Tick-borne haemoparasite occurrence and *Anaplasma* species diversity in selected South African wild rodents and domestic dogs

by

Samantha Kelly Wills-Berriman

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Supervisor: Prof Marinda C Oosthuizen

Co-supervisor: Dr Mamohale E Chaisi

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DEDICATION

To my husband who has provided me with endless love and support during this degree.

To my parents for always encouraging me to further my studies, this wouldn't have been possible without you.

To my brothers who have motivated me to be the best I can be.

To my friends, to show you if I can, then so can you.

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Co-Supervisor: Dr ME Chaisi
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SUMMARY

Since its first discovery, *Anaplasma phagocytophilum* has gained interest in both veterinary (and human) medicine as the causative agent of tick-borne fever in ruminants and granulocytic anaplasmosis in humans and in a wide variety of domestic animals. The enzootic cycle includes rodents, wild ungulates and possibly birds as reservoir hosts. Reports of human granulocytic anaplasmosis occurring in Africa have been few. In South Africa there have been no official diagnoses of *A. phagocytophilum* in humans. The first report of the molecular detection of a bacterium closely related to *A. phagocytophilum* in South Africa was in whole blood specimens from dogs in 2005, which was designated *Anaplasma* sp. South African dog strain. More recently, the same *Anaplasma* sp. South African dog strain as well as *A. phagocytophilum* and an *Orientia tsutsugamushi*-like partial 16S rRNA sequence were detected in several dog samples collected in the Mnisi community, Bushbuckridge, Mpumalanga, South Africa. Little is known about the significance of this finding. The aim of this study was, therefore, to determine the occurrence of *A. phagocytophilum* in selected small rodent species collected from four South African provinces and in dogs from the Mnisi community, and to molecularly characterize the *Anaplasma* species found.

Molecular analysis was carried out on 37 wild rodent blood samples (*Rhabdomys pumilio*, *Rhabdomys dilectus*, *Micaelamys namaquensis* and *Myotomys unisulcatus*) collected from four provinces within South Africa and 56 domestic dog blood samples collected from the Mnisi community, Bushbuckridge, Mpumalanga, South Africa. All samples were screened for the presence of *Anaplasma*, *Ehrlichia*, *Theileria* and *Babesia* species using the Reverse Line Blot (RLB) hybridization assay. The same samples were also subjected to real-time quantitative PCR (qPCR) analysis for the specific detection of *A. phagocytophilum*. The RLB results have shown single infections of *A. bovis* (3.3%), *Babesia microti* (3.3%) and *Ehrlichia ruminantium*

(3.3%) in *R. pumilio* (four-striped grass mouse) while *Anaplasma* sp. Omatjenne (33.1%) and *E. ruminantium* (33.1%) were detected in *M. namaquensis* (Namaqua rock mouse). There were 83.3% (n = 25) mixed infections in *R. pumilio;* of which *B. microti* was the highest (56.7%). There were no mixed infections in the other three rodent species. For both *R. dilectus* (mesic four-striped grass rat) and *M. unisalcatus* (Karoo bush rat), PCR products only hybridized with the genus-specific probes, and with none of the species-specific probes; indicating the presence of novel species or a variant of a species. In dogs, single infections of *B. microti* (3.6%), *B. rossi* (3.6%) and *E. canis* (21.4%) were detected. Mixed infections were present in 50% of the dog samples. The RLB assay only detected 4.3% *A. phagocytophilum* positive samples in both rodents and dogs whereas qPCR detected 57% *A. phagocytophilum* DNA positive samples in both rodents and dogs; most propably due to the *A. phagocytophilum* real-time PCR probe cross-reacting with *Anaplasma* sp. Zambian and South African dog strain DNA. The pathogen's 16S rRNA gene was subsequently amplified, cloned and sequenced from eight samples (7 dogs; 1 rodent) that tested positive by the qPCR assay. A total of 36 recombinant sequences were obtained from the eight samples. BLASTn homology searches showed that the obtained sequences had 98-100% sequence identity to published sequences of *A. phagocytophilum*, *A. platys*, *Anaplasma* sp. from Zambian and South African dogs; and *A. bovis*. The observed sequence similarities were confirmed by Neighbor-joining and Maximum Likelihood phylogenetic analyses.

The study highlighted the importance of wild rodents and dogs as reservoir species for haemoparasites that are of medical and veterinary importance. We have shown that: (i) wild rodents and domestic dogs in South Africa habour many tick-borne pathogens, some of which are animal and human pathogens, further studies should be carried out to determine the risk of these infections to human health; (ii) the qPCR assay was more sensitive than the RLB assay in detecting *A. phagocytophilum* infections, however, the specificity of the assay should be confirmed; (iii) sequence analyses confirmed the presence of *A. phagocytophilum* DNA in a dog; and (iv) *Anaplasma* sp. Zambian and South African dog strain seems to be a common species in South African dogs, more studies are needed to determine the taxonomic status and epidemiology of these species in South Africa.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1
INTRODUCTION

In the past 60 years it has been estimated that up to 65% of emerging infectious diseases have been attributed to zoonotic agents including bacteria, parasites, fungi, and viruses (Paweska, 2012). These frequently originate from zoonotic transmission events implicating wildlife reservoirs (Jones *et al*., 2008), with the highest risk for emergence being seen in tropical countries with high biodiversity and poor infrastructure (Wolfe *et al*., 2007); resulting in US$20 billion direct cost to the affected economies (World Bank, 2010). Zoonotic pathogens continue to emerge with >15 new tick borne pathogens identified since the discovery of Lyme disease in 1981 (Jongejan *et al*., 2004). The effects of climate change, urbanization and farming practices, together with an increase in travel and trade, are regarded as global drivers for the spread of infectious diseases. Of relevance in Africa is the extensive human/wildlife interactions, increased land-usage and the current socio-economic conditions. In South Africa, the public health burden of zoonotic agents is still poorly understood (Paweska, 2012). The identification and characterization of microorganisms in wildlife reservoirs and their vectors are, therefore, central to the development of in-country health surveillance systems to identify these potential pathogens before they enter the human population (Wolfe *et al*., 2007; Jones *et al*., 2008).

In South Africa, the role of ticks in the transmission of disease agents (especially among livestock) has been widely reported, however, their role as vectors of human diseases has been under-reported. In a review done by Hotez and Kamath (2009) on neglected tropical diseases, they stated that there was a lack of information on tick-borne zoonoses in sub-Saharan Africa; making it difficult to assess their impact. Chitanga *et al*. (2014), provided preliminary information on the tick-borne pathogens of potential zoonotic importance present in southern Africa, including Crimean-Congo haemorrhagic fever; ehrlichiosis (*Ehrlichia ruminantium*, *E. canis* and *Anaplasma phagocytophilum*); babesiosis (*Babesia microti*); relapsing fever (*Borrelia duttonii*) and rickettsioses (*Rickettsia africae*, *R. aeschlimannii* and *R. conorii*). A survey investigating the occurrence of zoonotic diseases among South African veterinarians indicated that 63.6% had suffered from one or more zoonoses (Gummow, 2003); African tick bite fever (*R. africae*) and Q fever (*Coxiella burnetii*) were amongst the 22 zoonotic diseases

listed. Many of these diseases could not immediately be diagnosed by general practitioners and only with the assistance of the veterinarian; highlighting the fact that zoonotic diseases are not only difficult to diagnose, but are also frequently misdiagnosed or missed. In malaria endemic areas, they can easily be misdiagnosed as malaria. It was hypothesized that the incidence and severity of certain zoonotic conditions are likely to increase at rates similar to the HIV epidemic, adding to an already severe situation (Gummow, 2003). Testing for zoonotic pathogens in ticks (n = 1634) collected from livestock in four South African provinces (Mtshali *et al*., 2015) indicated the presence of *A. phagocytophilum* (7%); *C. burnetii* (7%); *E. ruminantium* (28%) and *Rickettsia* spp. (27%) DNA, while *B. burgdorferi* DNA could not be confirmed. In a subsequent study, ticks (n = 318) collected from dogs and cats in four South African provinces (Mtshali *et al*., 2015), indicated the presence of *C. burnetti* DNA (41%), *Ehrlichia* or *Anaplasma* spp. (18%), *Rickettsia* spp. (37%), *A. phagocytophilum*-like bacterium (18%) and *E. canis* (19%). A study examining ticks (n = 205) collected from nine wildlife species and domestic animals from two provinces in South Africa (Halajian *et al.,* 2016) found *R. massiliae* (5.4%) DNA in *Amblyomma sylvaticum* and *Rhipicephalus simus,* and *R. africae* (3.4%) DNA in *A. hebraeum*; research to assess the impact of these pathogens and the risk of transmission to humans and domestic animals was recomemnded (Halajian *et al*., 2016). Recently, Essbauer *et al*. (2018) have shown a high diversity of *Rickettsia* spp. in small mammals with a prevalence of 15.7% (1616 samples from 23 species) in South Africa and Namibia; *R. conorii, R. massiliae, R. felis* and *R. helvetica*, known to be pathogenic for humans, were described. Based on these findings, the authors recommended further studies to determine the prevalence and significance of these pathogens (and their vectors) in the country. The impact of the newly described "*Candidatus* Rickettsia" and other genotypes on human health in South Africa and neighboring countries should also be determined.

Furthermore, wild rodent blood samples (n = 423) from four provinces in South Africa were recently screened for tick-borne haemoparasites using the Reverse Line Blot (RLB) hybridization assay (Troskie, 2017). The results revealed the presence of *B. microti* (11.3%), *A. phagocytophilum* (1.8%), *A. bovis* (3.5%), and *E. ruminantium* (3.5%) either as single or mixed infections. In another investigation, a bacterium closely related to *A. phagocytophilum* (designated *Anaplasma* sp. South African dog strain) was detected in dog blood samples collected from the Mnisi community, Bushbuckridge, Mpumalanga, South Africa as well as *A. phagocytophilum*, and an *Orientia tsutsugamushi*-like partial 16S rRNA sequence (Kolo *et al*., 2016). Little is known about the significance of these findings; highlighting the potential risk

of human infection with these pathogens. The aim of this study was, therefore, to determine the tick-borne haemoparasite diversity and specific occurrence of *A. phagocytophilum* in selected South African small rodent species and domestic dogs, and to molecularly characterize the *A. phagocytophilum* strains found. The specific objectives were:

(i) To screen blood samples collected from wild rodents from four provinces in South Africa, and from domestic dogs from the Mnisi community for the presence of haemoparasites using the RLB hybridization assay.

(ii) Specific detection of *A. phagocytophilum* infections using a previously described real-time quantitative PCR (qPCR) assay.

(iii) Molecular characterization of *A. phagocytophilum* using 16S rRNA gene sequencing and phylogenetic analysis.

CHAPTER 2
LITERATURE REVIEW

Anaplasma phagocytophilum, a tick-transmitted intra-granulocytic Gram-negative bacterium of the family Anaplasmataceae, is an emerging zoonotic pathogen in humans and animals worldwide. It is the causative agent of human granulocytic anaplasmosis (HGA) in humans, tick-borne fever (TBF) in ruminants, equine granulocytic anaplasmosis (EGA) in horses and canine granulocytic anaplasmosis (CGA) in dogs (Woldehiwet, 2010). The enzootic cycle includes rodents (Bown *et al*., 2003), wild ungulates and possibly birds (Bjoersdorff *et al*., 2001) as reservoir hosts. Dogs are considered as accidental hosts; *A. phagocytophilum* causes an acute febrile illness in dogs with lethargy and inappetence. To date, there are no case reports documenting fatalities in dogs (Carrade *et al*., 2009). Humans are accidental hosts (Parola *et al*., 2005; Carrade *et al*., 2009); the severity of HGA ranges from mild to more serious infections and in rare cases death. Reports of HGA occurring in Africa have been few (M'Ghirbi *et al*., 2009; 2012). In South Africa there have been no official diagnoses of *A. phagocytophilum* in humans.

2.1 History and taxonomic classification

There are 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 *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), however, is not a formally recognized species and not on the List of Prokaryotic Names with Standing in Nomenclature (www.bacterio.net/anaplasma.html).

Anaplasma phagocytophilum was first described in sheep from Scotland (Gordon *et al*, 1940; Foggie, 1951). During an experimental study on louping-ill, sheep contracted an unknown febrile reaction on tick-infested pastures. The disease was given the provisional name "tick-borne fever" (TBF); the causative agent was first classified as *Rickettsia phagocytophila* in 1951 (Foggie, 1951). Based on morphology, it was included in the genus *Cytoecetes*, tribe *Ehrlichia*, as *C. phagocytophila* in 1962 (Foggie, 1962). In 1969, a similar disease (equine

granulocytic ehrlichiosis) was described in horses in the USA (Gribble, 1969) caused by *E. equi* (now known as equine granulocytic anaplasmosis, EGA) (Woldehiwet, 2010). In 1974, *C. phagocytophila* was named *Ehrlichia phagocytophila* in Bergey's Manual (Philip, 1974). This was followed by the description of a distinct granulocytic agent causing a similar disease in dogs in the USA in 1982; canine granulocytic anaplasmosis (CGA) (Madewell and Gribble, 1982). The discovery of *E. chaffeensis* in 1986, causative agent of human monocytic ehrlichiosis (Maeda *et al*., 1987; Anderson *et al*., 1992), and the agent of human granulocytic ehrlichiosis (which is now reported as human granulocytic anaplasmosis, HGA) in 1994 in the USA (Chen *et al.*, 1994) prompted further studies on the taxonomy of the granulocytic *Ehrlichiae* (Ogden *et al*., 1998); this led to the genus *Ehrlichia* being divided into three genogroups, of which the granulocytic group contained *E. phagocytophilum, E. equi* and the agent of HGE. Later, a reclassification of the genus *Ehrlichia* was proposed. In 2001, the order Rickettsiales was reorganized and the genera within the families *Rickettsiaceae* and *Anaplasmataceae* were reclassified using phylogenetic analyses of 16S rRNA and *groESL* genes, supported by biological data (Dumler *et al*., 2001); the three species of granulocytic bacteria, *E. phagocytophila, E. equi* and the agent of HGE were united within the single species designation *Anaplasma phagocytophilum*. The current classification of this organism is as stated below (Dumler *et al*., 2001).

Kingdom: Bacteria / Prokaryota
Phylum: Proteobacteria
Class: Alpha Proteobacteria
Order: Rickettsiales
Family: Anaplasmataceae
Genus: *Anaplasma*
Type species: *Anaplasma phagocytophilum*

2.2 Morphology

As with other members of the family *Anaplasmataceae, A. phagocytophilum* is a small, Gram-negative, pleomorphic coccus enveloped by two membranes. The bacterial size is generally 0.4 to 1.3 µm, but it can be as large as 2 µm (Dumler 2005; Stuen, 2007; Rymaszewska and Grenda, 2008; Bowman *et al*., 2009; Carrade *et al*., 2009; St. Clair and Decker, 2012). The outer membrane of the bacterium is often ruffled, creating an irregular periplasmic space; there is no capsule layer.

Anaplasma phagocytophilum has a specific affinity for leukocytes where it is known to replicate within the vacuoles (Rymaszewska and Grenda, 2008; Bowman *et al*., 2009; St. Clair and Decker, 2012). The bacteria reside in early endosomes where they obtain nutrients for binary fission. Clusters of bacteria known as microcolonies, are formed within the vacuoles, and they are called "morulae" due to the characteristic mulberry-like structure (Foggie, 1951; Dumler 2005; St. Clair and Decker, 2012). These microcolonies exist as a survival strategy to overcome phagocytosis (St. Clair and Decker, 2012).

