Temporal dynamics of tick-borne haemoparasite infection in calves in the Mnisi communal area, Mpumalanga, South Africa

By

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Declaration

I hereby declare that this dissertation, which I submit in fulfilment of the degree of Master of Science at the University of Pretoria, South Africa, is my own work. This work has not been submitted previously for any degree or examination at any other university.

.....	.....
Sekgota Marcus Makgabo	**Date**

Dedication

This work is dedicated to my beautiful siblings, Lebogang and Mosima Makgabo, as well as my nephews and nieces. I hope this work inspires them to aim for greater things in life and work twice as hard to reach their dreams and goals.

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List of Abbreviations

AEGP	Addo Elephant Game Park
AHTs	animal health technicians
CCT	canine cyclic thrombocytopenia
CGA	canine granulocytic anaplasmosis
Cp	crossing point
DAFF	Department of Agriculture Forestry and Fisheries
dATP	deoxyadenosine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
EB	elementary bodies
ECF	East Coast Fever
ECL	enhanced chemiluminiscence
EDAC	1-ethyl-3-(-3-dimethylamininopropyl) carbodiimide
EDTA	ethylenediaminetetraacetic acid
EGA	equine granulocytic anaplasmosis
ELISA	enzyme-linked immunosorbent assay
EM’s	environmental monitors
FMD	foot-and-mouth disease
G	gamete
gDNA	genomic deoxyribonucleic acid
GLTFCA	Great Limpopo Transfrontier Conservation Area
HGE	human granulocytic ehrlichiosis
HGA	human granulocytic anaplasmosis
HHWRS	Hans Hoheisen Wildlife Research Station
HIP	Hluhluwe-IMfolozi Game Park
IFAT	indirect fluorescent antibody test
ITS2	internal transcribed spacer
KCl	potassium chloride
KNP	Kruger National Park

KZN	KwaZulu-Natal
M	merozoites
MCP	Mnisi Community Programme
$MgCl_2$	magnesium chloride
MSA	Mnisi study area
MSP	major surface protein
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NK	natural killer
OBP	Onderstepoort Biological Products
PBS	phosphate-buffered aaline
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction – restriction fragment length polymorphism
pH	potential Hydrogen
POD	peroxidase
qPCR	quantitative polymerase chain reaction
RB	reticulate bodies
RLB	reverse line blot
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
Sk	Stranhlenkorper
SSPE	saline–sodium phosphate–EDTA
St	sporoblast
Sz	sporozoites
T	trophozoites
TqM	TaqMan
TBDs	tick-borne diseases
Tris-HCL	Tris hydrochloride
UDG	uracil-DNA glycosylase
UNG	uracil-N-glycosylase
US	United States
USA	United States of America
VPN	veterinary procedural notice

STUDY SUMMARY

Temporal dynamics of tick-borne haemoparasite infection in calves in the Mnisi communal area, Mpumalanga, South Africa

by

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Supervisor : Dr NE Collins
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Anaplasmosis, babesiosis, and heartwater are the three most important tick-borne diseases of cattle in South Africa and result in a large number of mortalities. Endemic stability contributes to disease control, but little is known about the conditions required for maintenance of endemic stability. Through the on-going Health and Demographic Surveillance System in Livestock in the study area of the Mnisi One Health Platform, Mpumalanga, a great deal of information is being collected about cattle in the area, with the eventual aim of developing mathematical models to describe and predict infections. More than 15000 cattle have been identified for tick burden assessment, serological analyses and parasite identification. However, little is known about the time-course of infection of cattle with various tick-borne haemoparasites. Therefore, this study aimed to investigate the time-course of infection in new-born calves (n=10) and the presence of haemoparasites in adult ticks over a one year period using reverse line blot (RLB) hybridization and quantitative polymerase chain reaction assays. Blood samples and adult ticks were collected monthly from new-born calves in two areas of the Mnisi communal area: five located in a peri-urban area and five at the wildlife/livestock interface. A total of 119 blood samples and 805 adult ticks were collected. The RLB results confirm the exposure of most new-born calves in the Mnisi communal area to non-pathogenic and pathogenic tick-borne haemoparasites in the genera

Anaplasma, *Babesia*, *Ehrlichia* and *Theileria* in their first year of life. A total of 805 adult ticks were identified to species level using identification keys and molecular methods. Only two tick species, *Amblyomma hebraeum* and *Rhipicephalus microplus*, were found on the calves during the year. Non-pathogenic and pathogenic haemoparasites in the genera *Anaplasma*, *Babesia*, *Ehrlichia* and *Theileria* were detected in pooled DNA extracted from ticks that had digested their blood meal. Pathogen-specific qPCR results indicated that some of the pathogens could not be detected in the calves until six to seven months of age and *A. marginale* was not detected at all in three calves at the wildlife/livestock interface. These calves were either infected at levels below the detection limit of our assays, or they were not infected at all. If the latter, it is possible that exposure to related non-pathogenic haemoparasites might help to establish and maintain endemic stability. Factors such as cattle density and dipping methods within different areas in the Mnisi communal area may play a role in the number of infected tick vectors in an area, and thus in the time-course of infection in new-born calves. It is clear that detailed information for cattle in different localities in the Mnisi communal area will be required in order to build accurate mathematical models to describe and predict infections.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Ticks and tick-borne diseases (TBDs) are regarded as major obstacles to livestock farming worldwide, especially in Africa (Mbati et al., 2002; Smith and Parker, 2010). Tick-borne haemoparasites are distributed globally, occurring and affecting all domestic animals on every continent (Zwart, 1985; Uilenberg, 1995). The existence of ectoparasites such as ticks is considered as an important factor affecting livestock health because they inflict not only direct damage to their hosts, but they can transmit various infectious pathogens from infected hosts to healthy ones (Anderson et al., 2013).

Anaplasma, Babesia, Ehrlichia and *Theileria* are genera of paramount importance to the veterinary field with species causing various TBDs that affect cattle worldwide. The most important TBDs of cattle in southern Africa are anaplasmosis, babesiosis and heartwater, which cause major economic losses through mortality and morbidity, decreased milk and meat production and expensive control measures (Uilenberg, 1995; de Waal, 2000; Makala et al., 2003; Mtshali et al., 2004; Rikhotso et al., 2005; Eygelaar et al., 2015).

Although the last half century has seen an increasing interest in animal diseases, a better understanding of disease processes at the wildlife/livestock interface and how to manage them is required (Rhyan and Spraker, 2010). With increasing and expanding human populations, close contact between wild animals and livestock throughout the world is increasing dramatically and as a result, disease transmissions in such areas have a negative impact on people and livestock residing at the wildlife/livestock interface (de Garine-Wichatitsky et al., 2013).

The interactions occurring at the wildlife/livestock interface require an improved understanding of the ecology of pathogens and pathogen transmission together with the development of tools and methods to manage these pathogens (Miguel et al., 2014). Mathematical models can play a vital role, particularly in the field of ecology, as they provide general methods and strategies for

predicting and managing outbreaks of tick-borne diseases while identifying mechanisms within a system and highlighting areas where further empirical research is needed (Gaff and Gross, 2007).

Endemic stability is known to be one of the major factors involved in the natural control of tick-borne diseases worldwide, and is characterized by the lack of or low prevalence of clinical cases in a population with high levels of infection. For this epidemiological state to occur in a population, a stable and balanced relationship between the host, agent (disease-causing pathogen), vector and the environment is required. Establishment and maintenance of endemic stability to TBDs in a population requires large numbers of infected ticks and vertebrate hosts, and exposure of the vertebrate hosts to pathogens of interest during lack of or low prevalence of clinical cases (Norval et al., 1992). Disruption of endemic stability in a population can be witnessed by a sudden increase in the rate at which clinical cases are observed (Coleman et al., 2001).

1.2. Wildlife/livestock interface

The wildlife industry in South Africa has grown significantly over the past two decades and this was evident by the significant increase in the purchase and sale of wild animals despite their high prices during this time. Amongst these are eland (*Tragelaphus oryx*), Cape buffalo (*Syncerus caffer*), white and black rhinoceros (*Diceros bicornis* and *Ceratotherium simum*), sable antelope (*Hippotragus niger*) and roan antelope (*Hippotragus equinus*) (Horak et al., 2015). The increase in the purchase and sale of wild animals has in turn led to an increasing shift in land use from agricultural livestock farming to game farming in South Africa, which has consequently resulted in an increase in the wildlife/livestock interface in many parts of the country (Parker and Bernard, 2005; Smith and Parker, 2010).

The wildlife/livestock interface is a common boundary separating wild animals and livestock (Bengis et al., 2002). The Kruger National Park (KNP) in South Africa is one of the largest game reserves in Africa. The interface consists of a 750 km dividing fence-line erected in 1960 to separate cattle from Cape buffalo (*Synercus caffer*), creating the southern and western boundaries of the KNP, which border rural areas in South Africa (Jori et al., 2011). The main disadvantage of this interface is the risk of pathogen transmission between wildlife and livestock, because various tick vectors feed on wild animals, increasing the risk for occurrence, abundance

and distribution of ticks among livestock situated at the interface (Smith and Parker, 2010; Caron et al., 2013).

Most African wild animals such as the Cape buffalo have innate resistance to many tick-borne diseases. Cape buffalo are the natural reservoir host of various important pathogenic and non-pathogenic *Theileria* species (Horak et al., 1983; Uilenberg, 1995; Bengis et al., 2002). Various studies have reported that the buffalo population is commonly co-infected with different *Theileria* spp., namely, *T. buffeli*, *T. parva*, *Theileria* sp. (buffalo), *T. mutans* and *T. velifera* (Allsopp, et al., 1993; Gubbels, et al., 1999; Oura, et al., 2004; Pienaar, et al., 2011; Chaisi, et al., 2011). The presence of tick-borne haemoparasites of veterinary importance belonging to the genus *Anaplasma*, namely: *A. marginale*, *A. centrale*, *A. phagocytophilum* and *Anaplasma* sp. Omatjenne, have been reported recently in the South African buffalo population (Debeila, 2012; Eygelaar et al., 2015; Henrichs et al., 2016; Khumalo et al., 2016; Sisson et al., 2017). However, little is known about the prevalence and impact of *Anaplasma* species originating from Cape buffalo at the livestock/wildlife interface.

The Cape buffalo is not the only wild animal affected by tick-borne haemoparasites. Recent studies conducted in South African wildlife, including eland, roan and sable antelope, blesbok (*Damaliscus pygargus phillipsi),* blue and black wildebeest (*Connochaetes gnou* and *Connochaetes taurinus*, respectively), gemsbok (*Oryx gazelle*), giraffe (*Giraffa camelopardalis*), impala (*Aepyceros melampus*), nyala (*Tragelaphus angasii*) and waterbuck (*Kobus ellipsiprymnus*), have identified numerous tick-borne haemoparasites in the genera *Anaplasma, Babesia, Ehrlichia* and *Theileria* (Tonetti et al., 2009: Pfitzer et al., 2011; Berggoetz et al., 2014; Khumalo et al., 2016).

Given the presence of so many wildlife reservoir hosts, disease transmission by ticks is likely to have a negative impact on livestock and humans residing at the wildlife/livestock/human interface (Smith and Parker, 2010). Co-existence and contact between wild animals and livestock has also led to wildlife/human conflict because the increasing contact results in increased disease transmission from wildlife to livestock and, in some cases, to humans (Brahmbhatt et al., 2012; de Garine-Wichatitsky et al., 2013).

1.3. The Mnisi communal area

The Mnisi communal area in the Bushbuckridge Municipal Area, Mpumalanga Province, South Africa, is located at the heart of a wildlife/livestock/human interface as three quarters of its boundary is adjacent to wildlife areas, including the Andover and Manyeleti provincial game reserves and the Timbavati and Sabi Sand private game reserves. The Manyeleti Game Reserve and private game reserves in the area were incorporated into the Greater Kruger National Park in 1993 through the removal of the fence and these now form part of the important conservation initiative of the Great Limpopo Transfrontier Conservation Area (GLTFCA).

