Marinda Oosthuizen
Veterinary Tropical Diseases,
Faculty of Veterinary Science,
University of Pretoria

Email: Marinda.oosthuizen@up.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear Prof Oosthuizen

Your application, received on 7 October 2016, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him/her by any other Act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. Only rock sengi extracted DNA and tick samples collected during previous studies at the university, as outlined in the Section 20 application, may be utilised in the current study. No new field samples are permitted to be collected or utilised;
4. Please note that Section 20 approval is required to be obtained prior to the collection of field samples intended for research purposes;

Title of research/study: Tick-borne haemoparasite occurrence and *Anaplasma bovis* strain diversity in eastern rock sengis (*Elephantulus myurus*)
Researcher: Prof Marinda Oosthuizen
Institution: Department of Veterinary Tropical Diseases, University of Pretoria
Reference: 12/11/1/1/8

Kind regards,

Maja.

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2016 -12- 07

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8

Prof Marinda Oosthuizen
Veterinary Tropical Diseases,
Faculty of Veterinary Science,
University of Pretoria

Email: Marinda.oosthuizen@up.ac.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "TICK-BORNE HAEMOPARASITE OCCURRENCE AND *ANAPLASMA BOVIS* STRAIN DIVERSITY IN EASTERN ROCK SENGIS (*ELEPHANTULUS MYURUS*)"

Dear Prof Oosthuizen

A dispensation is hereby granted on Point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Extracted DNA, selected for long term storage, may be stored at the -80°C freezer at the Department of Veterinary Tropical Diseases, University of Pretoria;

ii) Stored samples may not be outsourced or used for further research without prior written approval from DAFF.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2016 - 12 - 07

Appendix III: Summary of results obtained within this study along with the results from the study of Harrison *et al*. (2013).

Sample No	Location	Harrison results with Parola primers	RLB Results	16S rRNA results from current study	*GroEL* results from current study
1	Goro	*A. bovis*	A/E catch-all, T. catch-all (vf)		
2	Goro	*A. bovis*	Negative		
3	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi) (vf)		
5	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi) (f)	Genotype B – Closest match is to Uncultured *Anaplasma* sp. clone ES1	
6	Goro	Negative	B. catch-all 1, B. catch-all 2		
7	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi) (vf)		
8	Goro	Negative	Negative		
9	Goro	Negative	Negative		
10	Goro	Negative	A/E catch-all (vf)		
11	Goro	Negative	Negative		
12	Goro	*A. bovis*	A/E catch-all		
13	Goro	Negative	Negative		
14 (22)	Goro	Negative	Negative		
15	Goro	Negative	A/E catch-all		
16	Goro	*A. bovis*	Negative		
17	Goro	Negative	Negative		
18 (20)	Goro	Negative	Negative		
19	Goro	Negative	Negative		
21	Goro	Negative	Negative		
23	Goro	Negative	Negative		
24	Goro	Negative	Negative		
25	Goro	Negative	Negative		
26	Goro	Negative	A/E catch-all		
27	Goro	Negative	A/E catch-all		
28 (31)	Goro	*A. bovis*	T. catch-all, B catch-all 1(f)	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone ES1	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone 499
29	Goro	Negative	Negative		

Sample No	Location	Harrison results with Parola primers	RLB Results	16S rRNA results from current study	*GroEL* results from current study
30	Goro	Negative	Negative		
32	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi) (vf)		
33	Goro	Negative	Negative		
34	Goro	Negative	Negative		
35	Goro	Negative	Negative		
36	Goro	Negative	A/E catch-all, T/B catch-all, B. catch-all 1, B. catch-all 2, *B. bovis*		
37	Goro	Negative	B. catch-all 1; B. catch-all 2		
38	Goro	*A. bovis*	A/E catch-all		
39	Goro	Negative	A/E catch-all		
40	Goro	Negative	Negative		
41	Goro	Negative	Negative		
4(42)	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi) (vf)	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone ES1	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone 499
43	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi)	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone ES1	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone 499
44	Goro	Negative	B. catch-all 1; B. catch-all 2		
46	Goro	*A. bovis*	A/E catch-all		
47	Goro	Negative	Negative		
48	Goro	Negative			
49	Goro	*A. bovis*	Negative		
50	Goro	Negative	B. catch-all 1; B. catch-all 2		
52	Goro	Negative	B. catch-all 1; B. catch-all 2		
53	Goro	Negative	Negative		
54	Goro	Negative			
55	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi) (f)	Genotype B – Closest match is to Uncultured *Anaplasma* sp. clone ES1	
56	Goro	Negative	Negative		
57	Goro	Negative	Negative		
58	Goro	Negative	Negative		
59	Goro	Negative	B. catch-all 1; B. catch-all 2		