2.3 Life cycle

Vertebrates act as the reservoir where the bacteria live and proliferate for many years (Rymaszewska and Grenda, 2008). Tick vectors form an essential part of the life cycle of the bacteria as they contribute to circulating the pathogen in the environment.

Larval, nymphal, or adult stage ticks acquire *A. phagocytophilum* strains through blood feeding on infected animals. Thereafter the pathogen enter the tick midgut epithelium, where primary replication takes place (Ueti *et al*., 2009). It then moves to the salivary epithelial cells where the bacteria undergo a second round of replication. After that it enters the salivary gland secretion when the tick feeds on the next vertebrate host. The bacteria are then transmitted to a susceptible host during the next feeding stage (Ismail *et al*., 2010; Rakihisa, 2011). For the bacteria to fulfil its purpose it is important that it can attach to and enter the susceptible host cells (Rakihisa, 2011). *Anaplasma phagocytophilum* is maintained mostly in circulating neutrophils (Rakihisa, 2011). Once the bacterium has successfully entered the host cell it will start to multiply within membrane bound inclusions where it will carry on dividing and proliferating until the inclusion body has nearly completely filled the cytoplasm of the infected cell (Ismail *et al*., 2010; Rakihisa, 2011). During this process, the bacterium interferes with vesicular trafficking to avoid being destroyed, it infects granulocytes resulting in their innate defences being altered resulting in the host being more susceptible to opportunistic infections (Rakihisa, 2011). The bacteria disrupts normal cellular processes to escape destruction providing enough time for it to multiply, and also has the ability to activate signals within the host cells to optimize its ability to infect the cells (Ismail *et al*., 2010).

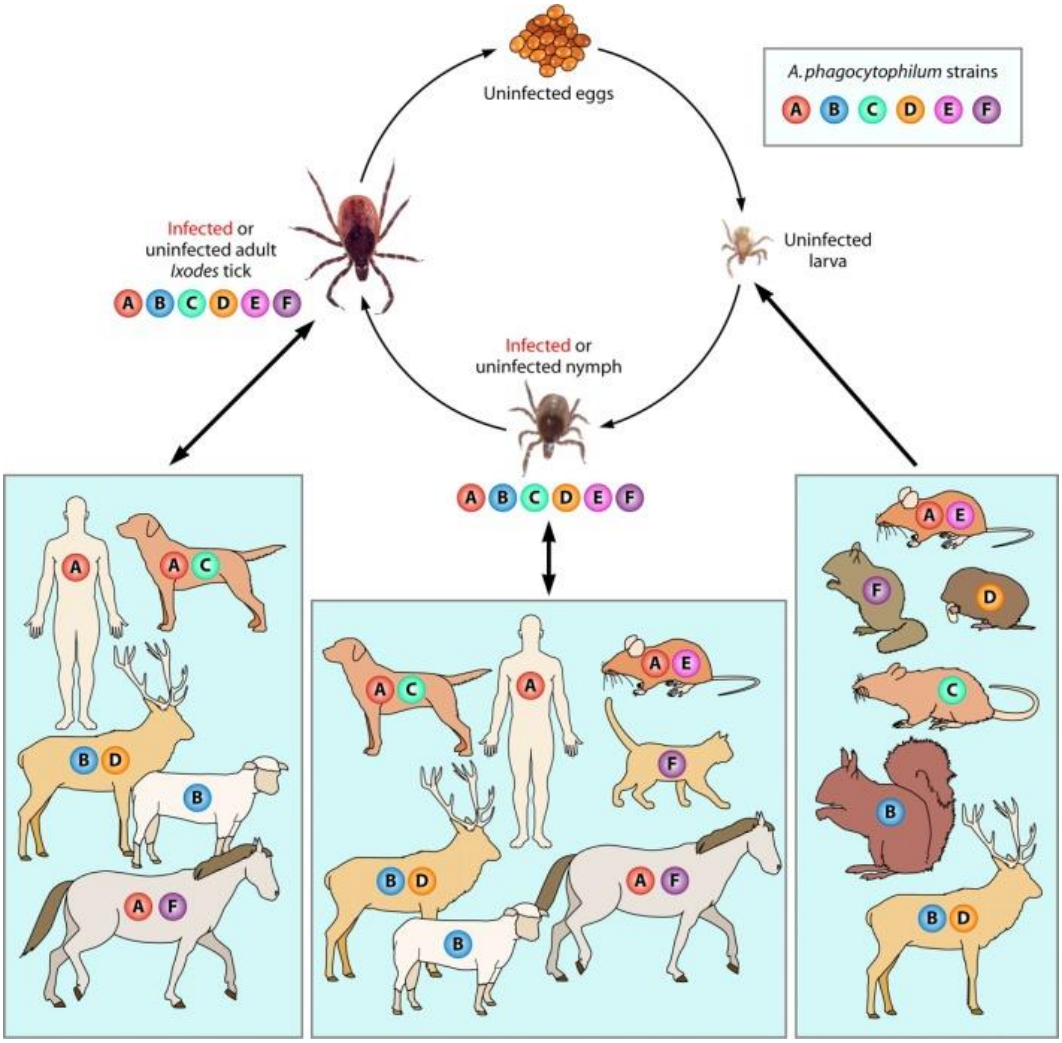

Figure 2.1: Life cycle of *A. phagocytophilum* showing different strains that infect different host species (Rikihisa, 2011).

Once the larval or nymphal stage of ticks are infected, *A. phagocytophilum* is maintained in the ticks through metamorphosis and moulting to the next life stage. It will then be transmitted to an animal via blood feeding when the animal host is susceptible to that particular strain. In case of humans: humans are susceptible to a limited number of strains, are considered dead-end hosts, and are not a normal part of the life cycle of *A. phagocytophilum* or ticks (Rikihisa, 2011).

2.4 Transmission

Pathogens are maintained in nature through transmission cycles between ticks and their vertebrate hosts, without the vectors the pathogens would not be able to survive (Hojgaard *et*

al., 2014). Recent studies have indicated that migrating birds could play an important role in dispersing *A. phagocytophilum* infected *Ixodes ricinus* ticks in Europe (Stuen, 2007).

2.4.1 Vectors

The tick genera involved in transmitting *Anaplasma* species are *Ixodes*, *Dermacentor*, *Rhipicephalus* and *Amblyomma* (Rymaszewska and Grenda, 2008; Carrade *et al*., 2009). According to Bowman *et al*, (2009) and Carrade *et al*. (2009), *A. phagocytophilum* is transmitted by *I. scapularis* in Northwest and upper Midwestern States while *I. pacificus* transmits the pathogen in the Western states, *I. ricinus* is the main European vector which is found universally and *I. persulcatus* and *I. trianguloceps* are important in South Europe and eastern borders of Europe and Asia (Stuen, 2007; Rymaszewska and Grenda, 2008; Carrade *et al*., 2009). The black legged tick (*I. scapularis*) is the principal vector of several pathogens that are important to public health, one of them being *A. phagocytophilum* (Courtney *et al*., 2004; Rymaszewska and Grenda, 2008; Hojgaard *et al*., 2014). While in North and Central America the lonestar tick (*A. americanum*) plays an important role in transmission (Rymaszewska and Grenda, 2008).

In Japan, India, Russia, China, Korea and Thailand as well as North America species from the *Haemaphysalis* genus are also important transmitters, especially *H. leporispalustris*, *H. lagrangei*, *H. longicornis* while *H. punctata* has been found to be important in the bordering regions of South Europe and eastern Europe and Asia (Stuen, 2007; Rymaszewska and Grenda, 2008; Carrade *et al*., 2009). Other tick vectors such as *Rhipicephalus sanguineus* have also been associated with the transmission of *A. phagocytophilum* but are more important in South Europe and eastern borders of Europe and Asia (Stuen, 2007; Rymaszewska and Grenda, 2008; Carrade *et al*., 2009).

According to Stuen (2007), vector species other than ticks that transmit *A. phagocytophilum* have not been found, however the bacterium has been identified in *Neutrombicula autumnalis* and *Syringophilidae* quill mites.

Norval and Horak (2004) stated that in South Africa around 90 ixodid tick species and 25 argasid tick species have been identified, only 35 have been associated with livestock while only approximately 15 are of economical importance. Mtshali *et al.* (2015) discussed that in South Africa the recognized vectors of *A. phagocytophilum* (I. *persulcatus*, *I. ricinus* and *I.*

pacificus) are absent and therefore the possibility of other tick species that are present in abundance should be considered to be possible vectors, such as, *Rhipicephalus* spp., *R. e. evertsi*, *R. decoloratus* and *A. hebraeum*. However *A. phagocytophilum* transmission was not mentioned specifically (Spikett *et al*., 2011) and therefore the tick species that transmits *A. phagocytophilum* in South Africa is still unknown.

2.4.2 Host range

The host range includes numerous animals such as dogs, sheep, cattle, horses, goats, cats and various wildlife species such as roe deer, red foxes, European bison, wild boars, moose and red deer (Stuen, 2007; Rymaszewska and Grenda, 2008; Bowman *et al*., 2009; Woldehiwet, 2010). According to Stuen (2007), and Rymaszewska and Grenda (2008), *A. phagocytophilum* can also cause clinical manifestations in humans. Antibodies for *A. phagocytophilum* have also been detected in hare and Eurasian lynx (Stuen, 2007).

It was only recently recognized that rodents harbour *A. phagocytophilum* variants; previously it was believed that the parasite was maintained in a ruminant-tick cycle (Woldehiwet, 2010). However, the primary reservoir host is now thought to be rodents (Stuen, 2007; Rymaszewska and Grenda, 2008; Bowman *et al*., 2009; Woldehiwet, 2010). According to Courtney *et al*. (2004) the white footed mouse (*Peromyscus leucopus*) is the major reservoir species of *A. phagocytophilum*. Ogden *et al*. (1998), Liz *et al,* (2000), Woldehiwet (2010) and Brown *et al*. (2003; 2006) stated that wood mice (*Apodemus sylvaticus*), yellow-necked shrew (*Apodemus flavicollis*), field voles (*Microtus agrestis*) and bank voles (*Clethrinomyces glareolus*) are competent hosts of *A. phagocytophilum*. Vertebrate hosts of the various tick species such as wood rats (*Neotama spp*.), deer mice (*Peromyscus leucopus*), prairie vole (*Microtus ochrogaster*), chipmunks (*Tamias spp*.), grey squirrels (*Sciurus carolinensis*), racoon (*Procyon lator*) and golden-mantled ground squirrel (*Spermophilus lateralis*) were also found to be infected with HGA variants (Woldehiwet, 2010).

2.5 Geographic distribution

The host range of *A. phagocytophilum* appears to vary according to geographical regions and one would suspect that the geographic region is related to that of the vectors (Woldehiwet, 2010). According to Stuen (2007) *A. phagocytophilum* has been detected by PCR in mammals and ticks in most of the European countries. *Anaplasma phagocytophilum* has been found to cause TBF in sheep and cattle in the United Kingdom (UK) (Hudson, 1950), Ireland (Collins

et al., 1970), Scandinavia (Thorshaug, 1940; Overas, 1962; Tuomi, 1967a) and other parts of Europe (Bool and Reinders, 1964; Hinaidy, 1973; Pfister *et al.*, 1987; Juste *et al*., 1989). Equine granulocytic anaplasmosis (EGA) was first recognized in horses in California (Gribble, 1969) and was later found to occur in other parts of the USA and Europe, while canine granulocytic anaplasmosis (CGA) was first recognized in the USA (Madewell and Gribble, 1982) and was recently described in dogs in Europe (Carrade *et al*., 2009). Human anaplasmosis was first reported in 1996 in Slovenia, followed by Poland in 2001 (Rymaszewska and Grenda, 2008).

2.6 Clinical disease

In humans, HGA was first reported in the USA in 1994 (Chen *et al.*, 1994). Subsequently, infections were reported in different parts of Europe, China and Japan (Lotric-Furlan *et al.*, 1998; Strle, 2004; Zhang *et al.*, 2008; Ohashi *et al.*, 2013). Several genetic variants of *A. phagocytophilum* have been characterized (Scharf *et al.*, 2011) but not all infect humans. Human granulocytic anaplasmosis is a multisystemic disease which is difficult to diagnose due to its symptoms being unspecific and resembling flu (Rymaszewska and Grenda, 2008). The clinical manifestations can range from mild, self-limited flu-like symptoms to life threatening infections in the most extreme cases, however, most human infections are either minimal infections or there are no clinical manifestations whatsoever (Stuen, 2007). Infection in people with compromised immune systems can be fatal (Rymaszewska and Grenda, 2008).

In animals, the general disease caused by *A. phagocytophilum* is characterised by symptoms such as high fever, depression, myalgia, anorexia, severe neutropenia and thrombocytopenia (Rymaszewska and Grenda, 2008; Bowman *et al*., 2009). In animals, direct losses (such as crippling conditions) and production losses have been observed (Stuen, 2007). *Anaplasma phagocytophilum* infections are very seldom fatal unless complications arise due to other infections (Stuen, 2007; Rymaszewska and Grenda, 2008). Complications may include abortions, impaired spermatogenesis has been reported in sheep and cattle, reduced body weight or body condition, reduced milk production, as well as susceptibility to predisposing infections caused by other pathogens (Stuen, 2007; Rymaszewska and Grenda, 2008). The severity of infection can be influenced by numerous factors which may include different strains of *A. phagocytophilum*, the presence of other pathogens, age, immune status and condition of the host, as well as climatic conditions and management (Stuen, 2007; Rymaszewska and Grenda, 2008).

Canine granulocytic anaplasmosis (CGA) is the term used for dogs infected with *A. phagocytophilum*, the first report of such an infection was in California in 1982 (Madewell and Gribble, 1982). Subsequently there have been reports of CGA in North America, Europe and in the UK (Carrade *et al*., 2009). Canine infections with *A. phagocytophilum* seem to correlate with the geographic distribution of human infections with *A. phagocytophilum* (Carrade *et al*., 2009). Humans and dogs have been said to be accidental hosts, whereas small mammals such as rats and mice act as reservoir hosts. It is very rare that transmission of *A. phagocytophilum* will occur in the absence of tick vectors and it seems that prevalence increases in seasons where ticks are more active, as well as seasons where people and their dogs are likely to spend more time outdoors (Carrade *et al*., 2009). According to Carrade *et al*. (2009) the symptoms commonly associated with CGA are lethargy, fever (39.2 – 41.4°C), inappetence, lameness, reluctance to move as well as thrombocytopenia. It is said that most naturally infected dogs are able to remain healthy, according to the review by Carrade *et al*. (2009) there have been no cases reporting fatalities due to CGA; it seems to be self-limiting in dogs.

2.7 Laboratory diagnosis

There is very little definitive data on anaplasmosis in Africa. Detecting members of this family is difficult due to the fact that they are fastidious intracellular bacteria that require sophisticated laboratory facilities and substantial funding in order to isolate and characterise them (Inokuma *et al*., 2005). However, serological studies can be carried out in less equipped laboratories but the results can be affected by antigenic cross-reactivity between etiological agents (Inokuma *et al*., 2005).