In the community, the University of Pretoria established the Mnisi Community Programme (MCP), a multidisciplinary platform for research, teaching, learning and community engagement within the 'One Health' philosophy. Research in the area is focused mainly at the human/livestock/wildlife ecosystem interface and includes disease ecology and emergence, zoonoses, livestock production and trade as well as natural resource utilisation.

The Mnisi communal area has a population of about 40,000 people living in 8,500 households. Livestock farming is the main agricultural activity in the area and a game-proof fence is the only barrier between livestock and the abundant wildlife populations in the reserves. Comprehensive disease surveillance measures are implemented in the area by local veterinary services, mainly in the form of cattle dip tanks built throughout the region, which every cattle herd must visit for dipping and inspection once a week. During the study period, the dip consisted of the Delete® X5 acaricide which is used on cattle, sheep and goats, for the prevention and treatment of ectoparasite infestations. Delete® X5 contains 5% deltamethrin that belongs to a family of pyrethroids. Synthetic pyrethroids are widely used for controlling tick infestation on animals (Mehlhorn et al., 2011; Buczek et al., 2014; Buczek et al., 2015).

1.4. Ticks and tick-borne diseases

Ticks are a group of invertebrate parasites known to be vectors of pathogens within the phylum *Arthropoda*, class *Arachnida* and order *Acari* (Walker et al., 2003). These invertebrate parasites are the second most important vectors of both pathogenic and non-pathogenic diseases in the world, with mosquitoes being the first (Parola and Raoult, 2001). They are divided into three major

families: the Ixodidae, commonly known as the hard ticks, the Argasidae, the soft ticks and the Nuttalliellidae, which consists of one tick species that is only found in southern Africa (Parola and Raoult, 2001; Walker et al., 2003).

Ticks feed on the blood of their hosts. During their feeding state, ticks attach to the skin of their host and thus transmit various pathogenic microorganisms from infected animals to healthy ones, resulting in detrimental effects (Jongejan et al., 2007). Like mosquitoes, ticks can also transmit pathogens that cause human diseases, but they are the most important blood-feeding arthropods able to maintain and transmit a variety of pathogenic microorganisms such as rickettsiae (causing anaplasmosis and heartwater), protozoa (causing theileriosis and babesiosis), viruses and spirochaetes (causing a number of severe toxic conditions such as paralysis, allergy and irritation) in livestock and wild animals (Jongejan and Uilenberg, 2004; Pfaffle et al., 2013).

Ticks are responsible for the biological transmission of pathogens in three different ways; these are 1) transstadial transmission, which occurs when a tick remains infected with the pathogen from one life stage to the next before transmission to the host, 2) intrastadial transmission, occurs when a pathogen is transmitted between two hosts by the same tick in the same life stage, or 3) transovarial transmission, which occurs when the pathogen is passed from parent ticks to offspring and transmission to the host only occurs through the larvae of the following generation.

1.4.1. Tick life-cycles

Ticks are invertebrate parasites that depend on various intrinsic and extrinsic factors for survival and growth. Some of these factors include the accessibility and abundance of vertebrate hosts, genotype of the tick and environmental conditions (Padgett and Lane, 2001). Their life stages are divided into four phases: egg, nymphal, larval and adult. The life cycle of most tick species from reproduction to adult involves a number of hosts. The life cycle of the ixodid tick species is characterised by a three-host life cycle where the ixodid tick species feed on three specific hosts, however, some species only feed on one or two hosts. The soft ticks (Argasid species) can feed on multiple hosts (Minjauw and McLeod, 2003).

Ticks associated with the one-host life cycle belong to the genus *Rhipicephalus* (formerly *Boophilus)*. These ticks are normally found on large animals (hosts) such as cattle and horses.

The one-host tick life cycle begins with the larva finding a suitable host; it feeds and moults on the same host until the adult stage. All the stages of the one-host ticks take place on the same host until the adult ticks mate, and the engorged adult female ticks then drop off from the host to lay eggs on the ground (Minjauw and McLeod, 2003).

The larval and nymphal stages of the two-host ticks occur on the same host, however, the nymphs leave the host and moult to the adult stage on the ground, where the adult tick will then search for a final suitable host. Ticks in the genera *Hyalomma* and *Rhipicephalus* have a two-host life-cycle (Minjauw and McLeod, 2003).

With three-host ticks, each tick life stage (larva, nymph and adult) occurs on different vertebrate hosts. The immature stages of three-host ticks only feed on rodents and birds, while adults ticks associated with this life cycle only feed on larger animals (Minjauw and McLeod, 2003).

According to Horak et al, (2015) the most important tick species known to be common to both cattle and wild animals are *Amblyomma hebraeum*, *Ixodes rubicundus*, *Rhipicephalus appendiculatus*, *Rhipicephalus decoloratus* and *Rhipicephalus microplus*. These ticks are very sensitive and vulnerable to climate change because their local survival, maintenance and reproduction strongly depend on environmental factors such as: temperature, sunlight and water or humidity (Leger et al., 2013). These tick species are known to transmit various TBDs of veterinary and human importance.

Rhipicephalus microplus is a tick species that occurs worldwide and has been shown to transmit bovine babesiosis and anaplasmosis (de Castro, 1997). Anaplasmosis, babesiosis and heartwater are the three most important TBDs of cattle in South Africa (Mtshali et al., 2004) while theileriosis is a state controlled disease of cattle. It is believed that TBDs become a problem when domestic livestock are in contact with wildlife and this may be due to movement of livestock to tick infested areas or movement of tick infested livestock to previously uninfested areas (Jongejan and Uilenberg, 2004).

1.5. Rickettsial pathogens and diseases

The order Rickettsiales was reorganised and reclassified into two families based on molecular phylogenetics. These families include the Anaplasmataceae, which are known to replicate within a vacuole in the eukaryotic host cell, and the Rickettsiaceae, which grow freely in the cytoplasm of the eukaryotic host cell (Dumler et al., 2001). Dumler et al. (2001) also reorganised the genus *Anaplasma* to include *Anaplasma bovis*, *Anaplasma phagocytophilum* and *Anaplasma platys*, which were formerly classified within the genus *Ehrlichia* (Dumler et al., 2001; Aubry and Geale, 2011). Pathogens in the genera *Ehrlichia* and *Anaplasma* can be transmitted by ticks and they can infect erythrocytes, leukocytes and platelets in the vertebrate host (Dumler et al., 2005).

Obligate intracellular parasites in the genera *Anaplasma* and *Ehrlichia* cause diseases such as bovine anaplasmosis and heartwater in cattle, and thus affect livestock farming throughout Africa (Jongejan and Uilenberg, 2004).

1.5.1. Bovine anaplasmosis

Bovine anaplasmosis is a tick-borne disease of cattle caused by obligate intra-erythrocytic, gram-negative pathogens in the genus *Anaplasma*, which reside within cytoplasmic vacuoles (Aubry and Geale, 2011). This disease of cattle is known to be one of the four most important and detrimental tick-borne diseases occurring in sub-Saharan Africa, and causes 3% of cattle mortalities in South Africa (De Waal, 2000). The anaplasmosis disease-causing organisms are also known to grow in tick vectors, providing a route for biological transmission. However, transmission can also occur transplacentally or mechanically (Dumler et al., 2001; Aubry and Geale, 2011; Potgieter and Stoltz, 1994).

Three tick-borne haemoparasites are known to cause clinical anaplasmosis in cattle: these are *Anaplasma marginale* which is highly pathogenic, *Anaplasma centrale* (originally named *Anaplasma marginale* variety *centrale*) which, in contrast to *A. marginale*, usually causes asymptomatic infections, and *Anaplasma bovis* which has been reported to cause infection in ungulates such as cattle and buffalo in different continents, such as Africa, South America, and Asia (Dumler et al., 2001; De La Fuente et al., 2005; Rymaszewska and Grenda, 2008; Joazeiro et al., 2015).

Anaplasmosis occurs predominantly in cattle; however, infection can also occur in ruminants such as African antelopes, Cape buffalo and some species of deer (Aubry and Geale, 2011). The disease is known to affect cattle in tropical and subtropical areas of the world, including Africa, United States (US), South and Central America, southern Europe, Asia and Australia, and causes a decrease in livestock production as a result of abortion, fever, weight loss, lower milk yields and death in up to 36% of clinical cases (Kocan et al., 2004; Aubry and Geale, 2011; Hammac et al., 2013).

1.5.1.1. *Anaplasma marginale*

Anaplasma marginale is a highly pathogenic intra-erythrocytic pathogen and causes anaplasmosis in ruminants, predominantly cattle. This pathogen is found in the family *Anaplasmataceae* of the order Rickettsiales (Aubry and Geale, 2011). Rymaszewska and Grenda (2008) report that *A. marginale* was first discovered by Salmon and Smith in 1894. Sir Arnold Theiler fully described the pathogen in 1910, when he detected and observed that the “marginal points” (inclusion bodies) in the cells of infected erythrocytes of calves were the causative agent of a specific disease, then named gallsickness, in South Africa. The inclusion bodies located at the margins of erythrocytes are actually small colonies containing four to eight individual organisms.

Bovine anaplasmosis caused by *A. marginale* is prevalent throughout the world and occurs in Africa, Australia, the Americas, Asia, the Caribbean and Europe (Kocan et al., 2003). It is regarded as one of the four most important TBDs of cattle in South Africa, and is widespread and endemic throughout the cattle-farming area of the country, with 99% of cattle at risk (De Waal, 2000). A recent study conducted by Mutshembele et al. (2014), reported on the molecular evidence of the prevalence and presence of *A*. *marginale* in South Africa. In this study, *Anaplasma marginale* was reported in all South African provinces except for the Northern Cape, which is free of the tick vectors. Mpumalanga, Gauteng, and Eastern Cape Province had the highest prevalence with 100% infection rate (Mutshembele et al., 2014). These findings were further confirmed by a study conducted by Hove et al., (2018), who also identified *A. marginale* in a larger sample size of cattle in all South African provinces except for Northern Cape. However, in contrast to the findings of Mutshembele et al., (2014), the highest prevalence of *A*. *marginale* was detected in KwaZulu-Natal (KZN), Western Cape and Mpumalanga (Hove et al., 2018).

1.5.1.1.1. Transmission of *A. marginale*

Anaplasma marginale is different from other tick-borne pathogens as it can be transmitted in two ways: biologically by ticks and mechanically by biting flies or blood-contaminated veterinary instruments including needles, castration instruments and ear tagging devices (Kocan et al., 2000). Recent studies (Aubry and Geale, 2011; Costa et al., 2016) suggest that *A. marginale* can also be transmitted transplacentally, where infected erythrocytes can pass through the placenta of infected cows to their offspring. The efficiency of transmission by biting flies has, however, been reported to be very low (Scoles et al., 2005), and biological transmission by tick vectors remains the most important means of transmission (Potgieter, 1979; Eriks et al., 1993; Kocan et al., 2000).

Male ticks are thought to serve as the principal reservoir of anaplasmosis and play a key role in the intrastadial transmission of anaplasmosis, as the period it takes for male ticks to become infected from an infected bovine is believed to be short and male ticks have the ability to repeatedly transmit the infection when they continuously feed on multiple susceptible cattle (Kocan et al., 2000). This is due to the fact that adult male ticks feed on more than one bovine host: they feed on a susceptible cattle host for a period of 4 to 8 days before detaching and moving to another host in search of a mating partner, thereby increasing the chances of intrastadial transmission of the parasite between cattle hosts (Eriks et al., 1993). In contrast, adult female ticks generally do not feed on more than one bovine host, they attach to one host and feed until they are completely engorged (Eriks et al., 1993).