Sample No	Location	Harrison results with Parola primers	RLB Results	16S rRNA results from current study	*GroEL* results from current study
60	Goro	Negative	Negative		
61	Goro	Negative	B. catch-all 1 (f)		
62	Goro	Negative	Negative		
63	Goro	Negative	Negative		
64	Goro	Negative	Negative		
65	Goro	Negative	Negative		
66	Goro	Negative	Negative		
67	Goro	Negative	Negative		
68	Goro	Negative	Negative		
69	Goro	Negative	Negative		
71	Goro	Negative	T. catch-all (vvf)		
72	Goro	Negative	Negative		
73	Goro	Negative	A/E catch-all		
75	Goro	Negative	A/E catch-all (vvf)		
76	Goro	Negative	Negative		
77	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi) (vf)		
78	Goro	Negative	T/B catch-all, B. catch-all 1, B. catch-all 2		
79	Goro	Negative	T/B catch-all (vf); B. catch-all 1; B. catch-all 2 (vf)		
80	Goro	Negative	Negative		
82	Goro	*A. bovis*	A/E catch-all		
83	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi) (f)		
84	Goro	Negative	Negative		
85	Goro	Negative	A/E catch-all		
86	Goro	Negative	Negative		
87	Goro	*A. bovis*	A/E catch-all (f)		
88	Goro	Negative	B. catch-all 1; B. catch-all 2 (vf)		
89	Goro	Negative	B. catch-all 1; B. catch-all 2 (vf)		
90	Goro	Negative	Negative		
91	Goro	Negative	B. catch-all 1; B. catch-all 2 (f)		
92	Goro	*A. bovis*	A/E catch-all		

Sample No	Location	Harrison results with Parola primers	RLB Results	16S rRNA results from current study	*GroEL* results from current study
93	Goro	Negative	A/E catch-all (vf)		
94	Goro	*A. bovis*	A/E catch-all (vf)		
51(95)	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi) (vf), T/B catch-all (vf), B. catch-all 1, B. catch-all 2	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone ES1	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone 499
96	Goro	*A. bovis*	A/E catch-all		
97	Goro	*A. bovis*	A/E catch-all		
98	Goro	Negative	Negative		
99	Goro	Negative	Negative		
100	Goro	Negative	B. catch-all 1, B. catch-all 2		
101	Goro	Negative	Negative		
102	Goro	*A. bovis*	Negative		
103	Goro	*A. bovis*	Negative		
104	Goro	Negative	Negative		
105	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi) (vf)	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone ES1	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone 499
106	Goro	Negative	Negative		
107	Goro	Negative	T. catch-all (vvf)		
108	Goro	Negative	Negative		
109	Goro	*A. bovis*	A/E catch-all, *A. bovis*-like (sengi) (f), T/B catch-all (f), B. catch-all 1, B. catch-all 2	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone ES1	Genotype B-D – Closest match is to Uncultured *Anaplasma* sp. clone 499
110	Goro	*A. bovis*	A/E catch-all, T. catch-all, B. catch-all 1		
111	Goro	Negative	B. catch-all 1; B. catch-all 2 (vvf)		
112	Goro	Negative	Negative		
113	Goro	*A. bovis*	A/E catch-all		
New samples from Hatyoka, 2018					
L27-14	Goro	ND	Negative		
L35-14	Goro	ND	Negative		
L43-14	Goro	ND	A/E catch-all (f), T/B catch-all, B. catch-all 1, B. catch-all 2		
L6-15	Goro		Negative		
L20-14	Goro		Negative		