2.7.1 Traditional diagnosis

According to Foggie (1951) the pathogen can be visualised by light microscopy of Giemsa –or Wright-stained peripheral smears as shown in the image below (Figure 1.2) which shows an intracytoplasmic inclusion in a polymorphonuclear neutrophil obtained from a human infected with *A. phagocytophilum.*

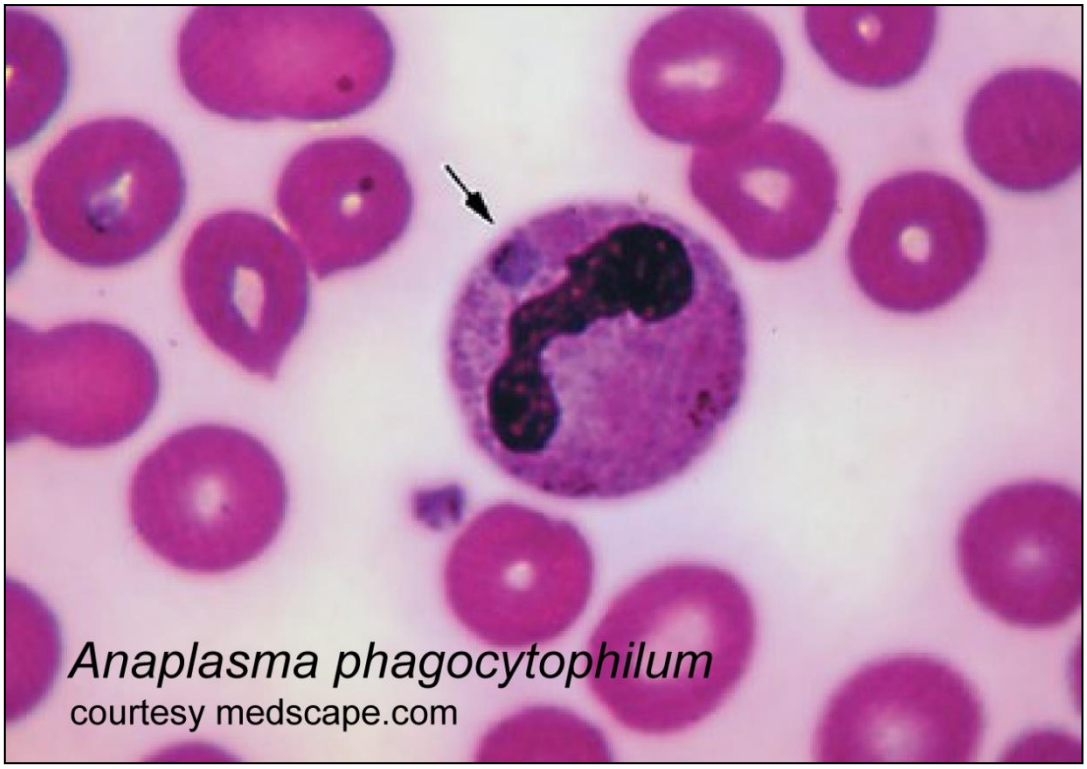

Figure 2.2: A peripheral blood smear, Wright-Giemsa of a human with anaplasmosis (x1000) (http://www.vetmed.auburn.edu/anaplasmosis_canine/feline#.U_Nok_mSwft)

2.7.2 Serological diagnosis

There are various antibody and antigen detection assays which can be used such as immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA) and western blotting (Courtney *et al*., 2004). To test for infection with the causative agent a single-use diagnostic test known as the SNAP® 4Dx® test (IDEXX Laboratories, Westbrook, ME) can be used, this method is already being used by many veterinarians in the US that are familiar with this approach (Bowman *et al*., 2009). Baker *et al*. (1987) and French and Harvey (1983) mentioned that the IFA used to detect *A. platys* could be put to use for the detection of *A. phagocytophilum.*

2.7.3 Molecular diagnosis

Molecular based techniques such as Polymerase Chain Reaction (PCR) assay are more sensitive and specific than microscopy and serological assays, and have been used to detect members of *Anaplasmataceae* from clinical and field samples (Courtney *et al*., 2004; Hojgaard *et al*., 2014). Multiplex PCR is more advantageous than conventional PCR due to simultaneous detection of multiple organisms. Similarly, the Reverse Line Blot (RLB) hybridization assay

can simultaneously detect and differentiate between various *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* species (Gubbels *et al*., 1999). Real-time PCR (qPCR) is an even more efficient way of doing PCR as it is more sensitive than conventional PCR and it eliminates the need to run an agarose gel as well as post-PCR processing. As such, several PCR techniques (conventional, nested and real-time PCR) have been developed for the identification of *A. phagocytophilum* primarily based on the 16S rRNA, *groEL* and *p44* genes (Chen *et al.*, 1994; Courtney *et al*., 2004; Alberti *et al*., 2005).

2.8 Control, treatment and prevention

Infection with tick-borne pathogens such as *Anaplasm*a may be prevented to some extent by using management options which eliminate vectors or controlling of vectors (Bowman *et al*., 2009). With animals, most control strategies are currently based on reducing the number of tick infestations that animals get from feeding out in pastures, this is done by using long acting antibiotics prophylactically before the animals are transferred from a tick-free area to tick-infested areas (Brodie *et al.,* 1986). The number of ticks on the animals are also reduced by regular dipping and pour-on treatments with pyrethroids (Brodie *et al*., 1986) however the use of acaracides and insecticides alone is ineffective and doesn't break the transmission cycle of the enzootic pathogens (Bowman *et al*., 2009). An alternate option is to keep ewes and lambs in tick-free areas until the lambs are at least seven weeks old, in order to prevent abortions pregnant ewes shouldn't be moved to fields that are infested with ticks (Jones and Davies, 1995).

Using antibiotics such as oxytetracyclines in general is effective for treating TBF in sheep and cattle, but more specifically doxycycline at a dosage of 10 mg/kg for 28 days (Woldehiwet and Scott, 1993) and HGA in humans (Bakken *et al*., 1996; Maurin *et al*., 2003). According to Woldehiwet (2010) cross protection is not an option due to high antigenic diversity amongst the different isolates of *A. phagocytophilum.* Studies done by Foggie (1951) and Tuomi (1967b) proved that the primed animals didn't resist subsequent infections with several heterologous strains. The development of prophylactic vaccines against *A. phagocytophilum* is considered to be the most effective strategy for disease control, however, there are no currently available vaccines (Woldehiwet, 2010).

CHAPTER 3
MATERIALS AND METHODS

3.1 Sample collection

3.1.1 Rodents

Thirty-seven rodent blood samples (Table 3.1) were obtained from the Department of Conservation Ecology and Entomology, Stellenbosch University. The samples were collected during various studies that focused on parasites of small mammals in South Africa between 2007 and 2012. The study was approved at the Animal Ethics Committee of the University of Stellenbosch (reference numbers: 2006B01007 and SU-ACUM11-00004 (P)). Rodents were trapped at several localities across South Africa (Figure 3.1), although the main sampling regions were the Western and Northern Cape Provinces. The samples obtained for this study originated from four provinces of South Africa (Western Cape, Eastern Cape, Northern Cape and KwaZulu-Natal). The animals were captured using Sherman-type live traps which were left for 4 – 7 days, the traps were checked twice a day and only adult rodents were selected for sampling while the non-target species were set free. The rodents were identified by means of Stuarts' Field Guide to Mammals of Southern Africa (Stuart and Stuart, 2001). The selected rodents were euthanized using sodium pentobarbitone at a dosage of 200 mg/kg. Data regarding location, sex, weight and morphological measurements were also collected. Blood was collected from the tongue immediately after the heartbeat stopped and spotted onto FTA filter paper (Merck, South Africa). For this study, blood was collected from four species of rodents, namely *Rhabdomys pumilio*, *R. dilectus*, *Micaelamys namaquensis* and *Myotomys unisulcatus*. Molecular identification of species within the genus *Rhabdomys* was done using sequencing of the mitochondrial COI (Cytochrome Oxidase I) gene (Du Toit *et al*., 2012).

Table 3.1: Rodent species, locality information and sample sizes of the animals included in the study.

SPECIES	PROVINCE	LOCALITY
Rhabdomys pumilio (n = 30) 	Western Cape (n = 30)	Gordon's Bay (n = 3) Somerset West (n = 7) Stellenbosch (n = 14) Beaufort West (n = 6)
Myotomys unisulcatus (n = 1) 	Northern Cape (n = 1)	Groblershoop (n = 1)
Rhabdomys dilectus (n = 3) 	Northern Cape (n = 1) KwaZulu-Natal (n = 2)	Groblershoop (n = 1) Not specified
Micaelamys namaquensis (n = 3) 	Eastern Cape (n = 3)	Gariep (n = 3)
Total Number of Rodents sampled:	**37**	**37**

Figure 3.1: A map of South Africa showing some locations from which samples were collected (stars). In close proximity to Cape Town is Gordons Bay, Somerset West, Stellenbosch and clearly marked is Beaufort West. Gariep Area is North West of Middelburg in the Eastern Cape Province, Groblershoop is situated between the points of Upington and Prieska in the Northern Cape, while KwaZulu-Natal regions were not specified.
(http://www.wineandvinesearch.com/africa/south_africa.png)

3.1.2 Domestic dogs

The Mnisi community, an area of high rural poverty and located in the north-eastern corner of the Bushbuckridge Municipality, Mpumalanga, South Africa, is nestled at the livestock/wildlife/human interface of the western boundary of the Kruger National Park (Figure 3.2). A Health and Demographic Surveillance System in Dogs (HDSS-Dogs) was established in the community in 2011 (Conan *et al*., 2015); and as part of this, blood samples were collected

from owned, free roaming, apparently healthy dogs present at households visited by the HDSS-Dogs field team during routine quarterly visits. Of these, 56 blood samples stored in EDTA tubes were made available for this study. Further description of the dog population and the HDSS-Dogs can be found in Conan *et al*. (2015).

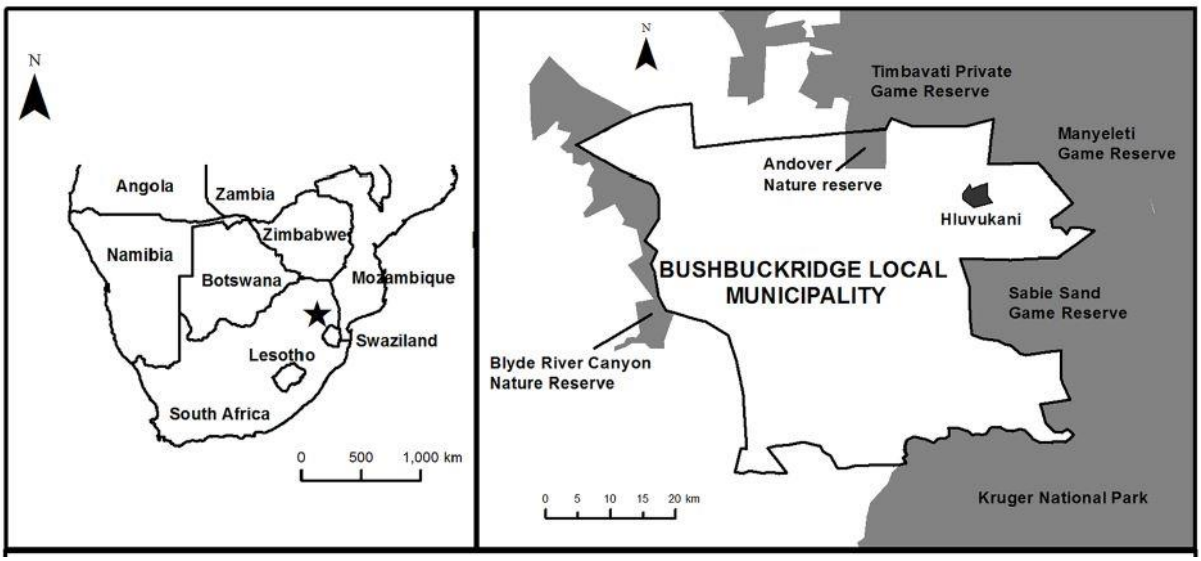

Figure 3.2: Map of the Mnisi community study area, Bushbuckridge Municipality, Mpumalanga, South Africa (Conan *et al*., 2015).

3.2 DNA extraction

The DNA was extracted from 3 – 5 punched circles of the FTA filter paper (rodents) and from whole blood (dogs) stored in EDTA using the QIAmp DNA Blood Mini Kit (QIAGEN, Southern Cross Biotechnologies, South Africa) as per manufacturer's protocol. In short, 1.5 ml microcentrifuge tubes were filled with 20 µl of QIAGEN Protease, 200 µl sample was added to the tube, followed by 200 µl of AL buffer. The contents of the tube were mixed for 15 seconds by pulse-vortexing and then incubated at 56°C for 10 minutes. After incubation the tube was briefly centrifuged and then 200 µl of ethanol (96–100%) was added to the sample, the sample was briefly vortexed and centrifuged again, as mentioned above. The mixture was transferred into a QIAamp Spin Column placed into a centrifuge tube where it was centrifuged on a desktop centrifuge for 1 minute. The spin column was placed into a clean 2 ml collection tube and the filtrate was discarded, 500 µl of the AW1 buffer was added to the spin column and it was centrifuged again in a desktop centrifuge for 1 minute. The last step was repeated once more and then the spin column was transferred to a new tube where 500 µl AW2 Buffer was added, the tube was centrifuged in a desktop centrifuge for 3 minutes, the filtrate was

discarded, the spin column was placed into a clean collection tube and the tube was centrifuged again for 1 minute and the filtrate was discarded. Finally the spin column was placed into a clean tube where the DNA was eluted in 100 µl elution buffer, the sample was then incubated at room temperature for 1 minute and then centrifuged for 1 minute. The DNA was stored at -20°C until further use.

3.3 Reverse Line Blot (RLB) hybridization assay

The RLB assay was done as described by Gubbels *et al*. (1999) and Bekker *et al*. (2002).

3.3.1 Polymerase chain reaction (PCR)

The V4 hypervariable region (460-520 bp) of the parasite 18S rRNA gene of *Theileria* and *Babesia* species was amplified using primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3') (Nijhof *et al.* 2005). For the simultaneous detection of *Anaplasma* and *Ehrlichia* spp., the hypervariable V1 region (492–498 bp) of the parasite 16S rRNA gene was amplified using primers Ehr-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') (Schouls *et al.,* 1999) and Ehr-R (5'-biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3') (Bekker *et al.,* 2002). The PCR master mix consisted of 12.5 µl Platium® Quantitative PCR SuperMix–UDG (containing 60 U/ml Platinum Taq DNA polymerase, 40 mM Tris-HCl (pH 8.4), 100 mM KCl, 6 mM $MgCl_2$, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 400 µM dUTP and 40 U/ml UDG) (Life Technologies™, South Africa), 0.2 µM of each primer, 2.5 µl template DNA, 2.5 µl of genomic DNA (~75 ng) and 9.5 µl molecular grade water to make up a total volume of 25 µl. Genomic DNA extracted from the *Anaplasma centrale* and *Babesia bovis* vaccines (obtained from Onderstepoort Biological Products, Pretoria) were used as positive controls and were included in each PCR run. The negative control contained the master mix with molecular grade water and no DNA template. PCR amplification was carried on the Gene Amp®PCR System 9700 (Life Technologies™, South Africa) using a touch-down protocol as indicated in Table 3.2.

Table 3.2: Thermocycler program for *Theileria/Babesia* and *Anaplasma/Ehrlichia* touchdown PCR.