Ixodid ticks are the principal vectors of *A. marginale* (Potgieter and Stoltsz, 1994; Eriks et al., 1993). *Dermacentor andersoni*, also known as the Rocky Mountain wood tick, is a three-host tick believed to be responsible for the transstadial (occurs from one life stage to the next in the presence of a pathogen) and intrastadial (as adult males) transmission of *A. marginale* in the northwestern United States (Kocan et al., 2000). *Dermacentor variabilis* transmits *A. marginale* in the eastern U.S. *Rhipicephalus decoloratus*, *R. microplus*, *R. evertsi evertsi*, *R. simus* and *Hyalomma marginatum rufipes* and are the five tick species implicated in the transmission of *A. marginale* in South Africa (De Waal, 2000; Potgieter, 1981; Potgieter & Stoltsz, 1994). In the case of *A. marginale*, transovarial transmission does not occur (Eriks et al., 1993; Kocan et al., 2000).

1.5.1.1.2. Life cycle of *A. marginale*

The life cycle of *A. marginale* is complex (Fig. 1.1). Biological transmission of *A. marginale* begins when the tick vector ingests infected erythrocytes from infected cattle. The parasite infects the tick gut cells, where it develops and then spreads to many other tissues of the tick including the salivary glands. The pathogen is transmitted to vulnerable vertebrates during the feeding state (Kocan et al., 2003). The severity of anaplasmosis depends on the strain of *A. marginale* and the susceptibility of the cattle host; acute infection is characterised by 10-90% parasitemia (Aubry and Geale, 2011).

The clinical symptoms of bovine anaplasmosis include fever, icterus, lethargy, weight loss, abortion and occasionally death, usually in animals older than two years (De Waal, 2000). Cattle that survive the anaplasmosis infection develop immunity and become resistant to clinical disease upon exposure to *A. marginale*. However, these cattle become carriers, and can serve as reservoirs of *A. marginale* (Kocan et al., 2003).

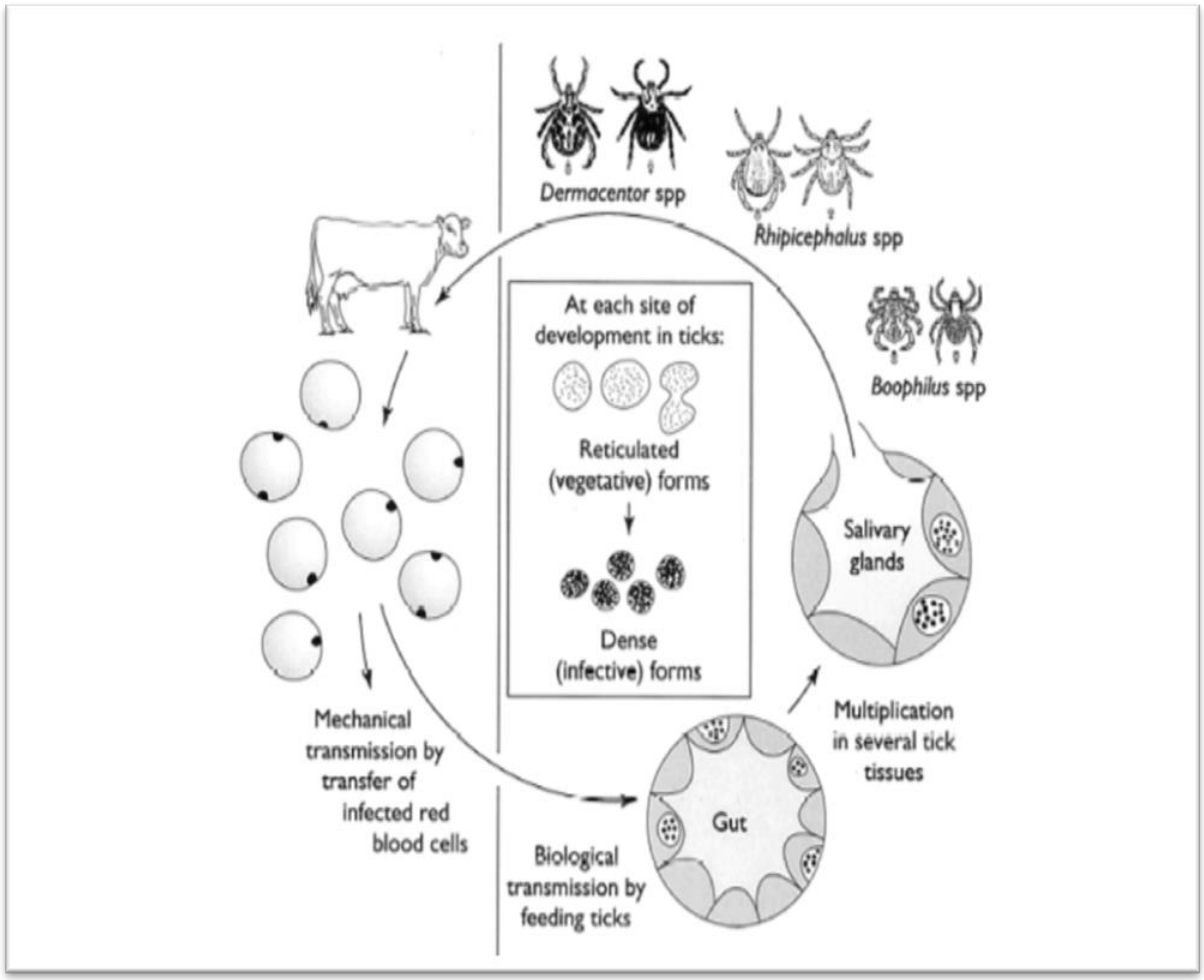


Figure 1.1: Life cycle of *Anaplasma marginale* in ticks and vertebrate hosts (Kocan et al., 2003).

1.5.1.2. *Anaplasma centrale*

Anaplasma centrale was discovered by Sir Arnold Theiler in 1911 and he named it *Anaplasma marginale* variety *centrale.* Theiler recognized that this organism was closely related to *A. marginale* although it was usually located in the centre of the erythrocyte and was less virulent in cattle (Theiler, 1911). The organism was later classified as a separate species (Ristic and Kreier, 1984), but this was based on an erroneous assumption by Ristic in 1968 that Theiler had considered the organism to be a separate species. Although it has long been recognized that *Anaplasma centrale* is known to be antigenically and genetically closely related to *A. marginale* (Carelli et al., 2008), its taxonomic position has been uncertain, due to insufficient evidence in terms of phylogenetic differences between *A. marginale* and *A. centrale* (Dumler et al., 2001).

However, recent work using phylogenetic analysis of 16S rRNA, *groEL* and *msp4* gene sequences obtained from several South African isolates of *A. marginale* and *A. centrale*, as well as differences in Msp1a/Msp1aS gene structure, and overall genome architecture has shown that *A. centrale* is indeed a separate species from *A. marginale* (Khumalo et al., 2016, Khumalo et al., 2018).

In 1911 Sir Arnold Theiler recognized the potential of *A. centrale* to provide active acquired immunity against bovine anaplasmosis. The *A. centrale* live blood vaccine remains the only effective vaccine currently in use in many countries including South Africa (Theiler, 1911; Aubry and Geale, 2011).

The epidemiology of *A. centrale* is poorly understood worldwide because detection of natural infection with *A. centrale* in cattle is usually thought to be due to vaccination against *A. marginale* and thus frequently reported as coinfection with *A. marginale* without any further investigations (Georges et al., 2001). However, the first known case of bovine anaplasmosis caused by *A. centrale* in Europe was reported in 2008 (Carelli et al., 2008). Hove et al. (2018) also detected the presence of *Anaplasma centrale* in cattle in all South African provinces, except for the Eastern Cape and Northern Cape provinces. This pathogen was also detected recently as single infections in Cape buffalo, blue and black wildebeest, waterbuck and eland populations in various national parks in the northern parts of Botswana and South Africa, suggesting that wildlife are a reservoir of *A. centrale* (Eygelaar et al., 2015; Khumalo et al., 2016).

Anaplasma centrale was shown to be transmissible to cattle by the African tick species, *Rhipicephalus simus* (Potgieter & van Rensburg, 1987). Although *A. centrale* has been demonstrated to infect numerous tick species such as *Rhipicephalus* (*Boophilus*) *annulatus, R. sanguineus* and *Hyalomma excavatum*, numerous attempts to try and transmit the organism to splenectomized calves failed (Shkap et al., 2009). Transmission studies by Ueti et al. (2007) further demonstrated that, like *A. marginale, A. centrale* has the ability to colonize and replicate in the midgut and salivary glands of *D. andersoni*, however, this was not sufficient to achieve transmission. Transmission of *A. centrale* could only be achieved through increasing the number of *D. andersoni* ticks because, unlike *A. marginale*, *A. centrale* is secreted into the saliva at a much lower rate. A greater number of ticks compensated for the low pathogen load in the saliva, resulting in transmission (Ueti et al., 2009).

1.5.1.3. *Anaplasma bovis*

Anaplasma bovis is a Gram-negative, obligate intracellular pathogen that is found in vacuoles of the host cells that causes infection in cattle and other mammals (Dumler et al., 2001). In contrast to *A. marginale* and *A. centrale*, which parasitize and replicate in the red blood cells, this pathogen infects monocytes and causes monocytic anaplasmosis. Although *A. bovis* infection is usually asymptomatic, it can cause clinical symptoms such as fever, weight loss and in some cases results in death (Rar and Golovljova, 2011).

Anaplasma bovis has been reported in cattle in Japan, as well as in African countries such as South Africa, Kenya and Uganda (Dumler et al., 2001; Ooshiro et al., 2008; Pfitzer et al., 2011; Njiiri et al., 2015; Byaruhanga et al., 2016). This pathogen has also been detected in cattle and ticks in Asia, Japan, Israel and South America (Dumler et al., 2001; Kawahara et al., 2006; Ooshiro et al., 2008; Harrus et al., 2011). A study conducted by Harrison et al. (2013) showed that Eastern rock sengis are natural reservoir hosts of *A. bovis* in South Africa.

This tick-borne pathogen has been reported to occur in *Rhipicephalus sanguineus* and *R. turanicus* ticks collected in Israel (Harrus et al., 2011). It can also be transmitted by African tick vectors such as *Hyalomma excavatum*, *R. appendiculatus* and *Amblyomma variegatum,* and possibly *A. cajennense* in Brazil (Dumler et al., 2001).

1.5.1.4. *Anaplasma* sp. Omatjenne

Anaplasma (formerly *Ehrlichia*) sp. Omatjenne is a non-pathogenic parasite that is detected mostly as a mixed infection with other haemoparasites (Pfitzer et al., 2011). It does not appear to cause disease. Little is known about *Anaplasma* sp. Omatjenne but it has recently been detected in wildlife such as nyala (*Tragelaphus angasii*) and Cape buffalo, and livestock such as cattle and goats in South Africa, northern Botswana, Mozambique, Ethopia, Uganda and Turkey (Bekker et al., 2002; Aktas et al., 2011; Pfitzer et al., 2011; Aktas et al., 2012; Eygelaar et al., 2015; Byaruhanga et al., 2016; Hailemariam et al., 2017). Teshale et al. (2015) recently detected *Anaplasma* sp. Omatjenne in *Amblyomma variegatum* and *A. lepidum* ticks collected from animals in central Ethiopia. Detection of this organism in ticks collected from animals implies that it circulates between mammalian hosts and ticks in the area.

1.5.1.5. *Anaplasma phagocytophilum*

Anaplasma phagocytophilum is a tick-borne pathogen, which causes various diseases such as human granulocytic anaplasmosis (HGA; formerly human granulocytic ehrlichiosis), equine granulocytic anaplasmosis (EGA; formerly equine granulocytic ehrlichiosis) and canine granulocytic anaplasmosis (CGA) by infecting granulocytes of the abovementioned mammals, amongst others (Dumler et al., 2005; Woldehiwet, 2010). Due to the reclassification of the order Rickettsiales into two families; Anaplasmataceae and Rickettsiaceae, the organism now called *Anaplasma phagocytophilum* encompasses three organisms formerly called *Ehrlichia equi*, *E. phagocytophila*, and the agent implicated in the cause of Human Granulocytic Ehrlichiosis (HGE) (Dumler et al., 2001; Woldehiwet, 2010).