Sample No	Location	Harrison results with Parola primers	RLB Results	16S rRNA results from current study	*GroEL* results from current study
L61-14	Goro		Negative		
L43-15	Goro		Negative		
L52-14	Goro		Negative		
L4	Ezemvelo		T. catch-all (vf)		
L5	Ezemvelo		Negative		
L6	Ezemvelo		A/E catch-all, *A. bovis*-like (sengi) (vf)		
L9	Ezemvelo		Negative		
L10	Ezemvelo		A/E catch-all, *A. bovis*-like (sengi) (vf)	Genotype A-C – Closest match is to Uncultured *Anaplasma* sp. clone ES1	Genotype A-C – Closest match is to Uncultured *Anaplasma* sp. clone 499
L11	Ezemvelo		A/E catch-all, *A. bovis*-like (sengi) (vvf)	Genotype A – Closest match is to Uncultured *Anaplasma* sp. clone ES1	
L14	Ezemvelo		Negative		
L15	Ezemvelo		Negative		
L16	Ezemvelo		T. catch-all, B. catch-all 1, B. catch-all 2 (f)		
L19	Ezemvelo		Negative		
L22	Ezemvelo		T. catch-all, B. catch-all 1, B. catch-all 2		
L27	Ezemvelo		Negative		
L28	Ezemvelo		Negative		
L29	Ezemvelo		Negative		
L42	Ezemvelo		Negative		
L43	Ezemvelo		Negative		
L44	Ezemvelo		Negative		
L47	Ezemvelo		A/E catch-all (vvf)		
L49	Ezemvelo		Negative		
L50	Ezemvelo		A/E catch-all, T/B catch-all, B. catch-all 1, B. catch-all 2 (f)		
L51	Ezemvelo		A/E catch-all (f), T/B catch-all, B. catch-all 1, B. catch-all 2 (f)		
L53	Ezemvelo		Negative		
L55	Ezemvelo		A/E catch-all		
L56	Ezemvelo		B. catch-all 1		
L57	Ezemvelo		T. catch-all		

Sample No	Location	Harrison results with Parola primers	RLB Results	16S rRNA results from current study	*GroEL* results from current study
L58	Ezemvelo		Negative		
L59	Ezemvelo		Negative		
L61	Ezemvelo		A/E catch-all, B. catch-all 1		
L62	Ezemvelo		A/E catch-all		
L63	Ezemvelo		A/E catch-all, T. catch-all, B. catch-all 1, B. catch-all 2		
L65	Ezemvelo		A/E catch-all, T. catch-all	Genotype B-C – Closest match is to Uncultured *Anaplasma* sp. clone ES1	Genotype B-C – Closest match is to Uncultured *Anaplasma* sp. clone 499
L67	Ezemvelo		Negative		
L68	Ezemvelo		A/E catch-all (f), T. catch-all (vvf)		
L73	Ezemvelo		Negative		
L74	Ezemvelo		Negative		
L75	Ezemvelo		A/E catch-all, *A. bovis*-like (sengi) (vf), T. catch-all (f)		
L77	Ezemvelo		A/E catchall, *A. bovis*-like (sengi) (vvf)	Genotype B – Closest match is to Uncultured *Anaplasma* sp. clone ES1	
L78	Ezemvelo		A/E catch-all, T. catch-all		
L79	Ezemvelo		Negative		
L83	Ezemvelo		Negative		
L85	Ezemvelo		T. catch-all, B. catch-all 1 (vf), B. catch-all 2 (vvf)		
L87	Ezemvelo		Negative		
L88	Ezemvelo		Negative		
L93	Ezemvelo		T. catch-all, B. catch-all 1 (vf), B. catch-all 2 (vvf)		
L94	Ezemvelo		Negative		
L97	Ezemvelo		Negative		
L100	Ezemvelo		A/E catch-all (vvf)		
L104	Ezemvelo		Negative		

A/E catch-all = *Anaplasma/Ehrlichia* genus-specific probe
B1 catch-all = *Babesia* genus-specific probe 1
B2 catch-all = *Babesia* genus-specific probe 2
T catch-all = *Theileria* genus-specific probe
Negative = Considered negative or below the detection limit of the assay

(f) = faint
(vf) = very faint
(vvf) = extremely faint
ND = Not Done
*Numbers highlighted with blue indicates those that were subjected to Genomiphi amplification