Cycle	Time	Temperature (°C)	Process
1	3 min	37	Activate UDG
1	10 min	94	Inactivate UDG & Activate *Taq*
2	20 sec	94	Denature double stranded DNA template
	30 sec	67	Anneal Primers
	30 sec	72	Extension of PCR products by *Taq* polymerase
2	20 sec	94	Denature double stranded DNA template
	30 sec	65	Anneal Primers
	30 sec	72	Extension of PCR products by *Taq* polymerase
2	20 sec	94	Denature double stranded DNA template
	30 sec	63	Anneal Primers
	30 sec	72	Extension of PCR products by *Taq* polymerase
2	20 sec	94	Denature double stranded DNA template
	30 sec	61	Anneal Primers
	30 sec	72	Extension of PCR products by *Taq* polymerase
2	20 sec	94	Denature double stranded DNA template
	30 sec	59	Anneal Primers
	30 sec	72	Extension of PCR products by *Taq* polymerase
40	20 sec	94	Denature double stranded DNA template
	30 sec	57	Anneal Primers
	30 sec	72	Extension of PCR products by *Taq* polymerase
1	7 min	72	Final Extension
		4	Storage/Keep

3.3.2 Membrane preparation

The RLB membrane was prepared by binding genus- and species-specific oligonucleotide probes (Table 3.3) onto a Biodyne C membrane (Separations, South Africa). The oligonucleotides were diluted in 150 µl 0.5 M $NaHCO_3$, pH 8.4. The membrane orientation was marked and then activated by 10 minute incubation at room temperature (25°C) in 10 ml 16% EDAC that was freshly prepared. The membrane was rinsed in demineralised water. The EDAC waste was disposed of in a disposal container. The activated membrane was placed on a support cushion in a clean MN45 miniblotter apparatus (Immunetics, Cambridge), the screws

were then fastened to be hand-tight. The miniblotter was placed in a vertical position and residual residue was removed by suction from the aspirator. Each slot was then filled with 150 µl diluted probes, the first and last slots were filled with drawing pen ink diluted 1:100 in 2 × SSPE. The membrane was incubated at room temperature for at least 1 minute. The probe solutions were removed by aspiration in the same order that they were applied. The membrane was removed from the blotter with forceps and placed into a washing tray to be inactivated in 100 ml 100 mM NaOH that was freshly prepared for 8 minutes. After inactivation the membrane was washed in 100 ml 2 × SSPE/0.1% SDS at 60°C for 5 minutes. The membrane was then prepared for storage until further use by washing with 20 mM EDTA, pH 8 for 15 minutes under gentle shaking at room temperature. The membrane was placed into a sealed container and stored at 4°C.

Table 3.3: Genus-specific and species-specific probes used in the RLB hybridization assay. R=A/G, W=A/T are the symbols used to indicate degenerate positions.

Species	**Probe Sequence (from 5' to 3')**	**Reference**
***Anaplasma* and *Ehrlichia* spp.**		
Anaplasma bovis	GTA GCT TGC TAT GRG AAC A	Bekker *et al*. (2002)
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 TAG AGA ATA GTT AGT GG	Bekker *et al*. (2002)
Anaplasma sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC	Bekker *et al*. (2002)
Anaplasma/Ehrlichia genus-specific	GGG GGA AAG ATT TAT CGC TA	Schouls *et al*. (1999)
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA	Schouls *et al.* (1999)
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT	Schouls *et al*. (1999)
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG	Bekker *et al.* (2002)
***Babesia* and *Theileria* spp.**		
Babesia 1 genus-specific	ATT AGA GTG CTC AAA GCA GGC	Dr A.M. Nijhof (unpublished)
Babesia 2 genus-specific	ACT AGA GTG TTT CAA ACA GGC	Dr A.M. Nijhof (unpublished)
Babesia bicornis	TTG GTA AAT CGC CTT GGT C	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 GTT TTC CGA CTG GC	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 vogeli	AGC GTG TTC GAG TTT GCC	Matjila *et al*. (2004)
Theileria/Babesia genus-specific	TAA TGG TTA ATA GGA RCR GTT G	Gubbels *et al.* (1999)
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*. (2000)
Theileria equi	TTC GTT GAC TGC GYT TGG	Butler *et al*. (2008)
Theileria lestoquardi	CTT GTG TCC CTC CGG G	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 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)

3.3.3 Reverse Line Blot (RLB) hybridization

The membrane was removed from the storage container in the fridge (4°C). It was then incubated at room temperature in 10 ml 2 × SSPE/0.1% SDS while the PCR products were prepared. The PCR products were diluted by adding 140 µl 2 × SSPE/0.1% SDS to each PCR reaction. The diluted PCR products were denatured for 10 minutes at 100°C in the thermal cycler, and immediately placed on ice thereafter. The membrane was placed on the mini blotter with the slots perpendicular to the line pattern of applied probes. The ink lanes were found directly under the slot openings. The residual fluid was removed by aspiration. The slots were filled with the diluted, denatured PCR products. The empty slots were filled with 2 × SSPE/0.1% SDS to avoid cross flow from occurring. The membrane was then hybridized on a horizontal surface for 60 minutes at 42°C. After hybridization the samples were removed by aspiration and the membrane was removed from the blotter. The membrane was washed twice in preheated 2 × SSPE/0.5% SDS for 10 minutes in a water bath heated to 50°C by gently shaking. The membrane was then incubated in 10 ml 2 × SSPE/0.5% SDS + 2.5 µl streptavidin-POD (peroxidise labelled) conjugate (1.25 U) (Roche Diagnostics, South Africa) for 30

minutes at 42°C. The membrane was washed twice in preheated 2 × SSPE/0.5% SDS for 10 minutes in a water bath heated to 42°C by gently shaking. The membrane was washed twice in 2 × SSPE for 5 minutes at room temperature by gently shaking. The 2 × SSPE was discarded and 10 ml of ECL (5 ml ECL1 + 5 ml ECL2) of enhanced chemiluminiscence (DNA ThunderTM, Separation Scientific, South Africa) was added to the membrane, it was gently shaken so that the membrane remained wet with ECL for 1 minute at room temperature. The ECL was discarded. The membrane was exposed onto an X-ray film (X-OMATTM Blue XB-1, Kodak, Separation Scientific, South Africa). The X-ray film was developed in a developer solution for 1 minute and fixed in a fixer solution for at least 30 seconds. Dark spots appeared where hybridization had occurred (Gubbels *et al*., 1999) as a result of a chemiluminescence reaction.

3.3.4 Stripping membrane

Before the membrane was re-used the PCR products were stripped from the membrane. The membrane was placed into a plastic container that contains 100ml of 1% SDS which was placed into a pre-heated water bath with a temperature of 80°C and shaken for 30 minutes. The process mentioned above was repeated. The third wash was done using 20 mM EDTA, shaking at a slow pace at room temperature (25°C) for 15 minutes. Once the stripping process had been completed the membrane was stored in 20 mM EDTA, at pH8 at 4°C until further use.

3.4 Real-time quantitative PCR (qPCR)

Ninety-three (37 rodent and 56 dog) samples were screened for the presence of *Anaplasma phagocytophilum* DNA using the Taqman real-time PCR assay targeting the *msp2* gene of *A. phagocytophilum* as described by Courtney *et al*. (2014) with minor modifications. Primers for the specific amplification of a 77 bp fragment of the *msp2* gene of *A. phagocytophilum* ApMSP2 forward (5'-ATG GAA GGT AGT GTT GGT TAT GGT ATT-3') and ApMSP2 reverse (5'-TTG GTC TTG AAGCGC TCG TA-3') were used together with the TaqMan probe ApMSP2p-FAM (5'-TGG TGC CAG GGTTGA GCT TGA GAT TG-3') TAMRA labelled at the 5' and 3' ends with fluorescein (FAM) and TAMRA, respectively. Each 20 µl reaction mixture contained 12.5 µl of iTaq probe supermix (containing DNA polymerase, dNTPs, $MgCl_2$, enhancers, stabilizers and a blend of passive reference dyes (including ROX) (Applied Biosystems) 0.72 µM of primers ApMSP2 forward and ApMSP2 reverse, 0.1 µM of probe and 2.5 µl of template DNA (approximately 400 ng) and 4 µl of molecular grade water. *Anaplasma phagocytophilum* DNA culture from dog strain L610 (obtained from Dr Erich Zweygarth, Freie

Universitőt Berlin, Berlin, Germany) was included as positive control and nuclease free water as a negative control.

Cycling conditions included an initial activation of *Taq* polymerase at 95°C for 10 minutes, followed by 40 cycles of a 15 second denaturation at 95°C followed by a minute annealing-extension step at 60°C (Courtney *et al.*, 2004). The reactions were run on an Applied Biosystems ABI StepOnePlus™ Real-Time PCR System. The qPCR results were analysed using the StepOne Plus software v2.2.

3.5 Statistical analysis

The Fisher's exact test was used to determine the association between the RLB and qPCR results. The Cohen's Kappa score (Viera and Garrett, 2005) is a measure of inter-rater reliability or inter-observer variation between two methods. It was used in this study to measure the level of agreement between the qPCR and RLB assays in detecting *A. phagocytophilum* infections in canine and rodent samples. Both statistics were computed using the GraphPad software (http://graphpad.com/quickcalcs).

3.6 Molecular characterization of the *Anaplasma phagocytophilum* 16S rRNA gene

3.6.1 Full length 16S rDNA amplification

Seventeen samples with the highest infection levels (rodent = 3; dogs = 14) (quantitative threshold, Ct values: 29 - 32) were selected for the amplification of an approximately 1 600 bp fragment of the 16S rDNA gene. Conventional PCR was carried out using the Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, South Africa) (containing Phusion Flash II DNA polymerase, dNTP's and $MgCl_2$ (https://www.thermofisher.com/order/catalog/product/F548S). The 25 µl reaction mix consisted of 0.4 µM of the universal primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3') (Weisburg *et al*. 1991), 12.5 µl of the Phusion Flash High-Fidelity PCR Master Mix and 2.5 of template DNA and nuclease free water to a total volume of 25 µl. Amplification was performed at 98°C for 10 seconds, 35 cycles of 98°C for 1 seconds, 60°C for 1 minute, 72°C for 38 seconds with a final extension of 72°C for 1 minute.

A 2% agarose gel was prepared to visualize the amplicons. Five microliters of the PCR product was mixed with 3 µl loading dye and run for 45 minutes at 120 volts using a Labnet GI XL Enduro machine (ENDURO ^{TM}). A Thermo Scientific GeneRuler (Thermo Fisher Scientific,

USA) 100 bp DNA ladder ready–to-use (50 µg) was used as a marker. The image was taken and analysed using Image Lab™ Software (BioRad).

Prior to cloning, PCR amplification products were purified to remove all primers, nucleotides, enzymes and any impurities using the QIAquick PCR Purification Kit (QIAGEN, Germany) according to the manufacturers' protocol. The DNA was eluted in 20 µl elution buffer and the purity and concentration of the DNA was determined spectrophotometrically.

3.6.2 Cloning of the parasite 16S rRNA gene

Depending on DNA concentration, 3 – 6 µl of the purified PCR 16S rDNA insert was ligated into the Clone JET PCR Cloning Kit (Thermo Fisher Scientific, USA). Positive and background (negative) controls (provided with the kit) were included in the set up. The ligated reactions were transformed into *E. coli* JM109 High-Efficiency Competent Cells (Promega, USA), each reaction was plated out in triplicate and incubated overnight at 37°C. White colonies represented successful transformation. At least 10 colonies per sample were screened by colony PCR using the vector specific primers (pJET1.2F) – (5'-CGA CTC ACT ATA GGG AGA GCG GC-3') and pJET1.2R (5'-AAG AAC ATC GAT TTT CCA TGG CAG-3'). Each pipette tip that was used to pick-up the colonies was suspended into the PCR master mix and then dipped into 5 µl LB broth and allowed to multiply overnight at 37°C. Plasmid DNA extraction was then carried out using the High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany) as per manufacturer's protocol. Colony PCR amplicons were analysed using 1.5% agarose gel electrophoresis; and plasmid DNA of selected colonies with the correct insert were sent for sequencing at Inqaba Biotechnologies (South Africa) using an ABI 3100 genetic analyser.

3.6.3 Sequencing and phylogenetic analyses

Sequencing was performed with approximately 350 ng of plasmid DNA, and 3.2 pmol of the vector primers (pJET1.2F and pJET1.2R) using the ABI BigDye^{TM} Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The obtained sequences were analysed using the CLC Bio Main Workbench 7 programme (http://www.clcbio.com). For each clone, the forward and reverse reads were assembled to obtain a consensus sequence. Consensus sequences were subjected to a Basic Local Alignment Search Tool (BLAST) search (Altschul *et al*. 1990) using the BLASTn function and aligned with homologous sequences (with 98 –

100% identity) from GenBank. Short sequences (< 1 400 bp) were omitted from the alignment. The alignment was edited and truncated to the size of the smallest sequence (1 400 bp). The evolutionary divergence was estimated by a similarity matrix of the aligned sequences using MEGA 7 (Kumar *et al*., 2016). Phylogenetic trees were generated by the Neighbour-joining and Maximum Likelihood methods using MEGA 7 software. Bootstrapping (Felsenstein, 1985) was applied using 1000 replicates/tree for the Neighbour-joining method and 100 replicates/tree for the Maximum Likelihood method. The sequences used in the phylogenetic trees, together with their GenBank accession numbers, locality and the source of the sample are listed in Table 3.4.

Table 3.4: GenBank accession numbers for reference sequences used in the phylogenetic analysis.

Accession number	**Taxonomic classification (strain)**	**Location**	**Source**	**Reference**
U54806	*Anaplasma* sp. Omatjenne	Namibia	Ticks	Allsopp *et al*., 1997
AY570539	*Anaplasma* sp. (South Africa dog-1076)	South Africa	Dog	Inokuma *et al*., 2005
LC269823	*Anaplasma* sp. (ZAM dog-181)	Zambia	Dog	Vlahakis *et al*., 2018
AY570539	*Anaplasma* sp. (South Africa dog-1108)	South Africa	Dog	Inokuma *et al*., 2005
U03775	*A. bovis*	South Africa	Ruminant	Visser and Allsopp, 1993 (unpublished)
KC811530	*A. bovis*-like	South Africa	Eastern rock sengi	Harrison *et al*., 2013
KP314237	*A. capra*	China	Ticks	Sun *et al*., 2015
AF309869	*A. centrale* (Israel)	Israel	Ticks	Rurangirwa, 2000 (unpublished)
AY048816	*A. marginale* (St. Maries)	USA	Cattle	Rurangirwa *et al*., 2002
AF414870	*A. ovis* (OVI)	South Africa	Sheep	Lew *et al*., 2003
CP006618	*A. phagocytophilum* (Dog2)	USA	Dog	Barbet *et al*., 2013
CP000235	*A. phagocytophilum* (HZ)	USA	Human	Dunning Hotopp *et al*., 2006
NR044762	*A. phagocytophilum* (Webster)	USA	Human	Chen *et al*., 1994
CP006616	*A. phagocytophilum* (HZ2)	USA	Human	Barbet *et al*., 2013
KC470064	*A. phagocytophilum* (HN)	China	Rodent	Zhao *et al*., 2013

M82801	*A. platys*	USA	Dog	Anderson *et al.*, 1992
LC269820	*A. platys* (ZAM dog-166)	Zambia	Dog	Vlahakis *et al*., 2018
LC269821	*A. platys* (ZAM dog-99)	Zambia	Dog	Vlahakis *et al*., 2018
U11021	*Rickettsia rickettsii*	USA	Ticks	Stothard *et al*., 1994

CHAPTER 4
RESULTS

4.1 Reverse Line Blot hybridization (RLB) assay

The RLB hybridization assay was used to screen rodents (n = 37) and domestic dog samples (n = 56) for the presence of *Theileria, Babesia, Ehrlichia* and *Anaplasma* species. The results of the RLB assay indicated the presence of various tick-borne blood-borne parasites either as single infections or mixed infections.