Anaplasma phagocytophilum appears to have a wide range of hosts worldwide including humans, cattle, sheep, rodents, carnivores, insectivores, reptiles and birds. However, the range of hosts can vary from one region to another (Woldehiwet, 2010; Battilani et al., 2017). This pathogen has been reported mainly in Asia, the USA, Europe and China, with few reports from Africa (Inokuma et al., 2005; Woldehiwet, 2010). This pathogen has been reported in cattle, horses and ticks in numerous African countries such as Ethiopia, Tunisia and Zambia (M'ghirbi et al., 2012; Teshale et al., 2018; Vlahakis et al., 2008). Although this pathogen has never been reported in South Africa, Inokuma et al. (2005) and Mtshali et al., (2017) detected a new *Anaplasma* sp. that is genetically closely related to *A. phagocytophilum* and *A. platys* in dogs and ticks collected from dogs and cats in South Africa. *Anaplasma phagocytophilum* is reported to be transmitted by ticks belonging in the *Ixodes* genus, namely: *Ixodes. persulcatus* in Asia and Russia, *I. pacificus* and *I. spinipalpis* in Western USA, *I. scapularis* in Eastern USA, *I. ricinus* in Europe, as well as *Dermacentor* tick species in France (Woldehiwet, 2010; Bonnet et al., 2013).

1.5.1.6. *Anaplasma platys*

Anaplasma platys (formerly *Ehrlichia platys*) is a tick-borne, Gram-negative, obligate intracellular bacterium belonging to the family Anaplasmataceae (Dumler et al., 2001). This pathogen is known to infect mainly canine platelets, causing canine cyclic thrombocytopenia (CCT). Although *A. platys* is known to infect dogs and humans, an *A. platys*-like pathogen has also been recently reported in ruminants worldwide (Inokuma et al., 2005; De La Fuente et al., 2006; Liu et al., 2012;

Yang et al., 2015; Zobba et al., 2015). Little is known about the transmission of this pathogen but the main vector is reported to be *Rhipicephalus sanguineus,* which is also known as the brown dog tick because of its host preference (Inokuma et al., 2000). However, *Dermacentor auratus* and *Hyalomma truncatum* tick species have also been implicated in the transmission of *A. platys* (Parola et al., 2003; Huang et al., 2005).

1.5.2. Heartwater

Heartwater (or bovine ehrlichiosis) is a tick-borne disease of wild and domestic animals. The disease was first documented in the beginning of the 19th century; however the causative agent was only identified as a rickettsial pathogen in 1925 (Pfitzer et al., 2004; Allsopp, 2010). Heartwater is caused by *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) (Dumler et al., 2001) and is transmitted by various species of the three-host ticks in the genus *Amblyomma* (van Winkelhoff and Uilenberg, 1981; Collins et al., 2005). This TBD results in high mortality of susceptible cattle, sheep and goats throughout sub-Saharan Africa, thus preventing the improvement of livestock production (van Winkelhoff and Uilenberg, 1981).

The causative agent of heartwater was initially named *Rickettsia ruminantium*, but was later renamed *Cowdria ruminantium.* For many years, *C. ruminantium* was the only species in the genus *Cowdria* but in 2001 the organism was reclassified within the genus *Ehrlichia* (Dumler et al., 2001) based on molecular and biological analysis of 16S rRNA and *groESL* gene sequences. The genus *Ehrlichia* is classified in the family *Anaplasmataceae*, order *Rickettsiales* (Dumler et al., 2001). The genus *Ehrlichia* is made up of five species, two of which, *E. canis* and *E. ewingii,* cause infection in dogs, *E. chaffeensis* causes infection in both humans and dogs*, E. muris* causes infection in humans, while *E. ruminantium* causes heartwater in ruminants (Dumler et al., 2001).

1.5.2.1. Transmission of *E. ruminantium*

Heartwater is transmitted by several species of tick vectors within the genus *Amblyomma*. The three host *Amblyomma* ticks are reported to become infected during the immature stages (larval and nymphal) when they feed on infected vertebrate hosts, and transmit the pathogen to other

susceptible vertebrate hosts during the nymphal and adult stages of development (transstadially) without losing their infective condition (Marcelino et al., 2012). Twelve species of *Amblyomma* have been reported to have the ability to transmit *E. ruminantium. Amblyomma variegatum* is regarded as the principal tick vector in the Caribbean and various Africa countries, *A. hebraeum* is implicated in southern Africa, *A. lepidum* in Sudan and East Africa, *A. astrion* in Sao Tome and Principe. *Amblyomma cohaerans, A. gemma*, *A. pomposum*, *A. sparsum*, *A. tholloni,* and *A. marmoreum* have been proven experimentally to have the capability of transmitting the infection, and finally *A. maculatum* and *A. cajennense* have been implicated experimentally in North America (Cowdry, 1925a; 1925b; Uilenberg et al., 1983; Bezuidenhout, 1987).

Amblyomma hebraeum is the main tick vector for the organism in South Africa and it occurs mainly in the KwaZulu-Natal¸ Eastern Cape and Limpopo provinces (Pfitzer et al., 2004). Heartwater in South Africa only occurs where the main vector, *A. hebraeum*, is present (Allsopp, 2010). Over 800 outbreaks of heartwater in goats have reported by veterinarians in South Africa between 1993 and 2014 (South African National Department of Agriculture, Disease database, 2014). Furthermore, a recent study conducted by Ringo et al. (2018) detected the presence of *E. ruminantium* in goats and sheep in two South African provinces (KwaZulu-Natal and Free State).

1.5.2.2. Life cycle of *E. ruminantium*

The general life cycle of *E. ruminantium* is shown in Fig. 1.2. The tick vector acquires the infection during the larval and nymphal stages when it feeds on an infected host. The *E. ruminantium* organisms invade the gut epithelial cells of the tick vector where they develop and replicate. The organisms then invade and infect the tick salivary gland cells (Marcelino et al., 2012). When the tick vector ejects infected saliva into a new vertebrate host during a blood meal, the new host becomes infected. *E. ruminantium* enters the host cells as the infectious and metabolically inactive form also known as elementary bodies (EBs). The elementary bodies then mature and develop into large colonies of reticulate bodies (RBs or morulae) through division by binary fission in membrane-bound vacuoles within the cytoplasm of endothelial cells. The RBs are the metabolically active form of *E. ruminantium*, however they are considered to be non-infectious. Binary fission of RBs then occurs, resulting in an increased number of progeny which subsequently reorganise into EBs and are released into the blood stream. EBs initiate the

infection again as vector ticks feed on the infected host (Marcelino et al., 2005; Marcelino et al., 2012).

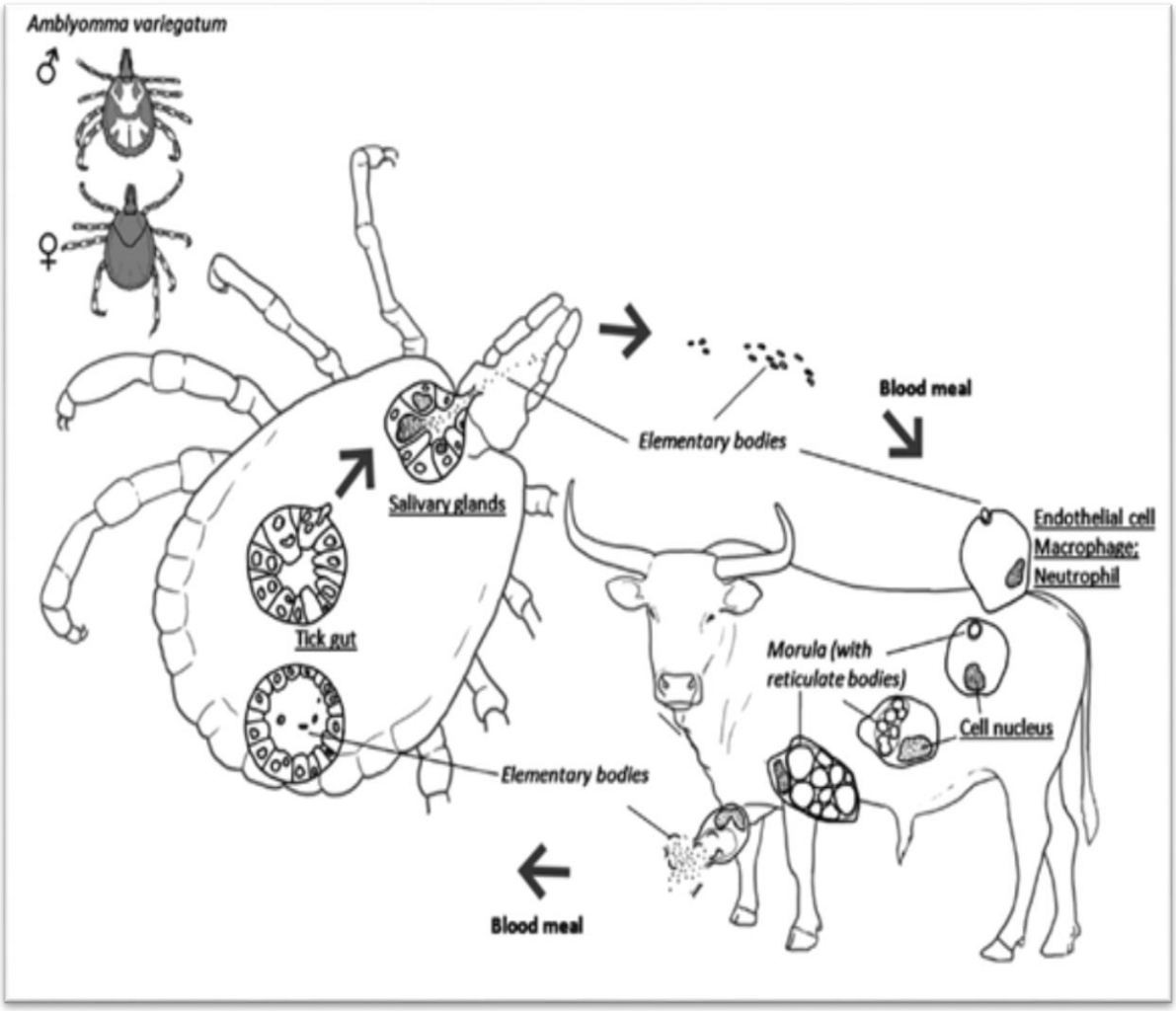


Figure 1.2: Development cycle of *Ehrlichia ruminantium* in tick and vertebrate hosts (from Marcelino et al., 2012).

1.6. Diseases caused by piroplasmid haemoparasites

Babesioses and theilerioses are haemoparasitic diseases caused by protozoan parasites in the genera *Babesia* and *Theileria*, respectively. These haemoparasitic diseases are commonly grouped together as piroplasmoses. Piroplasms are protozoan parasites of the phylum

Apicomplexa (Telford III et al., 1993). The name piroplasm originates from the pear-shape of the parasite (Telford III et al., 1993).

The phylum Apicomplexa is made up of a large group of eukaryotic microorganisms that are obligate parasites of vertebrates and invertebrates. These organisms are called apicomplexan parasites because of the presence of a unique secretory organelle called an apical complex that is involved in invasion and/or initiation of the parasite in the invertebrate or mammalian host cells (Bishop et al., 2004).

1.6.1. Bovine theileriosis

Theilerioses are haemoparasitic diseases of domestic animals including cattle, goats, sheep and wild animals, including Asian water buffaloes and Cape buffaloes, caused by tick-transmitted parasites of the genus *Theileria.* The ixodid tick species of the genera *Amblyomma*, *Hemaphysalis*, *Hyalomma* and *Rhipicephalus* transmit species of the genus *Theileria*. Theilerioses result in major economic losses amounting to hundreds of millions of dollars annually in Asia, Australia, Europe and as well as sub-Saharan Africa (Bishop et al., 2004; Hayashida et al., 2012; Sahinduran, 2012).

Species in the genus *Theileria* are divided into two groups, namely: transforming and non-transforming species based on their ability to transform the schizont of the host cell (Sivakumar et al., 2014). The most important and pathogenic transforming species of *Theileria* in Africa are *Theileria parva* (which causes East Coast fever, Corridor disease and January disease in sub-Saharan Africa) and *Theileria annulata* (causing tropical theileriosis in North Africa and Asia) (Norval et al., 1992).

1.6.1.1. *Theileria parva*

Theileria parva is a tick transmitted haemoprotozoan parasite which causes different forms of bovine theileriosis, East Coast fever (ECF), Corridor disease and January disease, in sub-Saharan Africa (Collins et al., 2002; Chaisi et al., 2011). Several studies have shown that the *T. parva* strains causing the different forms of theileriosis are morphologically and serologically

indistinguishable. However, several isolates demonstrated differences in virulence due to the numbers of schizonts and piroplasms, and do not provide cross-immunity in cross-protective immunity studies (Lawrence et al., 1994; Uilenberg, 1999).

Theileria parva is transmitted by *Rhipicephalus appendiculatus*, *R. zambeziensis* and *R. duttoni*. The natural host and reservoir of the parasite is the Cape buffalo which is regarded as an asymptomatic long-term carrier of the pathogen (Uilenberg, 1999; Sibeko et al., 2008). Although *T. parva* strains causing the three different forms of theileriosis are morphologically and serologically indistinguishable, ECF and January are observed when *T. parva* is transmitted by ticks from infected cattle to susceptible cattle, whereas corridor disease is observed when the parasite is transmitted from an infected buffalo to susceptible cattle.

Theileria parva transforms leukocytes of the infected vertebrate host thus causing a lympho-proliferative disease (Hayashida et al., 2012). The most important stages of *T. parva* infection are the schizont stage, where the parasite infects host lymphoid cells, and the piroplasm stage, where the parasite infects host erythrocytes.

1.6.1.1.1. East Coast fever (ECF)

East Coast fever is caused by cattle-derived *T. parva,* which is transmitted between infected and susceptible cattle and distributed in 12 countries in southern, central and eastern Africa including Zimbabwe, Zambia, Mozambique, Malawi, Burundi, Democratic Republic of Congo, South Sudan, Kenya, Rwanda, Tanzania and Uganda (Norval et al., 1992; Sahinduran, 2012; Olwoch et al., 2008; Thompson et al., 2008). Transmission and distribution of ECF depends directly on the main tick vector *Rhipicephalus appendiculatus* (Olwoch et al., 2008). This disease resulted in economic losses of about 170 million US dollars in 1989 alone and limits movement of the European (*Bos taurus*) breeds of cattle into central and eastern Africa (Bishop et al., 2004).

The clinical signs of ECF include fever, anorexia, lymphadenopathy and secondary bacterial respiratory infection with high levels of parasitemia, large number of schizonts without anaemia. Lacrimation, corneal opacity, nasal discharge and diarrhea are also observed resulting in cattle mortality if proper treatment is not administered (Mbassa et al., 1994; Gul et al., 2015).

The beginning of the 20th century marked an era where East Coast fever was first introduced to southern Africa due to imports of cattle from eastern Africa, where the disease had been endemic for centuries, and this resulted in cattle mortalities of about one million (Potgieter et al., 1988; Norval et al., 1992). After a massive campaign involving vastly expensive measures like quarantine, extensive dipping and herd slaughtering, the disease was eradicated from southern Africa in the 1950s.

1.6.1.1.2. Corridor disease

Although ECF has been eradicated from South Africa, buffalo-derived *T. parva* still remains endemic in the buffalo population, and the tick vectors *R. appendiculatus* and *R. zambeziensis*, are still prevalent. Thus, outbreaks of a different form of theileriosis of cattle, Corridor disease, still occur in South Africa (Collins et al., 1999; Uilenberg, 1999; Chaisi et al., 2011). The pathogen is transmitted when there is contact between Cape buffaloes and cattle in a vector endemic area. Because of the potential to cause epidemics, this disease is currently regulated by the South African Department of Agriculture, Forestry and Fisheries (Animal Disease Act 1984, Act No. 35) by controlling and constantly monitoring movement of buffalo outside areas that are known to be endemic, therefore preventing theileriosis in South African cattle, which are naïve to *T. parva* infection (Thompson et al., 2008; Pienaar et al., 2011).

The clinical symptoms of corridor disease includes enlargement of lymph nodes, lymphocyte proliferation, leukopaenia, pyrexia with or without pulmonary oedema, corneal opacity, subcutaneous oedema, gastrointestinal ulcerations leading to anaemia and diarrhoea (Norval et al., 1992). The clinical symptoms are the same as for ECF except that it is characterized by a low number of schizonts and piroplasms, accompanied by haemorrhagic anaemia. Corridor disease outbreaks result in more than 90% cattle mortality (Potgieter et al., 1988; Norval et al., 1992; Mbassa et al., 1994; Mbizeni et al., 2013).

The introduction of Cape buffalo onto an ever-increasing number of game farms around southern Africa has led to an increased risk of *T. parva* transmission. In South Africa, *T. parva* is prevalent in the buffalo population of the Kruger National Park, situated in the northern part of the Mpumalanga province and north-eastern part of the Limpopo province and neighboring private

game reserves, and in the Hluhluwe-iMfolozi Game Park in northern KwaZulu-Natal (KZN), South Africa (Collins et al., 2002; Thompson et al., 2008; Mbizeni et al., 2013).

Thompson et al. (2008) and Mbizeni et al. (2013) described seasonal outbreaks of Corridor disease in KZN. Mbizeni et al. (2013) reported that the incidence of Corridor disease in cattle at the wildlife/livestock interface in uPhongolo-Mkuzethe area of KZN increased in March to May and this observation correlated with the season when the adult *R. appendiculatus* ticks are the most active. However, some cases of Corridor disease were still encountered during the winter months (June–August), which is the adult R*. appendiculatus* late-feeding or nymphal season (Mbizeni et al., 2013). Cattle surviving the *T. parva* infection become permanent carriers of the parasite, which is transmissible to other susceptible hosts by ticks (Thompson et al., 2008). Although the phenomenon of lifetime carriers of carriers of *Theileria* in recovered animals is well documented, this is said to be strain dependant in the case of ECF. Animals infected with the Muguga strain of *Theileria parva* eventually lose the carrier-state due to the fluctuating parasitemia, which drops to a level no longer transmissible to ticks (Bishop et al., 1992; Skilton et al., 2002; Odongo et al., 2010). However, this is not true for all strains, because the carrier-state caused by the Marikebuni strain of *T. parva*, which is well maintained above the limit of detection (Bishop et al., 1992; Skilton et al., 2002). In the case of Corridor disease, cattle that survive *T. parva* infection only maintain the infection for three months followed by fluctuation of the parasitemia dropping to a level that is no longer transmissible to ticks leading to clearance of the *T. parva* infection (Mbizeni et al., 2013; Mans et al., 2015).

The carrier-state of cattle is reported to also be caused by immunisation and the use of anti-theilerial chemotherapeutic agents, which are known to lack the ability to clear and kill the *T. parva* parasite (Stoltsz, 1989; Potgieter et al., 1988); thus, treatment of and immunization against bovine theileriosis is not allowed in South Africa.

1.6.1.1.3. January disease

January disease, also known as Zimbabwe theileriosis, is a milder form of bovine theileriosis caused by *T. parva* infection and transmission usually occurs between infected cattle and susceptible cattle by adult *R. appendiculatus* and it can be experimentally transmitted by *R. zambeziensis* (Lawrence et al., 1994; Uilenberg, 1999). Although this disease is clinically

indistinguishable from ECF and corridor disease, it is characterized by few schizonts, and no or very few piroplasms without anaemia (Mbassa et al., 1994).This disease was first identified in 1936 when ECF was eradicated in Zimbabwe. This disease resembles ECF in terms of transmission (cattle-to-cattle) and clinical features; however, unlike ECF in East Africa which occurs throughout the year, January disease occurs seasonally during December to May and the course of infection is usually milder than that of ECF.

1.6.1.2. *Theileria annulata*

Theileria annulata is a haemoprotozoan parasite, transmitted by ticks belonging to the genus *Hyalomma*. The pathogen causes tropical theileriosis in cattle in Asia, South Europe and North Africa (Burridge et al., 1974; Gharbi and Darghouth, 2015). *Theileria annulata* does not occur in sub-Saharan Africa and the southern limit of its distribution is Sudan (Burridge et al., 1974).

Clinical signs for *T. annulata* infection include weight loss, lack of appetite, high fever, weakness, enlarged lymph nodes, anaemia, conjunctival petechia and diarrhea (Gul et al., 2015). After recovering from the infection, animals become lifetime carriers of the parasite in which the *Theileria* piroplasms persist in erythrocytes. Such carriers are thought to play a vital role in transmitting the parasite by infecting the tick vectors (D'Oliveira et al., 1995).

Clinical cases of *T. annulata* can be diagnosed by the use of Giemsa-stained lymph node biopsy smears to detect the macroschizonts, and serologically by employing the indirect immunofluorescent antibody test (IFAT) which detects circulating antibodies when using either piroplasms or cultured macroschizonts as antigens but since antibodies degrade over time, serologically negative subjects can still infect ticks that feed on them (D'Oliveira et al., 1995). Molecular tools such as the polymerase chain reaction (PCR) can also be used to diagnose the infection (D'Oliveira et al., 1995).

1.6.1.3. Other *Theileria* species known to occur in southern Africa

Other *Theileria* spp. reported in cattle in South Africa include *Theileria mutans*, *T. taurotragi* and *T. velifera*. While *Theileria velifera* is reported to be apathogenic, *T. mutans* and *T. taurotragi* are

parasites of the Cape buffalo reported to be mildly pathogenic in cattle and are transmitted by ticks in the genus *Amblyomma* (Mbassa et al., 1994). *Theileria taurotragi* has been implicated in the cause of cerebral theileriosis and Tzaneen disease in South Africa, while *T. mutans* is said to cause a haemolytic anaemia characterized by low levels of parasitemia and numerous large schizonts resulting in a prolonged carrier state (Uilenberg et al., 1982; Jongejan et al., 1986; Stoltsz, 1989; Mbassa et al., 1994). Although these three species are common causes of mild infection in cattle in South Africa, they are of little economic consequence in most cases (Stoltsz, 1989). *Theileria buffeli* is another *Theileria* species that has been detected in the Cape buffalo population based in the Addo Elephant Game Park (AEGP), Kruger National Park (KNP) and Hluhluwe–iMfolozi Game Park (HIP) in South Africa (Allsopp et al., 1999; Chaisi et al., 2013; Chaisi et al., 2014). *Theileria buffeli* belongs to a group of benign bovine *Theileria* parasites which includes *T. buffeli, T. orientalis* and *T. sergenti* which are buffalo-derived parasites that can affect the cattle population worldwide (Gubbels et al., 2000; Pfitzer et al., 2011); however, *T. buffeli* has not been reported in cattle in South Africa.

1.6.1.4. Life-cycle of *Theileria* species

The three main reproductive stages found within *Theileria* species are gamogony—the process of gamete formation and fusion in the tick gut, sporogony— sexual reproduction in salivary glands, and merogony— asexual reproduction in the vertebrate host (Homer et al., 2000). *Theileria* sporozoites do not invade the red blood cells directly, but penetrate and transform lymphocytes where they develop into schizonts (Uilenberg, 2006). However, not all *Theileria* species possess the ability to transform host leukocytes. The three “transforming *Theileria*” species are *T. parva, T. annulata and T. lestoquardi* (causes significant disease in sheep) (Hayashida et al., 2012). *Theileria orientalis*, *T. mutans*, *T. velifera* and *T. taurotragi* on the other hand belongs to the “non-transforming *Theileria* species that multiply in the bovine host as an intra-erythrocytic form causing anaemia and icterus (Hayashida et al., 2012).