4.1.1 Rodents

A total of 37 rodent samples from four provinces in South Africa were screened using the RLB hybridization assay. Single infections were detected for *A. bovis* (2.7%), *Anaplasma* sp. Omatjenne (2.7%), *B. microti* (35.1%) and *E. ruminantium* (5.4%). Rodent infections resulted in three different types of mixed infections as shown in Table 4.1; of which type one listed below seemed to be the most common occurring twice out of 37 rodents sampled:

(i) *A. phagocytophilum*, *B. microti* and *E. ruminantium*
(ii) *A. phagocytophilum* and *E. ruminantium*
(iii) *B. microti* and *E. ruminantium*

Out of all the rodent samples screened there were six samples that hybridized with only the *Anaplasma/Ehrlichia* genus-specific probe. There were no samples that completely failed to hybridize, indicating negative infections. Three rodents from the Western Cape tested positive for *A. phagocytophilum* all of which had mixed infections.

The majority of the samples were collected from *R. pumilio* (n = 30), originating from four localities in the Western Cape. PCR products hybridized with the *Anaplasma/Ehrlichia* genus-specific probe in 97.7% (n = 29) of the samples. The results indicated the presence of *A. bovis* (3.3%; n = 1), *A. phagocytophilum* (10%; n = 3) and *E. ruminantium* (10%; n = 3). PCR products furthermore hybridized with the *Theileria/Babesia* genus-specific probe in 77% (n = 23) of the samples. The results indicated the presence of *B. microti* in 56.7% (n = 17) of the samples.

Table 4.1: Tick-borne haemoparasite infections in wild rodents investigated in this study.

	Rhabdomys pumilio **(n = 30)**	***Rhabdomys dilectus*** **(n = 3)**	***Micaelamys namaquensis*** **(n = 3)**	***Myotomys unisulcatus*** **(n = 1)**	**TOTAL (n = 37)**
Single infections	**3 (10%)**	**0 (0%)**	**2 (66.7 %)**	**0 (0 %)**	**5 (13.5 %)**
Anaplasma bovis	1 (3.3%)	0	0	0	1 (2.7%)
Anaplasma sp. Omatjenne	0	0	1 (33.3%)	0	1 (2.7%)
Babesia. Microti	1 (3.3%)	0	0	0	1 (2.7%)
Ehrlichia. Ruminantium	1 (3.3%)	0	1 (33.3%)	0	2 (5.4%)
Mixed infections:	**25 (83.3 %)**	**0 (0 %)**	**0 (0%)**	**0 (0 %)**	**25 (67.5%)**
Anaplasma phagocytophilum	3 (10%)	0	0	0	3 (8.1%)
Babesia. Microti	17 (56.7%)	0	0	0	17 (45.9%)
Ehrlichia. ruminantium	5 (16.7%)	0	0	0	5 (13.5%)
Theileria/Babesia genus-specific ONLY	0	1 (33.3%)	0	0	**1 (2.7%)**
Anaplasma/Ehrlichia genus-specific ONLY	3 (10%)	3 (100%)	1 (33.3%)	1 (100%)	**8 (21.6%)**
Negative/Below detection limit	0	0	0	0	**0**

Three *R. dilectus* samples were obtained (n = 3); two from KwaZulu-Natal and one from the Northern Cape. PCR products hybridized with the *Anaplasma/Ehrlichia* genus-specific probe only in all of the samples; all *Anaplasma/Ehrlichia* species specific-probes tested negative. PCR products furthermore hybridized with the *Theileria/Babesia* genus-specific probe only in 33.3% (n = 1) of the samples.

Three *M. namaquensis* samples were obtained from the Eastern Cape (n = 3). PCR products hybridized with the *Anaplasma/Ehrlichia* genus-specific probe in all of the samples. The results indicated the presence of *Anaplasma* sp. Omatjenne (33.3%; n = 1) and *E. ruminantium* (33.3%; n = 1). PCR products failed to hybridize with the *Theileria/Babesia* genus-specific probe.

There was only one *M. unisulcatus* sample obtained from the Northern Cape (n = 1). PCR products hybridized with the *Anaplasma/Ehrlichia* genus-specific probe. All species specific-probes tested negative. PCR products also failed to hybridize with the *Theileria/Babesia* genus-specific probe.

4.1.2 Domestic dogs

A total of 56 canine samples from the Mnisi community in Mpumalanga, South Africa were screened using the RLB hybridization assay. Out of all the dog samples screened there were six samples (10.7%) that hybridized with only the *Anaplasma/Ehrlichia* genus-specific probe, while two samples (3.6%) hybridized with only the *Theileria/Babesia* genus-specific probe, while three samples (5.4%) hybridized with only the *Theileria* genus-specific probe in combination with other genus-specific and species-specific probes, while eleven samples (19.6%) hybridized with only the *Babesia* genus-specific probe in combination with other genus-specific and species-specific probes. Single infections were detected for *B. microti* (3.6%), *B. rossi* (3.6%) and *E. canis* (21.4%) as shown in Table 4.2**.** *Ehrlichia canis* was the most commonly occurring pathogen in dogs, both as single and mixed infections. There were five different types of mixed infections detected; four mixed infections with two pathogens only:

(i) A. phagocytophilum and *E. canis*

(ii) B. vogeli and *T. parva*

(iii)B. mictoti and *E.canis*

(iv)E. canis and *E. ruminantium*

(v) *Anaplasma* sp. Omatjenne, *B. rossi* and *E. canis*

Anaplasma phagocytophilum was present in 1.8% of the samples, and the sample was co-infected with *E. canis*. *Babesia vogeli* was detected in 5.4% of the samples. Unexpected findings included *E. ruminantium* which was identified in 8.9% of the samples, always in combination with *E. canis*, as well as *T. parva* which was identified in 5.4% of the samples. *Babesia* sp. (sable) was furthermore present in 1.8% of the samples, with only a very faint dot present on the membrane. A total of 15 samples (26.8%) failed to hybridize with any probes, these samples are considered negative or below the detection limit of the assay.

Table 4.2: Tick-borne haemoparasite infections in domestic dogs investigated in this study.

	Domestic dogs (n = 56)
Single infections	**16 (28.6%)**
B. microti	2 (3.6%)
B. rossi	2 (3.6%)
E. canis	12 (21.4%)
Mixed infections:	**28 (50%)**
Anaplasma sp. Omatjenne	1 (1.8%)
A. phagocytophilum	1 (1.8%)
B. microti	1 (1.8%)
B. rossi	8 (14.3%)
Babesia sp. (sable)	1 (1.8%)
B. vogeli	3 (5.4%)
E. canis	7 (12.5%)
E. ruminantium	5 (8.9%)
T. parva	1 (1.8%)
Theileria/Babesia genus-specific ONLY	2 (3.6%)
Anaplasma/Ehrlichia genus-specific ONLY	6 (10.7%)
Negative/Below detection limit	15 (26.8%)

4.2 *Anaplasma phagocytophilum* real-time PCR (qPCR)

All 93 blood samples (37 rodents and 56 canines) were tested for the presence of *A. phagocytophilum* using an *A. phagocytophilum*-specific qPCR assay (Courtney *et al*., 2004).

Amplification curves were obtained for the positive control and for positive field samples. No fluorescence was observed in the negative control and in negative field samples (Figure 4.1).

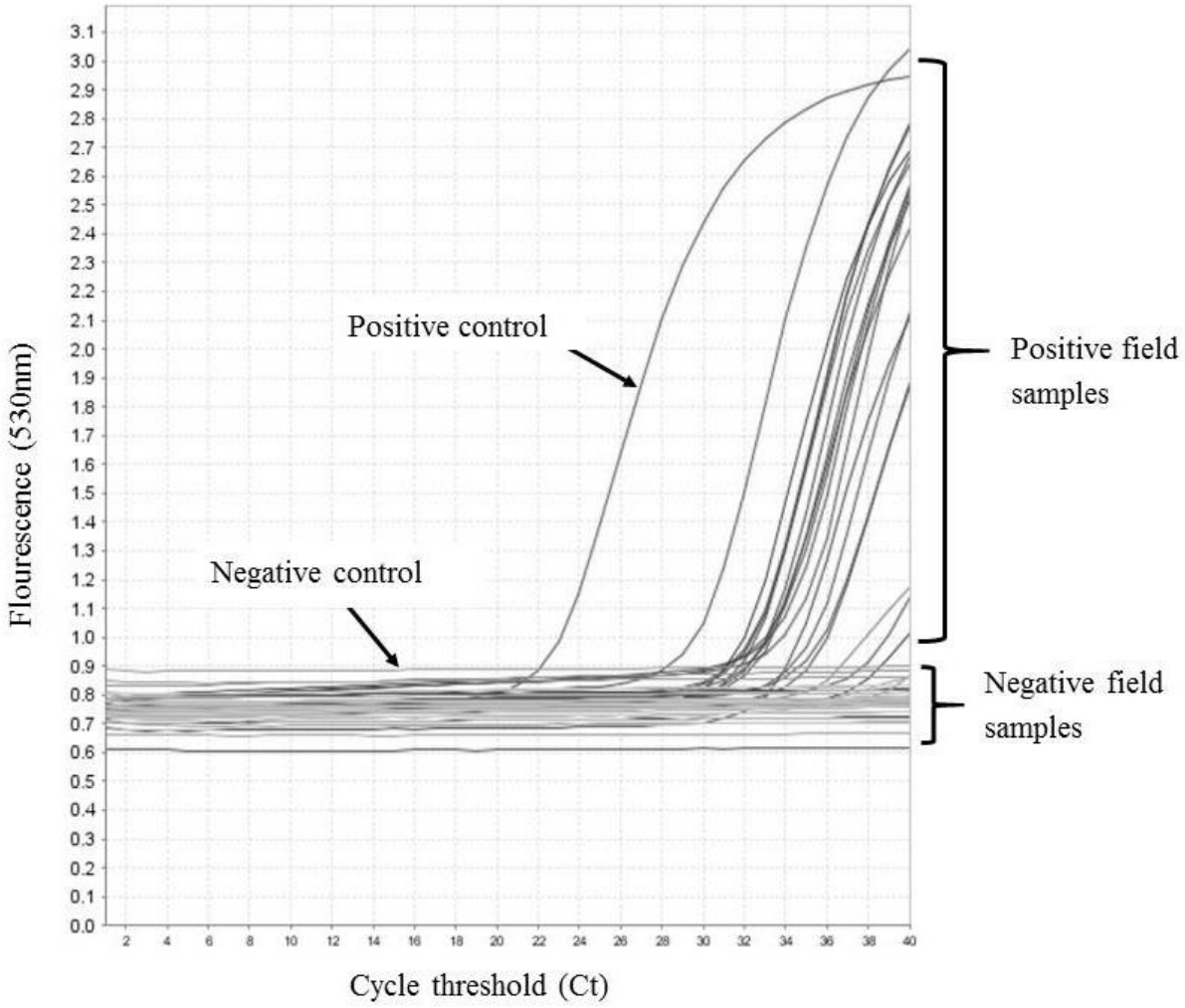

Figure 4.1: Amplification plot of a qPCR run showing the fluorescence (Rn) vs. the cycle number. The positive control has a Ct value of 19.62 while the negative control remained undetermined.

The results demonstrated that 53 samples (57.0%) tested positive for *A. phagocytophilum* DNA; 16.2% (n = 6) rodent blood samples (all *R. pumilio*; one from KwaZulu-Natal and 5 from Wesern Cape) and 83.9% (n = 47) canine blood samples (Figure 4.2). The CT values obtained from the rodent samples ranged between 28.42 and 37.60; indicative of low infection rates. The association between the qPCR and RLB results was not statistically significant ($p > 0.05$). Of the 53 positive samples, only one sample (canine) was positive by both assays while 52 samples were positive for the qPCR assay and negative by the RLB assay (Table 4.3). A

total of 37 samples were negative by both assays; an additional three samples were positive for *A. phagocytophilum* by the RLB assay but were negative by the qPCR assay. The Cohen's Kappa statistics indicated a slight agreement (0.049; 95% confidence interval) between the RLB and qPCR results (Table 4.3).

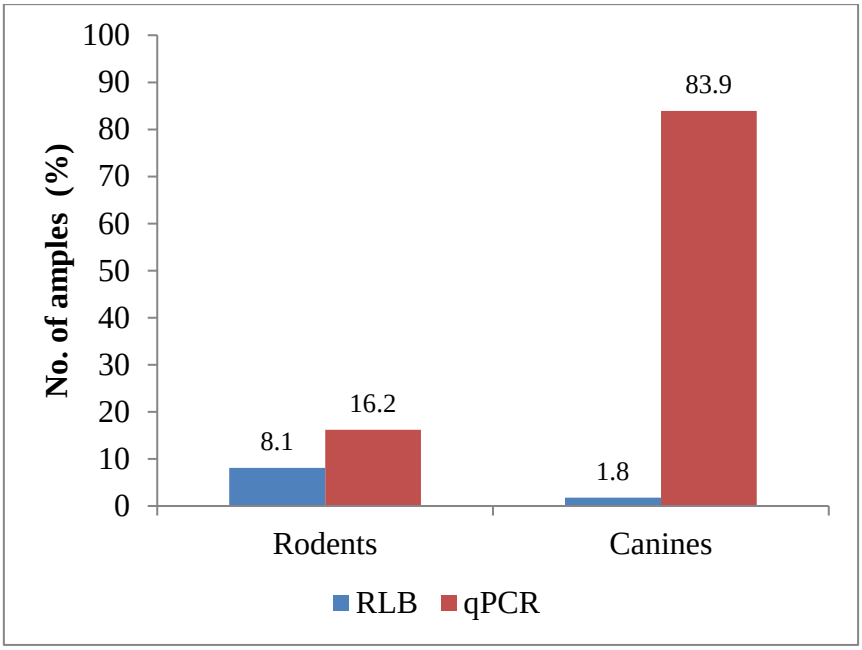

Figure 4.2: Detection of *A. phagocytophilum* in rodent and canine blood samples by the RLB hybridization and qPCR assays.

Table 4.3: Comparison of the RLB and qPCR assays in the detection of *A. phagocytophilum* in canines and rodents in South Africa

RLB	qPCR		TOTAL
	+	-	
+	1	3	**4**
-	52	37	**89**
TOTAL	**53**	**40**	**93**

Kappa value = 0.049; p > 0.05

Kappa agreements:

< 0 Less than chance agreement; 0.01–0.20 Slight agreement;

0.21– 0.40 Fair agreement; 0.41–0.60 Moderate agreement

0.61–0.80 Substantial agreement; 0.81–0.99 Almost perfect agreement

4.3 16S rDNA amplification, cloning and sequencing

A total of seventeen samples (rodent = 3; dogs = 14) were selected for further analysis. Of these, the near full length 16S rDNA of eight of these samples were successfully amplified, cloned and 36 recombinants were sequenced. The sequences were assembled, edited and aligned with sequences of related *Anaplasma* 16S rRNA sequences from Genbank. BLASTn homology searches showed that the obtained sequences had 98-100% sequence identity to published sequences of *A. phagocytophilum* (n = 5, obtained from 1 dog), *A. platys* (n = 4, obtained from 1 dog), *Anaplasma* sp. from Zambian and South African dogs (n = 20, obtained from 5 dogs); and *A. bovis* (n = 5, obtained from one *R. dilectus*). Of the 36 recombinant sequences obtained, 12 sequences from four dog samples were short (< 1400 bp) and were excluded from further phylogenetic analysis. One recombinant sequence (Ap33_1) was determined to be a chimera using the DECIPHER's Find Chimeras web tool (Wright *et al*., 2012) and also excluded from further analysis. The origin of the samples, RLB assay and qPCR results and phylogenetic classification for the obtained sequences are listed in Table 4.4.