1.6.1.4.1. In the vertebrate host

The general life development of the genus *Theileria* using *T. parva* as an example (Fig. 1.3) involves the secretion of infective sporozoites from an infected tick after three to seven days of the tick feeding on a vertebrate host. The sporozoites enter the host's blood stream, then penetrate the host's lymphocytes and develop into schizonts. The host cell is transformed and the parasite and cell divide synchronously, resulting in lymphoproliferation.

By the process of merogony, schizonts undergo asexual reproduction and multiply, resulting in merozoites being released into the blood stream. Merozoites then invade red blood cells resulting in development of the piroplasm (Bishop et al., 2004; Uilenberg, 2006; Mans et al., 2015).

1.6.1.4.2. In the tick host

When larval or nymphal vector ticks feed on an infected vertebrate host, they ingest piroplasms which initiate infection of the tick host by formation of gametes in the gut of the tick, which then fuse to form a diploid zygote. The zygote divides into motile kinetes that invade the haemolymph of the tick and move towards the salivary glands. Unlike the *Babesia* genus, infection or invasion of other organs like the ovaries does not occur. Initiation of sporogony (the multiplication and maturation of sporozites in the salivary glands) occurs when the tick (having moulted to the next stage) attaches to the vertebrate host. It is believed that 40–50 000 infective sporozoites may be secreted as the tick attaches and feeds on the vertebrate host (Bishop et al., 2004; Mans et al., 2015). This results in transstadial transmission of the parasite when the tick injects infected saliva into the vertebrate host during a blood meal (Bishop et al., 2004; Uilenberg, 2006; Mans et al., 2015). It is believed that the tick loses its theilerial infection after transmitting it to the vertebrate host; the infection does not persist to the next stage let alone the next generation (Uilenberg, 2006).

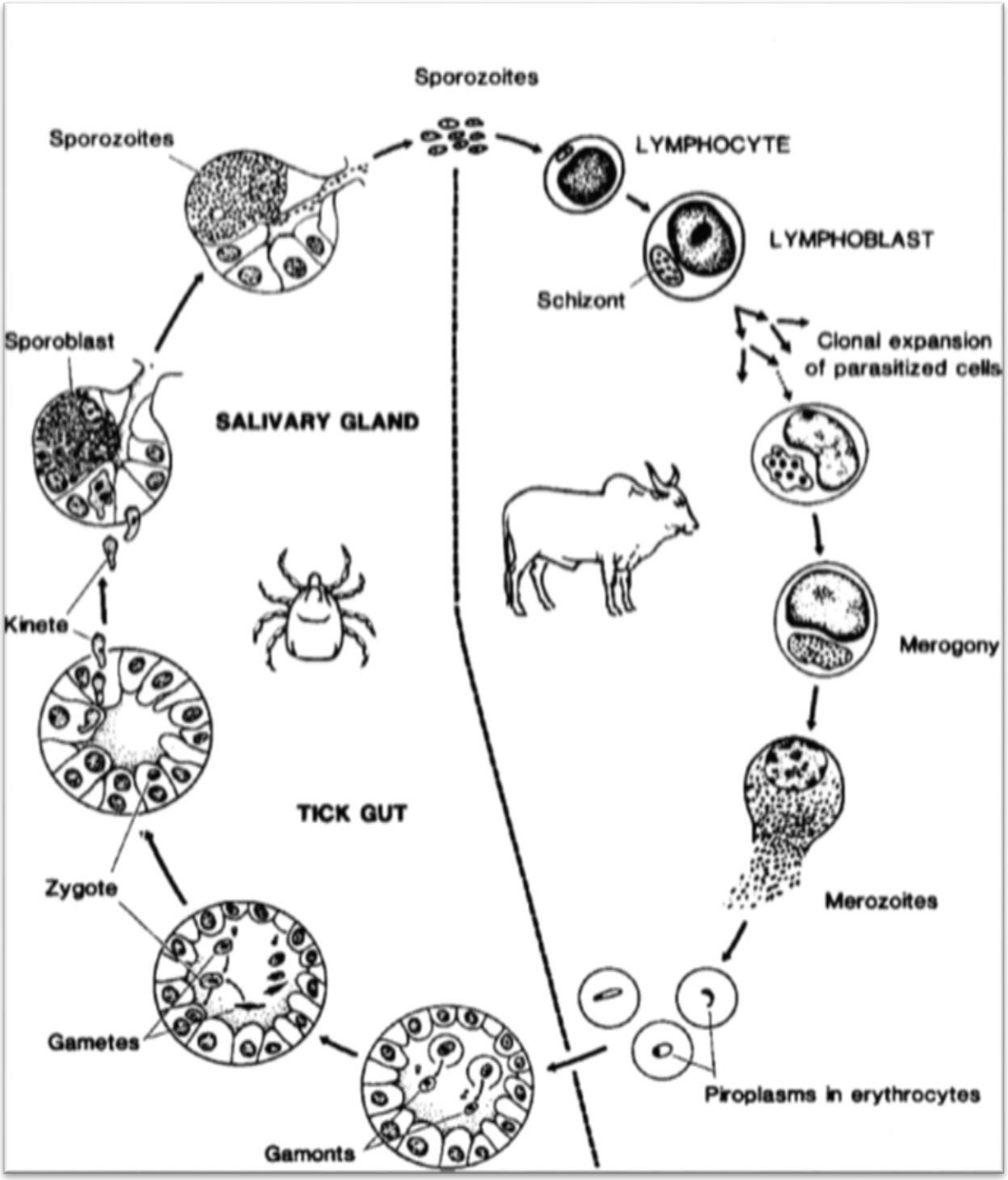


Figure 1.3: Development of *Theileria parva* in tick and vertebrate hosts (from Bishop et al., 2004).

1.6.2. Bovine babesiosis

Babesioses are tick-borne diseases of domestic and wild animals, as well as humans, particularly in immunocompromised individuals, caused by haemoprotozooans of the genus *Babesia* (Bock et al., 2004; Gray, 2006). Bovine babesiosis is of great health and economic importance as it has been estimated that 400 million cattle worldwide are at risk of being affected (Homer et al., 2000; Zintl et al., 2005). Although bovine babesiosis affects cattle worldwide, the USA was the first country to have successfully eradicated the disease and associated parasites in the 1950s, resulting in estimated savings of at least three billion US dollars annually for the livestock industry (Schnittger et al., 2012).

The genus *Babesia* was discovered in the late 19th century when Victor Babes associated micro-organisms in erythrocytes of cattle in Romania with bovine haemoglobinuria or red water fever (Uilenberg, 2006). The causative agent of Texas fever was named *Pyrosoma bigeminum* by Smith and Kilborne in the USA, who also reported on the first arthropod transmission of the parasite (Smith and Kilborne, 1893). The parasites were then renamed *Babesia bovis*, *Babesia ovis* and *Babesia bigemina* in 1983 by Starcovici (Uilenberg, 2006; Mihalca et al., 2010).

Bovine babesiosis, or redwater as it is commonly known, is caused by *Babesia bigemina*, *Babesia bovis* and *Babesia occultans* in southern Africa; the economically important species being *B. bigemina* and *B. bovis* (de Vos et al., 1994). Infection with these tick-borne pathogens is generally characterized by fever, anorexia, dehydration, hypotension, respiratory stress and neurological symptoms because infected erythrocytes may accumulate in lung and brain capillaries. In addition haemoglobinuria and haemoglobinemia may result in jaundice. The severity of this disease depends on the amount and levels of parasitaemia and cattle that are acutely infected with bovine babesiosis may have more than 40% of their erythrocytes infected (Zintl et al., 2005; Schnittger et al., 2012). However, the clinical signs of bovine babesiosis are only seen in mature cattle when they become exposed for the first time (Zintl et al., 2005). Animals that recover from the infection become carriers of the pathogen, although the parasitaemia is virtually undetectable by microscopy (Buling et al., 2007).

Bovine babesiosis is thought to affect up to 1.2 billion cattle worldwide (Bock et al., 2004). The economic importance of bovine babesiosis is associated with losses due to mortalities, abortions,

decreased meat and milk production, control measures, as well as losses of potential production, and cattle trade restrictions (Schnittger et al., 2012).

McLeod & Kristjanson estimated that losses and control of babesiosis and anaplasmosis in the Australian cattle industry in 1999 amounted to US$16.9 million per annum with tick control adding US$6.4 million to annual losses (Schnittger et al., 2012). They further estimated the costs in US $M/annum of losses and control of babesiosis and anaplasmosis for the following countries as China: 19.4; India: 57.2; Indonesia: 3.1; Kenya: 5.1; South Africa: 21.6; Tanzania: 6.8; Philippines: 0.6 and Zimbabwe: 5.4 (Schnittger et al., 2012). Although eradication of bovine babesiosis in the USA has resulted in estimated savings of at least three billion dollars annually for the cattle industry, maintenance of the *R. microplus*-free status and the "buffer zone" still results in financial losses (Schnittger et al., 2012).

1.6.2.1. *Babesia bovis*

Babesia bovis was first recorded in southern Africa in 1941, however, clinical evidence for redwater in South Africa was first reported in 1905 (de Vos et al., 1994). In southern Africa, *B. bovis* is transmitted by the Asian blue tick, *R. microplus*. Transovarial transmission of *B. bovis* occurs between generations of ticks; an adult tick ingests the parasite which is then transmitted to the host by larval ticks of the next generation (de Vos, 1979). *Babesia bovis* is less widely distributed in South Africa than *B. bigemina* because its tick vector, *R. microplus*, only occurs in the higher rainfall areas of the Eastern Cape, KZN, and the eastern parts of Mpumalanga and Limpopo Provinces (de Vos, 1979).

Babesia bovis causes mortality in more than half the susceptible cattle that it infects (Zintl et al., 2005). *Babesia bovis* infection is characterised by low levels of parasitaemia (0.04–0.2%), allergic reactions, cerebral dysfunction and pulmonary oedema (Zintl et al., 2005). European breeds of cattle (*Bos taurus*) are thought to be highly susceptible to the infection; however, the disease can also affect other breeds such as Zebu and Sanga (de Vos, 1979).

Infection with *B. bovis* is characterised by progressive haemolytic anaemia followed by depression, weakness, increased respiratory rate and a reluctance to move. The disease is known as redwater due to the fact that haemoglobinuria is often a clinical sign of the disease.

Muscle wasting, tremors and recumbency develop in advanced cases followed terminally by coma (de Vos et al., 1994). Abortion is likely to occur due to fever during the infection (Bock et al., 2004). It is believed the cattle that survive acute infection by *B. bovis* remain persistently infected without any clinical disease and this immunity to clinical disease is called concomitant immunity (Brown et al., 2006).

1.6.2.2. *Babesia bigemina*

In southern Africa, *Babesia bigemina* is transmitted by *Rhipicephalus microplus*, *R. decoloratus* and *R. evertsi evertsi.* It is thought that *B. bigemina* is more widespread than *B. bovis* in the region, due to the ability of *B. decoloratus* and *R. evertsi evertsi* to transmit the parasite in addition to *R. microplus* (de Vos, 1979; Bock et al., 2004). *Babesia bigemina* is thought to occur in most South African provinces except in the drier parts of the Western and Northern Cape, Limpopo and western Free State provinces (de Vos, 1979; Regassa et al., 2003).

Babesia bigemina infection is characterised by early haemoglobinuria, due to the fact that infection of red blood cells by the pathogen directly leads to erythrocytic destruction. However, affected cattle are usually not as severely affected as those with *B. bovis* infections (Bock et al., 2004). There is no cerebral involvement and recovery in non-fatal cases is usually rapid and complete. However, in some cases the disease can develop very rapidly with sudden and severe anaemia, jaundice and death, which may occur with little warning (de Vos, 1979; Bock et al., 2004). Animals that recover from *B. bigemina* infection remain infective for ticks for four to seven weeks and carriers for only a few months (Regassa et al., 2003; Bock et al., 2004).

1.6.2.3. Life-cycle of *Babesia* species

In contrast to the closely related genus *Theileria*, *Babesia* parasites invade the red blood cells directly upon injection into the vertebrate host (Uilenberg, 2006). With both pathogens, the feeding tick does not immediately transmit the parasite to the vertebrate host after attachment, the transmission occurs a few days after attachment, as sporozoites have to multiply and mature first before they are infective (Uilenberg, 2006).

1.6.2.3.1. In the vertebrate host

Sporozoites (Sz), as shown in Fig.1.4, initiate the transmission of *Babesia* parasites when they are transferred into a new host as the tick feeds. In the vertebrate host, the sporozoites infect the red blood cells and develop into trophozoites (T), also known as piroplasms. Multiplication of trophozoites occurs by binary fission, resulting in daughter cells known as merozoites (M), which re-initiate the replication cycle by infecting other red cells. This replication of merozoites continues either until death of the vertebrate host or until the immune system controls the infection (Homer et al., 2000; Uilenberg, 2006; Hunfeld et al., 2008).

1.6.2.3.2. In the tick host

When vector ticks feed on an infected vertebrate host, they become infected by ingestion of blood containing piroplasms, which develop and differentiate into gametocytes (G) in the tick gut. These gametocytes are also known as the arrowhead-shaped organelles called ‘‘Strahlenkorper” (Sk), also known as ray bodies. The ray bodies are thought to fuse with gametes and ultimately form a zygote (Z), which then multiplies and infects numerous organs of the tick, such as the ovaries and salivary glands. Infection of the ovaries is the basis of transovarial transmission because it can pass to the egg, and then the next tick generation which is then infective. Sporogony is the process of multinucleated sporoblast (St) and finally sporozoite (Sz) formation which occurs in the female larval, nymphal and/or adult ticks of the next generation that infects the vertebrate host during a blood meal when the tick attaches (Homer et al., 2000; Uilenberg, 2006; Hunfeld et al., 2008).

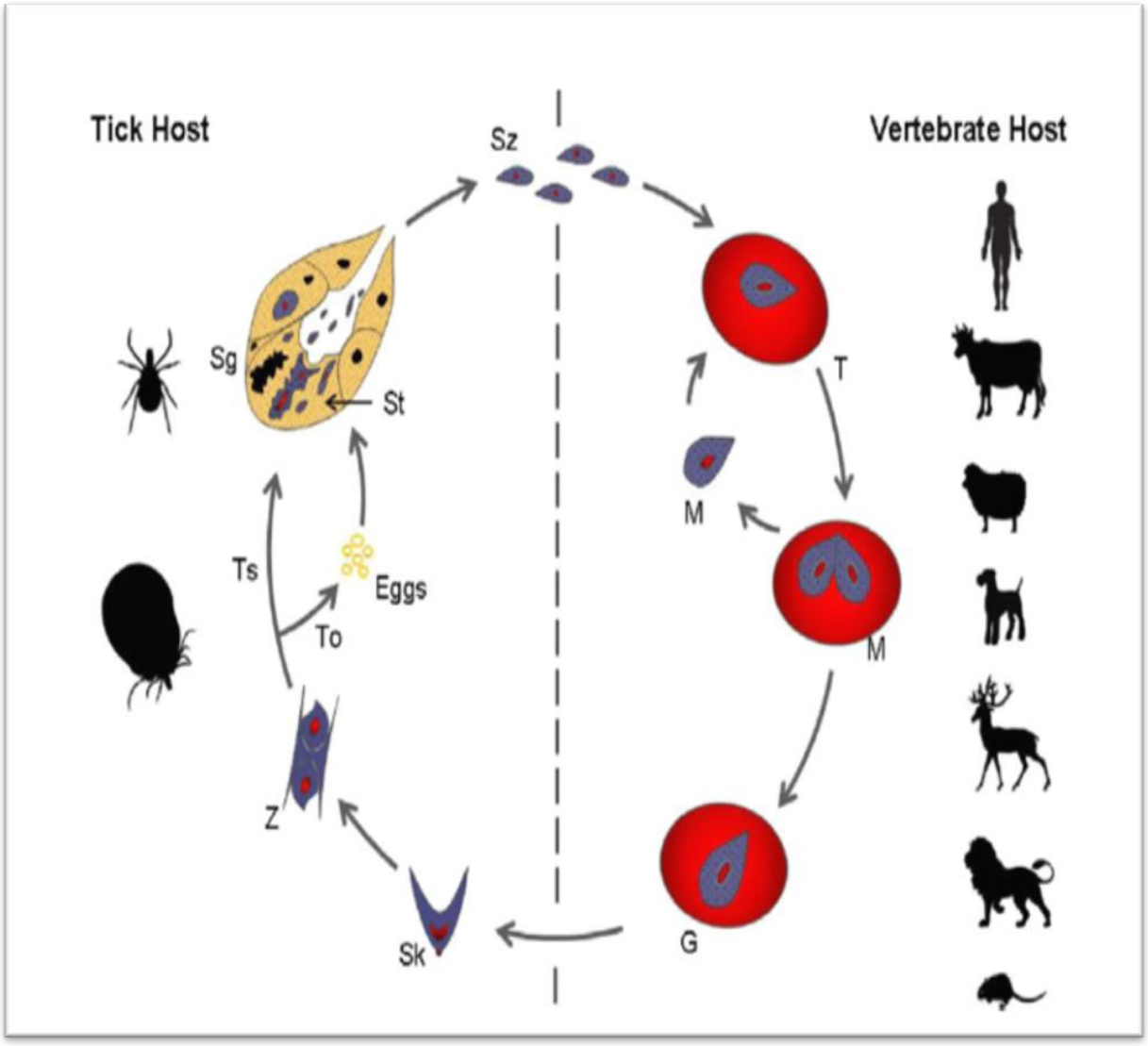


Figure 1.4: Life developments of *Babesia* species in tick and vertebrate hosts (from Schnittger et al., 2012).

1.7. Control of TBDs

1.7.1. Endemic stability

The most common factor that has been playing a significant role in the epidemiological state of tick-borne diseases worldwide for many years is endemic stability (Deem et al., 1996; Jonsonn et al., 2012). Endemic stability is characterized by the presence of high numbers of ticks infected with various tick-borne haemoparasites and lack of or low incidents of clinical cases (Jonsonn et al., 2012). For establishment of endemic stability in a herd, 75% of calves less than nine months

of age must be exposed to TBDs (Norval et al., 1984). Establishment and maintenance of endemic stability in a population requires: large numbers of infected ticks, a large number of vertebrate hosts and exposure of vertebrate hosts to pathogens of interest when resistance to infection is high (Norval et al., 1995).

It is generally accepted that cattle born in TBD endemic areas develop life-long immunity to tick-borne diseases. One of the critical factors contributing significantly to endemic stability of bovine anaplasmosis and babesiosis is inverse age immunity (Bock et al., 2004; Jonsonn et al., 2012), where calves are not clinically affected by anaplasmosis and babesiosis until 12 months of age despite being exposed. This allows these calves to develop a solid long-lasting immunity against the pathogens and clinical diseases.

In terms of babesiosis and anaplasmosis, mature animals only show clinical signs of the disease if they are exposed to the disease for the first time. However, calves less than nine months of age possess immunity to the diseases. This immunity is due to maternal antibodies (obtained from the colostrum) that protect newborns against disease for two months. This is then followed by innate immunity from three to nine months of age and this innate immunity to clinical diseases has been shown to be regulated by the presence of high levels of natural killer (NK) cells in the spleen (Zintl et al., 2005; Brown et al., 2006; Goff et al., 2001; Jonsson et al., 2012). Therefore calves six to nine months old do not show any clinical signs of bovine babesiosis and develop a long-lasting immunity against the disease (Bock et al., 2004; Jonsson et al., 2012). Although inverse age immunity is well documented for infection with *Anaplasma marginale*, *B. bovis* and *B. bigemina*, little is known about *E. ruminantium* and *T. parva* infection.

Endemic stability to heartwater was thought to have been achieved and maintained by the age-related resistance to *E. ruminantium* possessed by newborn ruminants, a high infection rate in *Amblyomma* ticks and the long-term carrier state of the pathogen in ruminants (Neitz et al., 1947; Andrew and Norval, 1989; Norval et al., 1990, Deem et al., 1996). A study conducted by Neitz et al. (1947) showed that the age-related immunity to heartwater exists in neonatal sheep. His findings showed that immunity to heartwater in neonatal sheep following *E. ruminantium* infection lasts for at least 12 months, after which immunity gradually decreases with time but can still protect against fatal infection for up to four years. These results were further confirmed by Deem et al. (1996), who also showed that newborn calves possess an age-related immunity to heartwater which lasts beyond four weeks of age and further suggested that maternal colostrum

played a significant role in the passive immunity possessed by newborn calves. It is therefore of paramount importance to expose herds to *E. ruminantium,* through the *Amblyomma* tick vectors in order to be able to control it (Deem et al., 1996).

Endemic stability to *T. parva* infections is defined as “the state in a cattle population in which the majority of that population becomes immune by six months of age and little or no disease occurs” (Kivaria et al., 2004). It is believed that endemic stability exists, when the cattle population is immune to *T. parva* infection, and it is achieved when the majority of calves younger than six months of age are continuously exposed to a low *T. parva* challenge and when there is little or no variation in the calving season so as to provide susceptible calves to the transmitting vector *R. appendiculatus* throughout the year (Kivaria et al., 2004; Norval et al., 1992). It is therefore possible for endemic stability for *T. parva* to establish in parts of Africa where *R. appendiculatus* can produce two generations in a year. This situation is reported in the East African highlands, a region where the local Zebu cattle are raised and maintained. However, in South Africa, there is endemic instability as far as *T. parva* infection is concerned. This is thought to be attributable to a number of factors including: 1) the single yearly rainy season and the cold winters in South Africa are not adequate for more than one generation of *R. appendiculatus* to occur each year, as compared to the frequent rainfall and warmer conditions observed in eastern Africa, which is adequate for two to three generations of *R. appendiculatus* each year (Kivaria et al., 2004; Norval et al., 1992; Perry and Young, 1995; Olwoch et al., 2008); 2) South African strains of *T. parva* differ genetically from those causing pathogenic infections of cattle in East Africa; and 3) The natural host and carrier of *T. parva* (Cape buffalo) is more widely distributed in eastern Africa than in southern Africa.

1.7.2. Tick control

The integrated use of acaricides and vaccines in South Africa is used for the strategic control of TBDs (de Waal, 2000). It is generally accepted that the use of acaricides on cattle worldwide in tick-infested areas has improved the viability of cattle farming. According to Masika et al. (1997) one of the major strategies contributing significantly to the control of ticks and TBDs is cattle dipping, where cattle are made to pass through an acaricide-filled dip tank. Measures of controlling ticks and TBDs, including cattle dipping, were introduced and made compulsory by the South African Department of Agriculture in the early 20th century, to eradicate the deadly disease,

ECF (Norval, 1992). As a result, this form of disease control was widely adopted and remains one of the major interventions by the state in communal livestock production in South Africa (Norval, 1992; Masika et al., 1997). However, even though the dipping method of ticks and TBD control remains practical and effective, the main disadvantage which causes major concern is that animals living under tick-free conditions are naïve to all TBDs and this disrupts endemic stability, thus resulting in high mortality rates on exposure to infections (Norval et al., 1992; Uilenberg et al., 1993). The other major concern resulting from effective dipping methods is development of resistance by ticks to acaricides, which differs according to tick species and countries (Dolan, 1999).