A comparison of estimated evolutionary divergence between the observed gene sequences and those of closely related *Anaplasma* 16S rRNA sequences was subsequently compared by determining the number of base differences per near full-length 16S rRNA gene sequence. All positions containing gaps and missing data were eliminated. There were a total of 1325 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The five recombinant sequences obtained from dog Ap2 were all identical and identical to *A. phagocytophilum* human strains Webster (Accession nr NR044762), HZ (CP000235) and HZ2 (CP006616), as well as the *A. phagocytophilum* Dog2 strain (CP006618) (Table 4.5). Recombinant Ap2_1 was used as representative Ap2 sequence in the phylogenetic analysis.

Table 4.4: Origin and results of the rodent and domestic dog samples selected for 16S rRNA gene characterization. Sequences in bold were included as representative sequences in the phylogenetic analysis.

Sample nr	Host	Place of Origin (Locality)	RLB results	qPCR result (Ct value*)	Clone nr	Sequence length (bp)	Phylogenetic classification
Ap2	Dog	Mpumalanga	*E. canis, E. ruminantium*	*A. phagocytophilum* (29.55)	**Ap2_1**	1469	*A. phagocytophilum*
					Ap2_2	1469	*A. phagocytophilum*
					Ap2_4	1471	*A. phagocytophilum*
					Ap2_5	1471	*A. phagocytophilum*
					Ap2_8	1471	*A. phagocytophilum*
Ap3	Dog	Mpumalanga	*T. parva*	*A. phagocytophilum* (29.18)	Ap3_1	1467	*Anaplasma* sp. Zambian dog
					Ap3_5	1466	*Anaplasma* sp. Zambian dog
					Ap3_7	1467	*Anaplasma* sp. Zambian dog
					Ap3_F	1467	*Anaplasma* sp. Zambian dog
Ap5	Dog	Mpumalanga	*Anaplasma/Ehrlichia* genus-specific	*A. phagocytophilum* (29.80)	Ap5_1	1466	*Anaplasma* sp. Zambian dog
					Ap5_A	1466	*Anaplasma* sp. Zambian dog
Ap27	Dog	Mpumalanga	*B. rossi*	*A. phagocytophilum* (32.96)	Ap27_2	1050	*Anaplasma* sp. Zambian dog
					Ap27_5	647	*Anaplasma* sp. Zambian dog
					Ap27_9	1466	*Anaplasma* sp. Zambian dog
					Ap27_11	1542	*Anaplasma* sp. Zambian dog
					Ap27_13	1614	*Anaplasma* sp. Zambian dog

Ap33	Dog	Mpumalanga	*A.* sp. Omatjenne*; E. canis; B. rossi*	*A. phagocytophilum* (29.31)	Ap33_1	1465	Chimera, sequence discarded
					Ap33_3	1063	*A. platys*
					Ap33_4	1467	*A. platys*
					Ap33_5	1526	*A. platys*
					Ap33_7	1063	*A. platys*
Ap36	Dog	Mpumalanga	*B. rossi*	*A. phagocytophilum* (33.55)	Ap36_9	1128	*Anaplasma* sp. Zambian dog
					Ap36_10	1049	*Anaplasma* sp. Zambian dog
					Ap36_A	648	*Anaplasma* sp. Zambian dog
					Ap36_C	941	*Anaplasma* sp. Zambian dog
					Ap36_J	937	*Anaplasma* sp. Zambian dog
Ap37	Dog	Mpumalanga	*Anaplasma/Ehrlichia* genus-specific	*A. phagocytophilum* (33.27)	Ap37_1	929	*Anaplasma* sp. Zambian dog
					Ap37_4	899	*Anaplasma* sp. Zambian dog
					Ap37_5	647	*Anaplasma* sp. Zambian dog
					Ap37_8	1466	*Anaplasma* sp. Zambian dog
Ap273/12	*R. dilectus*	KwaZulu-Natal	*Anaplasma/Ehrlichia* genus-specific	*A. phagocytophilum* (37.60)	**Ap273_1**	1468	*A. bovis*
					Ap273_2	1467	*A. bovis*
					Ap273_3	1468	*A. bovis*
					Ap273_4	1467	*A. bovis*
					Ap273_6	1469	*A. bovis*
					Ap273_8	1468	*A. bovis*

*Ct – quantitative threshold

The 10 recombinant sequences obtained from dogs Ap3, Ap5, Ap27, Ap37 were all identical and also identical to *Anaplasma* sp. Zambian dog (Dog-181) (LC269823) described from Zambia (Vlahakis *et al.,* 2018) and differed by 4-5 nucleotides with *Anaplasma* sp. South African dogs SA1076 and SA1108 (AY570538, AY50539) (Inokuma *et al*., 2005) (Table 4.5). Sequence Ap27_11 was selected as representative sequence for the phylogenetic analysis.

The two near full-length recombinant sequences obtained from dog Ap33 were identical to each other and also identical to *A. platys* Zam Dog-99, Zam Dog-166 and Zam Dog-72 (LC269821, LC269820, LC269822) previously described from Zambia (Vlahakis *et al*., 2018). It differed by two nucleotides from the *A. platys* type strain (M82801) and by three nucleotides from *Anaplasma* sp. Omatjenne (U54806) (Table 4.5). Ap33_4 was selected as representative sequence for the phylogenetic analysis.

Six recombinant sequences were obtained from one *R. dilectus,* all of which were identical. It differed by 23 nucleotides from the *A. bovis* type strain (U03775) and by 24 nucleotides from the *A. bovis*-like strain previously described from eastern rock sengi (*Elephantulus myurus*) (Harrison *et al*., 2013) (Table 4.5). Sequence Ap273_1 were included as representative sequence in the subsequent phylogenetic analysis.

The observed sequence similarities were subsequently confirmed by phylogenetic analyses. Neighbour-joining and Maximum Likelihood techniques were used to reveal the phylogenetic relationships between the near full-length 16S rDNA sequences obtained from this study to related *Anaplasma* species previously deposited in GenBank. The topologies of both trees were similar. A representative tree obtained by the Maximum Likelihood method is shown in Figure 4.3.

Table 4.5 Estimates of evolutionary divergence between 16S rRNA sequences of *Anaplasma* spp. The number of base differences per sequence from between sequences are shown. The analysis involved 43 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1325 positions in the final dataset. Evolutionary analyses were conducted using MEGA7 (Kumar *et al*., 2016).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
1. *A. ovis* (AF414870)																																										
2. *A. marginale* (St Maries)(AY048816)	6																																									
3. *A. centrale* (Israel)(AF309869)	7	9																																								
4. *A. capra* (KP314237)	20	20	19																																							
5. Ap27_11	50	50	51	52																																					
6. Ap5_A	50	50	51	52	0																																				
7. Ap27_9	50	50	51	52	0	0																																			
8. *Anaplasma* sp. Zam Dog-181 (LC269823)	50	50	51	52	0	0	0																																		
9. Ap37_8	50	50	51	52	0	0	0	0																																	
10. Ap3_1	50	50	51	52	0	0	0	0	0																																
11. Ap3_F	50	50	51	52	0	0	0	0	0	0																															
12. Ap3_5	50	50	51	52	0	0	0	0	0	0	0																														
13. Ap3_7	50	50	51	52	0	0	0	0	0	0	0	0																													
14. Ap27_13	50	50	51	52	0	0	0	0	0	0	0	0	0																												
15. Ap5_1	50	50	51	52	0	0	0	0	0	0	0	0	0	0																											
16. *Anaplasma* sp. SA Dog-1076 (AY570539)	49	49	50	51	4	4	4	4	4	4	4	4	4	4	4																										
17. *Anaplasma* sp. SA Dog-1108 (AY570538)	48	48	51	50	5	5	5	5	5	5	5	5	5	5	5	1																									
18. Ap2_8	34	36	37	44	20	20	20	20	20	20	20	20	20	20	20	20	19																								
19. Ap2_5	34	36	37	44	20	20	20	20	20	20	20	20	20	20	20	20	19	0																							
20. Ap2_2	34	36	37	44	20	20	20	20	20	20	20	20	20	20	20	20	19	0	0																						
21. Ap2_4	34	36	37	44	20	20	20	20	20	20	20	20	20	20	20	20	19	0	0	0																					
22. Ap2_1	34	36	37	44	20	20	20	20	20	20	20	20	20	20	20	20	19	0	0	0	0																				
23. *A. phagocytophilum* Dog2 (CP006618)	34	36	37	44	20	20	20	20	20	20	20	20	20	20	20	20	19	0	0	0	0	0																			
24. *A. phagocytophilum* HZ (CP000235)	34	36	37	44	20	20	20	20	20	20	20	20	20	20	20	20	19	0	0	0	0	0	0																		
25. *A. phagocytophilum* HZ2 (CP006616)	34	36	37	44	20	20	20	20	20	20	20	20	20	20	20	20	19	0	0	0	0	0	0	0																	
26. *A. phagocytophilum* Webster (NR044762)	34	36	37	44	20	20	20	20	20	20	20	20	20	20	20	20	19	0	0	0	0	0	0	0	0																
27. *A. phagocytophilum* HN (KC470064)	35	37	38	45	22	22	22	22	22	22	22	22	22	22	22	22	21	6	6	6	6	6	6	6	6	6															
28. Ap33_4	39	41	42	48	23	23	23	23	23	23	23	23	23	23	23	21	20	13	13	13	13	13	13	13	13	13	17														
29. Ap33_5	39	41	42	48	23	23	23	23	23	23	23	23	23	23	23	21	20	13	13	13	13	13	13	13	13	13	17	0													
30. *A. platys* Zam Dog-166 (LC269820)	39	41	42	48	23	23	23	23	23	23	23	23	23	23	23	21	20	13	13	13	13	13	13	13	13	13	17	0	0												
31. *A. platys* Zam Dog-72 (LC269822)	39	41	42	48	23	23	23	23	23	23	23	23	23	23	23	21	20	13	13	13	13	13	13	13	13	13	17	0	0	0											
32. *A. platys* Zam Dog-99 (LC269821)	39	41	42	48	23	23	23	23	23	23	23	23	23	23	23	21	20	13	13	13	13	13	13	13	13	13	17	0	0	0	0										
33. *A. platys* (M82801)	41	43	44	50	25	25	25	25	25	25	25	25	25	25	25	23	22	15	15	15	15	15	15	15	15	15	19	2	2	2	2	2									
34. *Anaplasma* sp. Omatjenne (U54806)	38	40	41	47	25	25	25	25	25	25	25	25	25	25	25	24	23	15	15	15	15	15	15	15	15	15	17	3	3	3	3	3	3								
35. Ap273_6	53	55	50	67	48	48	48	48	48	48	48	48	48	48	48	48	49	43	43	43	43	43	43	43	43	43	43	44	44	44	44	44	46	44							
36. Ap273_2	53	55	50	67	48	48	48	48	48	48	48	48	48	48	48	48	49	43	43	43	43	43	43	43	43	43	43	44	44	44	44	44	46	44	0						
37. Ap273_1	53	55	50	67	48	48	48	48	48	48	48	48	48	48	48	48	49	43	43	43	43	43	43	43	43	43	43	44	44	44	44	44	46	44	0	0					
38. Ap273	53	55	50	67	48	48	48	48	48	48	48	48	48	48	48	48	49	43	43	43	43	43	43	43	43	43	43	44	44	44	44	44	46	44	0	0	0				
39. Ap273_3	53	55	50	67	48	48	48	48	48	48	48	48	48	48	48	48	49	43	43	43	43	43	43	43	43	43	43	44	44	44	44	44	46	44	0	0	0	0			
40. Ap273_8	53	55	50	67	48	48	48	48	48	48	48	48	48	48	48	48	49	43	43	43	43	43	43	43	43	43	43	44	44	44	44	44	46	44	0	0	0	0	0		
41. *A. bovis* (U03775)	53	53	50	63	40	40	40	40	40	40	40	40	40	40	40	39	40	39	39	39	39	39	39	39	39	39	37	41	41	41	41	41	43	40	23	23	23	23	23	23	
42. *A. bovis-like* (KC811530)	53	53	50	63	37	37	37	37	37	37	37	37	37	37	37	37	38	38	38	38	38	38	38	38	38	38	34	41	41	41	41	41	43	41	24	24	24	24	24	24	5

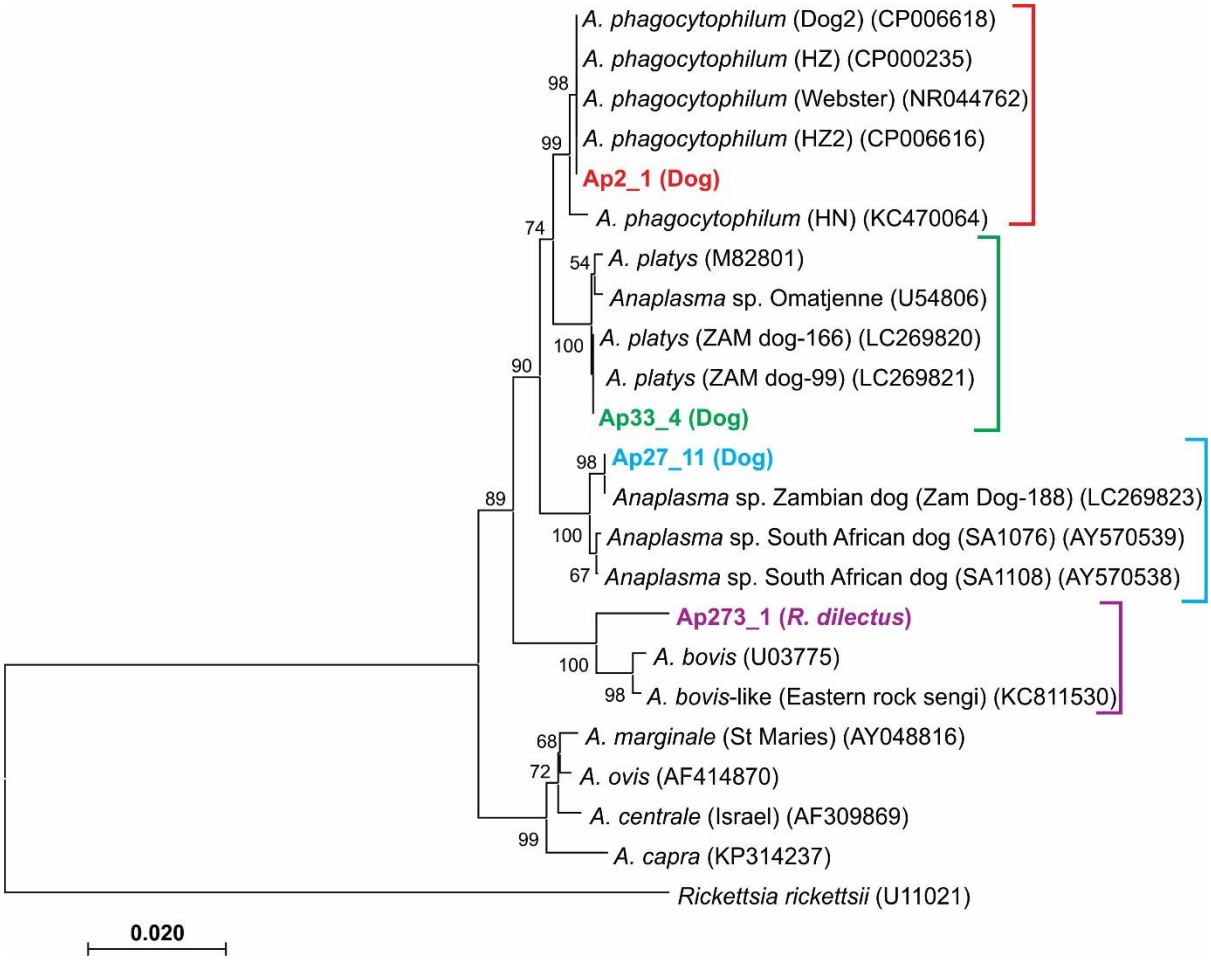

Figure 4.3: Maximum likelihood tree showing the evolutionary relationships of the *Anaplasma* 16S rDNA sequences obtained from South African dogs and *Rhabdomys dilectus*, with published sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa *et al.*, 1985). The tree with the highest log likelihood (-3787.3278) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3901)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 56.6964% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 23 nucleotide sequences. There were a total of 1386 positions in the final dataset. The 16S rRNA gene sequence of *Rickettsia ricketsii* was used as an outgroup. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

CHAPTER 5
DISCUSSION

The aim of this study was to determine the tick-borne haemoparasite diversity and the occurrence of *A. phagocytophilum* in selected rodent species collected from four different provinces in South Africa as well as in domestic dogs from the Mnisi community, Bushbuckridge, Mpumalanga, South Africa; and to characterize the *A. phagocytophilum* strains found in these samples by PCR and sequencing.

The results of the Reverse Line Blot (RLB) hybridization assay indicated that the four rodent species (*Rhabdomys pumilio*, *R. dilectus*, *Micaelamys namaquensis* and *Myotomys unisulcatus*) and the domestic dogs that were investigated harboured various tick-transmitted rickettsial and parasitic infections, either as single or mixed infections. *Anaplasma phagocytophilum* DNA was detected in both rodents and dogs, some of which was confirmed by sequence and phylogenetic analyses of the 16S rDNA.

5.1 Rodents

Rodents (Order: Rodentia) are small-sized mammals with a worldwide distribution, and they account for over 40% of all mammalian species (Huchon *et al.*, 2002; Mihalca *et al*., 2012). Rodents are both widespread and abundant, as are their associated ecto-parasites (i.e., ticks, fleas, mites and lice). By causing damage to crops, stored food, spreading diseases, and in destroying man-made infrastructures, rodents, particularly those of the family Muridae have been a menace to humankind for centuries worldwide. They play an important role as wildlife reservoirs of zoonotic infectious diseases; it is estimated that more than 10% of rodent species (244/2,220) are hosts of zoonotic infections, and carry 85 unique zoonotic diseases (Han *et al*., 2016). Many zoonotic pathogens that are endemic and avirulent in rodents are virulent in humans. These may be transmitted either directly or indirectly and cause diseases like bubonic plague, rickettsiosis, babesiosis, human granulocytic anaplasmosis, human monocytic ehrlichiosis, leptospirosis, *Bartonella* illness, tick-borne relapsing fever, Lyme borreliosis, just to name a few (Durden, 2006; Dantas-Torres *et al*., 2011).

Rhabdomys pumilio, R. dilectus and *M. namaquensis* are well-documented human commensal species (Eccard *et al*., 2000; Pedó *et al*., 2010; Rautenbach *et al*., 2014); and are generally found living in close associations with humans and very often dependent upon human habitats for the essential elements of food, water, shelter and space. These represent potential threats to both public and animal health through transmission of a variety of diseases (Gratz, 1994).

Rhabdomys species are locally abundant and regionally widespread, and occur endemically throughout southern Africa (De Graaf, 1981). They are habitat generalists and known to have successfully adapted to both unspoiled natural areas and human-modified landscapes, increasing their exposure to a large number of parasite species (Matthee *et al*., 2007). *Rhabdomys pumilio* has been shown to be an important host for a number of ectoparasite species and is of medical and veterinary importance especially with regards to the transmission of pathogens such as *Anaplasma marginale, Babesia caballi* and *B. canis* to domestic animals; *Rickettsia conori, Yersinia pestis,* and the viral disease Crimean-Congo hemorrhagic fever to humans (Matthee *et al*., 2007).

In our study, the majority of the blood samples (n = 30) were collected from *R. pumilio*, originating from four localities in the Western Cape. The RLB hybridization assay results revealed that *R. pumilio* harboured *A. phagocytophilum, A. bovis, Anaplasma* sp. Omatjenne, *Ehrlichia ruminantium* and *Babesia microti* DNA. The latter was the most prevalent species detected (56.7%). This is in concordance with a recent study (Troskie, 2017) that recorded pathogen diversity in 423 wild rodents and insectivore species collected from four provinces in South Africa; RLB results indicated the presence of *B. microti* (15.7%), *A. phagocytophilum* (2.3%), *A. bovis* (2%) and *E. ruminantium* (4.3%) DNA in 298 *R. pumilio* captured in the Western Cape and Northern Cape provinces of South Africa. In addition, *B. bovis* (1.3%) and *Theileria buffeli* (1%) were reported from this sample set. The presence of *B. microti*-like parasites was subsequently confirmed by 18S rDNA gene sequence analysis.

For *R. dilectus,* PCR products of the three available samples failed to hybridize with the species-specific probes, and only hybridized with the *Anaplasma/Ehrlichia* and *Theileria/Babesia* genus-specific probes. This could indicate the presence of novel species or a variant of a species. In the study by Troskie (2017), a larger sample set was investigated (n = 32) and reported the presence of *B. microti* (3.1%), *A. bovis* (9.3%) and *A. phagocytophilum* (3.1%) in *R. dilectus* from the Eastern Cape and KwaZulu-Natal provinces of South Africa.

Micaelamys namaquensis, a habitat generalists, has a wide distribution range and occurs across Africa south of the 18° latitude (with few exceptions in eastern Mozambique) (Skinner and Chimimba, 2005). They prefer rocky outcrops or hillsides, although they are quite flexible in their habitat requirements (Fagir *et al*., 2014). A large number of ectoparasite species have been reported to infest *M. namaquensis*; including the flea *Xenopsylla brasiliensis* that has been implicated as a vector for *Y. pestis* (Fagir *et al*., 2014). Furthermore, they are the preferred hosts for *Haemaphysalis elliptica* that transmits *B. rossi* to dogs and wild canids, and *Rickettsia conorii* to humans (Fourie *et al*., 1992; Lewis *et al*., 1996; Walker *et al*., 2000; Horak *et al*., 2005). They also sustain significant numbers of *Rhipicephalus warburtoni* which are vectors of *A. bovis* and may also cause paralysis in goats (Fourie *et al*, 1988; Harrison *et al*., 2011; Harrison *et al*., 2013). Furthermore, *M. namaquensis* has been shown to carry different *Bartonella* spp. including *B. elizabethae*, a *Bartonella* species with known zoonotic potential (Pretorius *et al*., 2004; Brettschneider *et al*., 2012).

Only three *M. namaquensis* samples were available for testing; RLB results indicated the presence of *Anaplasma* sp. Omatjenne and *E. ruminantium*. In the study by Troskie (2017), *A. bovis* (15%) and *Anaplasma* sp. Omatjenne (5%) were detected in 25 *M. namaquensis* captured in the Northern Cape province of South Africa.

Myotomys unisulcatus is a habitat generalist and occurs throughout the semi-arid Succulent Karoo and Nama-Karoo of South Africa (Monadjem *et al.*, 2015), specifically in the Eastern, Northern and Western Cape provinces, with some limited occurrence in the Fynbos Biome (Vermeulen and Nel, 1988). This species is not regarded as a human commensal species. Also, not much is known about the possible pathogens that this rodent species can harbour apart from the Arena virus (Merino Walk virus) that was described in 1985 (Palacios *et al*., 2010).

In our study, only one *M. unisalcatus* sample was available for testing; PCR products failed to hybridize with the species-specific probes, and only hybridized with the *Anaplasma/Ehrlichia* genus-specific probe. Troksie (2017) also reported PCR products of 14 *M. unisalcatus* samples that only hybridized with the *Anaplasma/Ehrlichia* and *Theileria/Babesia* genus-specific probes, indicating the presence of novel species or species variants.

Although only a limited number of rodent samples were analysed, the RLB hybridization assay results highlighted the importance of these rodent species as reservoir hosts for haemoparasites

that are of medical and veterinary importance. It furthermore confirms the findings of Troskie (2017) that South African rodent species are carriers of *Babesia, Anaplasma* and/or *Ehrlichia* species.

The detection of *B. microti* DNA in *R. pumilio* is of importance as it may have significant health implications for the human population living in contact with this generalist rodent species. However, since the focus of our study was on the *Anaplasma* species, *B. microti* results were not further confirmed with gene sequence analysis. *Babesia microti*, a common rodent pathogen, is one of the main causes of human babesiosis (Western *et al*., 1970). The white-footed mouse (*Peromyscus leucopus*), in addition to several other wild rodent species, is regarded as a reservoir host for *B. microti* in the United States, Europe and Asia (Van Peen *et al*., 1977; Watkins *et al*., 1991; Burkot *et al*., 2000; Duh *et al*., 2003; Siński *et al.*, 2006; Beck *et al*., 2011). Troskie (2017) speculated that *Rhabdomys* species may be the primary host for *B. microti* in South Africa. Other zoonotic *Anaplasma* and *Ehrlichia* species (causing Lyme disease, human granulocytic anaplasmosis and human monocytic ehrlichiosis) are known to occur concurrently in the same reservoir as *B. microti* and share the same tick vectors (Barbour, 1998). The vector for the transmission of *B. microti* parasites in South Africa is unknown, however, Troskie (2017) speculated that *H. elliptica* may be considered as a potential vector in the South African context.

Ehrlichia ruminantium infection was detected by RLB in *R. pumilio* and *M. namaquensis*; it was, however, not confirmed with gene sequence analysis. *Ehrlichia ruminantium* is an infectious, non-contagious rickettsial disease of sheep, goats and cattle (Cowdry 1925; Provost & Bezuidenhout, 1987; Dumler *et al*., 2001). It causes a disease commonly known as heartwater. Heartwater is considered to be one of the main tick-borne diseases in southern Africa and has a significant impact on livestock in the sub-Saharan region and neighbouring islands (Uilenberg, 1983), causing economic losses due to its high mortality rate (Uilenberg, 1983). It is transmitted by the tick vector *Amblyomma hebraeum* of which the immature stages have been known to feed on several rodent species including *R. pumilio* (Theiler, 1962). Howell *et al*. (1989) indicated that *R. pumilio* is not a suitable host reservoir for *E. ruminantium* and is unlikely to play a role in the transmission of heartwater.

We furthermore detected three *Anaplasma* species (*A. bovis, Anaplasma* sp. Omatjenne and *A. phagocytophilum*) in *R. pumilio*, and one *Anaplasma* species (*Anaplasma* sp. Omatjenne) in *M.*

namaquensis. Anaplasma bovis, the causative agent of monocytic and bovine anaplasmosis, is a small gram-negative obligatory intracellular bacteria (Dumler *et al.,* 2001). It infects the monocytes (Rar and Golovljova, 2011) of ruminants, including deer, goats, buffalo and cattle in Africa, Brazil, Japan, and the Middle East (Uilenberg, 1997; Kawahara *et al*., 2006; Santos and Carvalho, 2006; Ooshiro *et al*., 2008; Jilintai *et al*., 2009). *Rhipicephalus appendiculatus* and *Amblyomma variegatum* are considered possible tick vectors for *A. bovis* in Africa (Uilenberg, 1993; Dumler *et al*., 2001). Troskie (2017) found *R. pumilio, R. dilectus, M. namaquensis, Mastomys natalensis* and *Mus musculus* to be infected with *A. bovis* using the RLB hybridization assay and stated that to her knowledge, this was the first report of *A. bovis* infection in wild rodent species in South Africa. This was apart from an *A. bovis*-like organism previously detected in eastern rock sengi (*Elephantulus myurus*) and *Rhipicephalus* sp. near *Warburtoni* (in its possible vector) in Limpopo Province, South Africa (Harrison *et al*., 2011; Harrison *et al*., 2013). In a more recent study (De Boni, 2018), 40 wild rodents captured in the Mnisi community, Mpumalanga, South Africa were screened for the presence of haemoparasites using the RLB hybridization assay; *Lemniscomys rosalia* (5%), and *M. natalensis* (2.5%) tested positive for *A. bovis* DNA. During 16S rDNA phylogenetic analysis, we obtained six recombinant 16S rDNA sequences from one *R. dilectus* captured in KwaZulu-Natal, all of which were identical. It differed from the *A. bovis* type strain and the *A. bovis*-like strain previously described from eastern rock sengi (Harrison *et al.,* 2013) by 23-24 nucleotides, indicating the presence of a novel genetic variant of this pathogen. This was confirmed by phylogenetic analysis and warrants further investigation to confirm the taxonomic status of this *A. bovis*-like variant.

During the RLB hybridization screening of this *R. dilectus* sample, PCR amplicons hybridized with the *Anaplasma/Ehrlichia* genus-specific probe only, and with none of the species-specific probes. It also tested positive for *A. phagocytophilum* DNA using the real-time PCR (qPCR) assay, although with a high CT value of 37.60. When comparing the novel *A. bovis*-like 16S rRNA gene sequence obtained from *R. dilectus* to the *A. bovis* RLB probe region; it was found that the novel sequence differed by three nucleotides from that of the *A. bovis* RLB probe sequence. This might explain why the PCR amplicons only hybridized with the *Anaplasma/Ehrlichia* genus-specific probe and not with the *A. bovis* species-specific RLB probe. It also highlights the need for the development of an RLB probe that will allow for the specific detection of the *R. dilectus* associated *A. bovis*-like variant.

Anaplasma sp. Omatjenne was detected in *R. pumilio* and *M. namaquensis;* it was, however, also not followed-up with gene sequence analysis. *Anaplasma* (formerly *Ehrlichia*) sp. Omatjenne was described by Du Plessis (1990) as an apathogenic *Ehrlichia*-like parasite, which was isolated from a *Hyalomma truncatum* tick from Omatjenne in the Otjiwarongo district of Namibia. This is an area free of *Amblyomma* ticks and thus free of *Ehrlichia ruminantium*. However, a strain of *Anaplasma* sp. Omatjenne has been shown experimentally to produce disease indistinguishable from heartwater in sheep (Du Plessis, 1990). *Anaplasma* sp. Omatjenne has subsequently been found in cattle and dogs in Nigeria (Du Plessis, 1990; Adamu *et al*., 2014; Lorusso *et al*., 2016). The significance of our finding is unknown.

We then also detected *A. phagocytophilum* in *R. pumilio,* although only in three samples using the RLB hybridization assay. The samples were subsequently analysed with the qPCR assay for the specific detection of *A. phagocytophilum*; six of the *R. pumilio* blood samples tested positive for *A. phagocytophilum* DNA. This result could unfortunately not be confirmed by gene sequence analysis, probably due to low parasitaemia (as evident by the high CT values obtained). *Anaplasma phagocytophilum,* a tick-transmitted Gram-negative obligate intracellular pathogen, is an emerging tick-borne pathogen in humans and animals worldwide. In South Africa, in the absence of the *Ixodes* ticks 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 as possible vectors/reservoirs of the pathogen. The primary reservoir host is thought to be rodents (Stuen, 2007; Rymaszewska and Grenda, 2008; Bowman *et al*., 2009; Woldehiwet, 2010), with the white footed mouse (*Peromyscus leucopus*) the major reservoir species of *A. phagocytophilum*. In South Africa, Troskie (2017) recorded *A. phagocytophilum* in *R. pumilio* and in *R. dilectus* using the RLB hybridization assay. De Boni (2018) reported *A. phagocytophilum* DNA in *Aethomys ineptus* (2.5%) using the RLB hybridization assay, while 35% of their sample set (including *M. natalensis, Rattus rattus, R. tanezumi, Tatera leucogaster, Saccostomus campetris* and *L. rosalia*) tested positive for *A. phagocytophilum* DNA using the *A. phagocytophilum* qPCR assay.

5.2 Domestic dogs

There has always been a close relationship between humans and dogs; this is regarded as a risk factor for the transmission of pathogens (Otranto *et al*., 2009). Dogs thus act as good sentinels for human tick-borne infections (Hornok *et al*., 2013).

The Mnisi community is a rural community located in the north-eastern corner of the Bushbuckridge Municipality, Mpumalanga, South Africa; it consists of approximately 29,500 ha of communal land of which >75% borders private and provincial conservation areas. Smallholder agro-pastoralist farming (~1,300 farming households) is the primary subsistence activity, and ownership of domestic animals is common. Ownership of domestic dogs has also become more common in the community, although a number of dogs are free roaming and not confined to a specific household. Dogs are taken along for hunting or cattle herding; increasing their exposure to ticks and other ectoparasites. *Rhipicephalus sanguineus* (brown dog tick) is one of the main tick species that has been found in this community. It is almost an exclusive parasite of domestic dogs and is well adapted to living in kennels or human dwellings with its canine host (Walker *et al*., 2000), where it may also bite people in the safety of their own homes (Horak *et al*., 2002).

A recent study (Berrian *et al*., 2016) developed a One Health profile of the Mnisi agro-pastoralist population at the interface. The data summarized agricultural practices, food consumption and water-use habits, illness in animals and people, and access to health care which can be used to tailor education efforts for priority diseases and pandemic prevention. The study found that the participants' perception of animal species that are of most concern with regard to disease transmission to humans was dogs, followed by cattle, and cats.

In our study, the RLB hybridization assay results revealed the presence of *B. rossi, B. vogeli, E. canis, B. microti* and *A. phagocytophilum* DNA in the dog samples analysed, with *E. canis* being the most prevalent species. Canine babesiosis and ehrlichiosis are endemic in South Africa; our finding of B. *rossi, B. vogeli* and *E. canis* was thus not unexpected and were not further confirmed with gene sequence analysis. The *A. phagocytophilum* qPCR assay furthermore detected *A. phagocytophilum* DNA in 83.9%, which was higher than expected.

Babesia species are tick-transmitted apicomplexa parasites that infect a wide range of vertebrate hosts and cause severe diseases in wild and domestic animals (Kuttler, 1988). Canine babesiosis is one of the most important tick-borne diseases of dogs worldwide (Boozer and Macintire, 2003; Bourdoiseau, 2006; Jacobson, 2006). In South Africa, canine babesiosis is caused by *B. rossi* and *B. vogeli;* with *B. rossi* being more prevalent than *B. vogeli. Babesia rossi* is transmitted by the tick *H. elliptica* and cause a usually fatal infection in domestic dogs even after treatment. *Babesia vogeli*, transmitted by *R. sanguineus*, is known to cause less

severe disease than *B. rossi*, however infections can be fatal in young dogs and puppies (Solano-Galleho and Baneth, 2011).

Canine ehrlichiosis is common and widespread in dogs in tropical and sub-tropical regions, with infections ranging from asymptomatic to fatal disease (Donatien and Lestoquard, 1936). This disease is commonly found in South Africa and co-infections with *B. rossi* commonly occur in domestic dogs (Allsopp and Allsopp, 2001; Matjila *et al*., 2008a). Although *E. canis* and *B. vogeli* are transmitted by the same vector, *R. sanguineus*, co-infection with *B. rossi* and *B. vogeli* in domestic dogs is less frequent, than co-infection of *B. rossi* and *E. canis* (Matjila *et al.*, 2004; 2008a). Other small piroplasms capable of causing disease in dogs are *B. conradae*, (Kjemtrup *et al*, 2006), and the canine *Babesia microti*-like "Spanish isolate" or *Theileria annae* (which is phylogenetically close to zoonotic *B. microti* of humans). The latter is endemic in Galicia, north-western Spain (Camacho-García, 2006), but was also sporadically found in asymptomatic dogs from Croatia (Beck *et al*., 2011) and Mississippi (Yeagley *et al*., 2009).

Upon 16S rDNA sequence and phylogenetic analyses of selected dog samples which exhibited moderate Ct values (29 - 32) during the *A. phagocytophilum* qPCR assay, sequences of *A. phagocytophilum*, *A. platys* and *Anaplasma* sp. Zambian dog were obtained.

Studies in other parts of the world suggest that multiple strains (or variants) of *A. phagocytophilum* may be circulating in wild and domestic animals. These strains may have differential host tropisms and pathogenicity. Also, the degree to which genetic variation contributes to altered pathogenicity of different strains of *A. phagocytophilum* is poorly understood. This should be investigated in the South African context.

When studying *A. phagocytophilum* genetic diversity, the *groESL* operon, 16S rRNA locus, *ankA*, and *msp2* genes are the most frequently utilized markers (Sumner *et al*., 1997; Caturegli *et al*., 2000; Courtney *et al.*, 2004). It has, however, been shown that the 16S rRNA locus does not have the required level of discrimination to distinguish between pathogenic and non-pathogenic variants or between geographical variants (Huhn *et al*., 2014). In our study, five recombinant 16S rDNA sequences were obtained from one dog (all identical) that were identical to *A. phagocytophilum* human strains Webster, HZ and HZ2, as well as to the *A.*

phagocytophilum Dog2 strain. It is recommended that future studies should focus on more suitable markers to elucidate the possible *A. phagocytophilum* diversity in South Africa.

Anaplasma phagocytophilum causes canine granulocytic anaplasmosis in dogs (Woldehiwet, 2010). Dogs (and humans) are considered as accidental hosts and are not important in disease transmission to other host species. Dogs act as sentinels for human exposure and can also be a source of infection by bringing infected ticks into contact with people (Carrade *et al*., 2009). *Anaplasma phagocytophilum* causes an acute febrile illness in dogs with lethargy and inappetence. To date, there are no case reports documenting fatalities in dogs (Carrade *et al*., 2009).

Reports of human granulocytic anaplasmosis occurring in Africa have been few (M'Ghirbi *et al*., 2009; 2012). In South Africa there have been no official diagnoses of *A. phagocytophilum* in humans. Molecular detection of a bacterium closely related to *A. phagocytophilum* was made from specimens from three South African dogs (Inokuma *et al*., 2005) and designated *Anaplasma* sp. South African dog strain. More recently, the same *Anaplasma* sp. (SA dog strain) as well as *A. phagocytophilum*, and an *Orientia tsutsugamushi*-like partial 16S rRNA sequence were detected in dog samples collected in the Mnisi community, Mpumalanga, South Africa (Kolo *et al*., 2016). While *A. phagocytophilum* has also been reported in ticks collected from cattle, sheep, goats, dogs and cats (Mtshali *et al*., 2015; 2017), this finding should be verified since close inspection of the primers used in those studies indicates that they will amplify any *Anaplasma* species.

Anaplasma sp. Zambian dog was recently described from dogs in Lusaka, Zambia (Vlahakis *et al*., 2018) and is closely related to *Anaplasma* sp. South African dog (Inokuma *et al*., 2005). In our study, 10 recombinant sequences obtained from four dogs that tested positive for *A. phagocytophilum* DNA using the qPCR assay (although at very high Ct values) were all identical to *Anaplasma* sp. Zambian dog. Very little is known about the significance and epidemiology of these species. There are currently no probes for their detection by the RLB assay, and it is possible that the genus-specific only signals obtained by the RLB assay were for these species/genotypes. This highlights the need for the development of species-specific assays in order to differentiate infections by these important *Anaplasma* spp. Furthermore, the specificity of the *A. phagocytophilum* qPCR assay should be investigated to ensure that the primers will not also amplify *Anaplasma* sp. Zambian dog and/or *Anaplasma* sp. South African

dog DNA. If this would be the case, it could explain the high *A. phagocytophilum* qPCR detection rate in the dogs samples tested.

Anaplasma platys is a cosmopolitan species of dogs with a worldwide distribution and zoonotic potential (Matei *et al*., 2016). It is thought to be transmitted by *R. sanguineus.* It infects platelets of dogs and is the causative agent of infectious canine cyclic thrombocytopenia (ICCT) (Inokuma *et al*., 2002). Due to recent confirmed cases of human infections, *A. platys* is now regarded as a newly emergent zoonotic agent (Maggi *et al*., 2013; Arraga-Alvarado *et al*., 2014). In our study, the recombinant sequences we obtained were identical to *A. platys* Zam Dog-99, Zam Dog-166 and Zam Dog-72, previously described from dogs in Chilanga District, Lusaka Province, Zambia (Vlahakis *et al*., 2018).

Our RLB hybridization results also revealed some unexpected findings of *E. ruminantium, Theileria parva, Anaplasma* sp. Omatjenne and *Babesia* sp. (sable) DNA. It remains speculative whether these are true findings or perhaps due to cross-reaction of the RLB probes with previously unknown targets. Contamination with other target DNA from another sample can also not be ruled out. An incidental infection, which can occur when a non-host is accidentally infected with a parasite through a tick bite, should also be considered. 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 16S or 18S rDNA genotypes) that might have cross-reacted with the RLB probes could also explain some of these results. 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 16S and 18S rRNA genes should be amplified and sequenced.

Various African wild ruminants are said to be the reservoirs of *E. ruminantium* (Neitz, 1967; Peter *et al*., 2002; Allsopp *et al*., 2003); however, it has been shown that domestic livestock can also act as reservoirs of the bacterium (Uilenberg, 1983). In 2001, Allsopp and Allsopp reported on the detection of *E. ruminantium* in dogs where the symptoms ranged from asymptomatic to symptomatic. The authors also suggested further investigation to elucidate their finding.

Theileria parva the most pathogenic and economically significant *Theileria* sp. in eastern, central and southern Africa (Norval *et al.*, 1992), and causes East Coast fever, Corridor disease and January disease in cattle (Uilenberg *et al*. 1982; Perry *et al*. 1991). Whilst causing only subclinical infection in buffalo, *T. parva* causes fatal disease in cattle. There are no reports in literature of *T. parva* ever being reported from dogs, although, other *Theileria* species have been identified from dogs in South Africa (Matjila *et al*. 2008b; Rosa *et al*., 2014).

As mentioned before, *Anaplasma* sp. Omatjenne has been detected in cattle and dogs in Nigeria (Du Plessis, 1990; Adamu *et al*., 2014; Lorusso *et al*., 2016). The significance of our finding remains unknown.

Babesia sp. (sable) is a member of the *Babesia* sensu stricto clade, and was identified in a sable antelope (*Hippotragus niger*) in South Africa that presented with a sudden onset of disease and that subsequently died (Oosthuizen *et al*., 2008). The tick vector remains unknown. We would suggest that the presence of *Babesia* sp. (sable) is unlikely to be a true finding and we speculate that it was due to cross-reaction of the RLB probe or due to contamination with other target DNA.

5.3 *Anaplasma phagocytophilum* diagnostic assay performance

The qPCR assay was shown to be more sensitive than the RLB hybridization assay in detecting *A. phagocytophilum* DNA in both rodent and dog blood samples. This phenomenon has been shown before by several authors, to name a few: Eygelaar *et al.* (2015) found that the qPCR and IFAT were far better at detecting *T. parva* positive samples in buffalo from northern Botswana than the RLB assay. In a study by Chaisi *et al*. (2017), the RLB assay was found to be less sensitive than nested PCR and qPCR when detecting *A. marginale* and *A. centrale* infections in cattle in South Africa. Nevertheless, the RLB assay remains a valuable screening tool for the simultaneous detection and differentiation of infections using species- and genus-specific probes (Gubbels *et al*. 1999; Bekker *et al*. 2002). It has therefore widely been used to reveal *Anaplasma, Erhlichia, Babesia* and/or *Theileria* infections in different hosts and vectors and in identifying novel species and species variants (Nijhof *et al*. 2005; Bhoora *et al*. 2009; Oosthuizen *et al*. 2009; Bosman *et al*. 2010; Chaisi *et al.* 2011; Ceci *et al*. 2014).

As mentioned before, the specificity of the *A. phagocytophilum* qPCR assay should be investigated as it cannot be ruled out that the *A. phagocytophilum* real-time PCR probe cross-

reacted with *Anaplasma* sp. Zambian and South African dog strain DNA.. It is therefore recommended that *A. phagocytophilum, Anaplasma* sp. Zambian dog and *Anaplasma* sp. South African dog *msp*2 gene sequence data be generated and checked against the current qPCR primer and probe sequence sets to ensure the specificity of the assay. Once confirmed, more extensive sampling is encouraged to determine the true prevalence of *A. phagocytophilum* in South Africa. More gene sequence markers should also be utilized to determine the *A. phagocytophilum* strain diversity that might exist in the country.

IN SUMMARY:

The study highlighted the importance of wild rodents and dogs as reservoir species for haemoparasites that are of medical and veterinary importance. We have shown that: (i) wild rodents and domestic dogs in South Africa habour many tick-borne pathogens, some of which are animal and human pathogens, further studies should be carried out to determine the risk of these infections to human health; (ii) the qPCR assay was more sensitive than the RLB assay in detecting *A. phagocytophilum* infections, however, the specificity of the assay should be confirmed; (iii) sequence analyses confirmed the presence of *A. phagocytophilum* DNA in a dog; and (iv) *Anaplasma* sp. Zambian and South African dog strain seems to be a common species in South African dogs, more studies are needed to determine the taxonomic status and epidemiology of these species in South Africa.

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APPENDICES

Appendix 1: Letter approval for the MSc study 'Occurrence of *Anaplasma phagocytophilium* in wild rodent samples from South Africa and domestic dog samples collected from the Mnisi Community, Bushbuckridge, Mpumalanga, South Africa' issued by the Animal Ethics Committee, University of Pretoria, South Africa.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Occurrence of Anaplasma phagocytophilium in wild rodent samples and domestic dog samples collected from the Mnisi community area, Bushbuckridge, Mpumalanga SA**
PROJECT NUMBER	**V062-16** ***(Part of V46-14 and V030-14)***
RESEARCHER/PRINCIPAL INVESTIGATOR	**S Will-Berriman**

STUDENT NUMBER (where applicable)	**UP_12076067**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Wild rodent**	**Canine**
NUMBER OF ANIMALS	**Report blood samples**	**50**
Approval period to use animals for research/testing purposes		**June 2016 –June 2017**
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 27 June 2016
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 'Discovering emerging tick-borne pathogens that could impact on human health and livestock production in South Africa: Developing real-time PCR assays for the specific and sensitive detection of these pathogens', 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 Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6; 12/11/1/1

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

Dear Prof Oosthuizen,

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 19 October 2015, requesting an amendment to permission granted on 23 December 2015 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 amended research/study, with the following conditions :

Conditions:

1. All conditions stipulated within the approval dated 2014-12-23 must still be complied with;
2. The only allowable deviation from the research protocol for which Section 20 approval was granted on 2014-12-23 is the inclusion of the following postgraduate students to part take in the study:
   (i) Dr Agatha Kolo
   (ii) Dr Liesl De Boni
   (iii) Ms Samantha Wills

Title of research/study: Discovering emerging tick-borne pathogens that could impact on human health and livestock production in South Africa: Developing real-time PCR assays for the specific and sensitive detection of these pathogens
Researcher (s): Prof Marinda Oosthuizen, Dr Agatha Kolo, Dr Liesl De Boni, Ms Samantha Wills
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, UP
Your Ref./ Project Number:
Our ref Number: 12/11/1/1; 12/11/1/1/6

Kind regards,

Maja.

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2015 -11- 13