1.7.3. Immunisation

Immunisation by vaccination involves the injection of attenuated or virulent parasites into susceptible animals, followed by antibiotic treatment. This approach has been successfully applied in the past to the control of several TBDs including bovine anaplasmosis and babesiosis, heartwater and theileriosis (Kocan et al., 2000; Jenkins, 2001; Nuttall et al., 2006). Although vaccines are recommended and have been shown to be the effective way of controlling TBDs, there are several disadvantages, which include: 1) cost of production because of the need to use live cattle or sheep in preparation of the vaccine; 2) lack of protection of the vaccine strain against all field strains; 3) the need for careful maintenance of a cold storage chain; 4) the threat of unintentionally introducing other pathogens which may result in morbidity and mortality in vaccinated animals; 5) the risk of attenuated organisms reverting to a more pathogenic state; 6) difficulties in vaccine dose standardization; and 7) poor animal monitoring after vaccine administration, which may result in losses. (Anderson & May, 1990, Jenkins, 2001; Nuttall et al., 2006; Shkap et al., 2007).

Immunisation methods to several TBDs such as anaplasmosis, babesiosis, heartwater and theileriosis includes: 1) the use of *Anaplasma centrale* as a live blood vaccine which is currently in use in numerous countries including South Africa (Theiler, 1911; Aubry and Geale, 2011); 2) the use of attenuated, live strains of *Babesia bigemina* and *B. bovis* which are provided either chilled or cryopreserved in Australia, South Africa and a number of other countries (de Vos & Bock, 2000); 3) the use of *E. ruminantium* (Ball 3) stock that is currently used in South Africa, however, this does not fully protect against virulent field strains (Shkap et al., 2007); 4) the use of

infective sporozoites derived from ticks and subsequent chemotherapy to control *T. parva* in cattle (Dolan, 1989; Young et al., 1990).

To reduce the disease burden caused by various tick borne haemoparasites, it is of immense importance to thoroughly understand and investigate novel “Omics” approaches (genomics, proteomics, transcriptomics) to fully understand biological processes associated with protective immune response that can result in safer and less expensive vaccines with improved efficacy (Marcelino et al., 2012).

1.7.4. Chemotherapy

Several drugs have been used and/or developed to kill pathogens implicated in the cause of clinical anaplasmosis, babesiosis, heartwater and theileriosis.

Tetracycline compounds including chlortetracycline, oxytetracycline and tetracycline, chemotherapeutic agents which have been in use since the 1950s in many countries, are reported to be effective in the treatment of anaplasmosis (Kuttler, 1980; Ristic, 1981; Blouin et al., 2002). The use of tetracycline has been demonstrated to kill *A. marginale* organisms in cell culture (Blouin et al., 2002). Although the use of tetracycline is widely recommended for treatment of anaplasmosis, there are some disadvantages, such as cost, continuous administration and the risk of development and spread of resistant strains (Kocan et al., 2000). Furthermore, in contrast to older findings that tetracycline compounds were effective in the treatment of anpalasmosis, Coetzee et al. (2005) demonstrated failure of tetracycline compounds to clear *A. marginale* infection in persistently infected cattle. This study is further supported by Goff et al. (1990) who detected *A. marginale* in naturally infected cattle that were treated with tetracycline. The use of enrofloxacin and imidocarb proved to be more effective than tetracycline compounds in the treatment of *A. marginale* infection (Coetzee et al., 2006).

Chemotherapeutic agents that are commonly used to treat and control field cases of bovine babesiosis are imidocarb, diminazene aceturate, nerolidol, artesunate, triclosan, epoxomicin, gossypol, atovaquone, quinuronium sulphate, pentamidium (Kuttler, 1980; Mosqueda et al., 2012). Imidocarb is reported to be effective enough to remove parasites from carrier animals (Mosqueda et al, 2012).

In the past, infection with acute *E. rumantium* was treated with doses of 2.5 mg/kg of oxytetratcycline, repeated 24 hours later. However, it is now recommended to administer two treatments of 20 mg/kg of oxytetracycline on successive days (Uilenberg, 1983; Purnell et al., 1989; Allsopp, 2015).

The first effective chemotherapeutic treatment for the deadly disease theileriosis was reported by Neitz (1950) and this involved the use of 8-aminoquinoline compounds consisting of primaquin, pentaquin and pamaquin (Dolan, 1981). Tetracyclines, specifically oxytetracyclines are also reported to be highly effective against theileriosis due to their suppressive effect but only if given in large doses during the incubation stage of the disease (Neitz, 1953; McHardy, 1984). After intensive investigations (McHardy, 1979; Schein and Voigt, 1979), naphthoquinone parvaquone and halofuginone were found to be two highly effective compounds with high activity against *T. annulata* and *T. parva*. The efficacy of parvaquone was confirmed by Dolan (1981) when he demonstrated that only one dose of 20 mg/kg of the compound was highly effective in the treatment of cattle infected both with a *T. parva* stabilate and *T. parva* infected ticks. Parvaquone was also reported to be a very safe compound causing little or no discomfort (McHardy, 1984). Halofuginone in turn was also reported to be a highly effective compound for treating theileriosis (Schein and Voigt, 1979), of which a single dose of 1.2mg/kg of halofuginone hydrobromide was reported to be effective enough to cure theileriosis during the incubation stage of the disease and clear schizonts from the lymph node. However, Dolan (1989) reported that halofuqinone is not effective enough in the early stages of theileriosis.

The use of chemotherapeutic agents on cattle against infection of *T. parva* in South Africa is prohibited by law due to the fact that this treatment does not completely eliminate the *T. parva* parasite in the hosts and can result in carrier animals which remains infective to other animals (Stoltsz, 1989; Potgieter et al., 1988). The only methods of controlling corridor disease in South Africa depend on restricting movement of *T. parva*-infected buffaloes by maintaining the game proof fences which act as a barrier between cattle and buffaloes, as well as intensive tick control (Pienaar et al., 2011; Stoltsz, 2012).

1.8. Detection of haemoparasites

1.8.1. Microscopy

Microscopic detection of haemoparasites remains the most sustainable and best diagnostic method for acute infections. The preparation, staining and examination of thick and/or thin blood and lymph node biopsy smears is the least time consuming and inexpensive method available for the on-site diagnosis of tick-borne diseases. The preparation of thin blood films remains the only microscopic technique for examining morphologic details of the parasites, thereby allowing species identification. Thick blood films in turn are most useful for detecting the presence of pathogens, because they examine a larger sample of blood (Bose et al., 1995).

Anaplasma marginale can be identified microscopically by the dense, rounded and deeply stained intraerythrocytic bodies of about 0.3–1.0 µm in diameter located on or near the margin of erythrocytes. In contrast to *A. marginale*, which is located near the margin*, A. centrale* organisms have a more central location in the erythrocyte (Kocan et al., 2004).

Babesia bovis can be identified microscopically by the presence of a pair of parasites located centrally in the erythrocyte at an obtuse angle to each other measuring approximately 1.0–1.5 µm long and 0.5–1.0 µm wide. *Babesia bigemina*, typically pear-shaped, is usually a much bigger parasite (3.0– 3.5 µm long and 1.0–1.5 µm wide), and is found often as pairs at an acute angle to each other (Bock et al., 2004).

Ehrlichia ruminantium organisms are found often close to the nucleus and occur as clumps of reddish-purple to blue, coccoid to pleomorphic organisms in the cytoplasm of capillary endothelial cells on brain smears. However, it is highly difficult if not impossible to find colonies in animals that have been treated with antibiotics (Allsopp et al., 2004; Loftis et al., 2006).

The microscopic technique is widely used for early and rapid diagnosis of *Theileria* infection. However, limitations of using this method for the diagnosis of *Theileria* infections include the difficulty of differentiating the schizonts and piroplasms of different *Theileria* species and the low sensitivity in diagnosing carrier cattle (Norval et al., 1992).

1.8.2. Serology

A number of serological tests have been developed with the aim of detecting persistently infected animals. Serological methods used include the card agglutination test, complement fixation test, indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA).

Numerous tests such as the the complement fixation, card agglutination and ELISA are in use currently for the diagnosis of anaplasmosis in many laboratories. A validated competitive ELISA test based on the *A. marginale* major surface protein 5 (MSP5) is now used routinely in South Africa. However, major disadvantages of using the test are that it is not sensitive enough to diagnose all chronically infected animals and there is a high degree of serological cross-reactivity between *A. marginale* and *A. centrale* (De Waal, 2000).

Barry et al. (1982) and O'Donoghue et al. (1985) evaluated an ELISA test previously for detection of antibodies against *B. bovis* and *B. bigemina*. This test was recommended over the IFAT test because of its sensitivity, specificity, standardization and reproducibility in diagnosing bovine babesiosis. However, Fujinaga et al. (1980) reported a high degree of cross-reaction between *B. bovis* and *B*. *bigemina* isolates.

Ehrlichia ruminantium can be detected serologically by numerous tests such as the IFAT, ELISA and Western blots. However, all serological tests developed to date are of limited value to the diagnosis of heartwater because of cross-reactions with one or more *Ehrlichia* spp., (Yunker, 1996).

Diagnosis of Corridor disease has been achieved by various indirect methods based on serology, such as capillary tube agglutination (Ross & Lohr, 1972), conglutination (Cawdery et al., 1968), IFAT (Burridge and Kimber, 1972), indirect hemagglutination assay (Duffus & Wagner, 1974) and ELISA (Katende et al., 1998). ELISA and IFAT are regarded as good diagnostic assays, however, they do not detect the parasites directly and at times the animals may remain seropositive even when the pathogens have been cleared (Dolan, 1986; Bishop et al., 1992). Although the ELISA technique is a cheap and fast method for screening large numbers of samples and allows for high throughput, the IFAT remains the gold standard assay recommended by the OIE for most economically important parasites (OIE, 2014; Mans et al., 2015). However, disadvantages of

using the test include cross-reactivity between *Theileria* species, subjective operator-dependent interpretation of results and low throughput (Norval et al 1992; Katende et al., 1998).

1.8.3. Methods based on detection of nucleic acid sequences

Although various serological assays for detection of tick-borne haemoparasites have been developed, these assays are not always sensitive and specific enough (due to cross-reactions with related parasites) to detect the causal organisms in samples obtained from animals suspected to be infected, and this revealed the need for more sensitive and specific diagnostic methods (Figueroa and Buening, 1995). Nucleic acid-based tests that are more sensitive and specific have been developed and validated for detection and characterization of tick–borne haemoparasites (Salih et al., 2015).

1.8.3.1. Conventional polymerase chain reaction (PCR)

Assays involving hybridisation of labelled DNA probes and conventional PCR methods targeting the genes encoding the immunodominant antigens, have been developed and validated to identify *A. marginale* with high sensitivity and specificity (Eriks et al., 1989; Gale et al., 1996). In the diagnosis of anaplasmosis, different species of *Anaplasma* such as *A. marginale* can be distinguished from *A. centrale* by using PCR assays targeting *msp4* and/or *msp1α* genes (de la Fuente et al., 2001; Lew et al., 2002). Conventional PCR method for detection of *B. bovis* and *B. bigemina* targeting the cytochrome *b* gene have been developed and reported to be sensitive (Fahrimal et al., 1992; Salem et al., 1999). *Ehrlichia ruminantium* can be detected by the use of PCR assays targeting the pCS20 sequence (Yunker et al., 1993; Peter et al., 1995).

Bishop et al. (1992), Watt et al. (1998) and Ogden et al. (2003) developed and evaluated a conventional PCR test for detection of *Theileria parva* in experimentally infected animals and *R. appendiculatus* ticks by targeting the multi-copy *TPR* gene. Another *T. parva* species-specific conventional assay that has been previously validated and proved highly sensitive has been developed for detection of *T. parva* in asymptomatic carrier cattle (Skilton et al., 2002; Odongo et al., 2010). The assay is based on the highly conserved region of the *T. parva* antigen gene, *p104* (Skilton et al. 2002).

These techniques have improved the diagnostic industry in terms of the sensitivity and specificity of pathogen detection. However, these assays do not quantify the levels of parasitaemia in the animals and are often laborious and time-consuming.

1.8.3.2. Reverse-line blot (RLB) hybridisation assay

The reverse-line blot hybridisation (RLB) assay is a technique for simultaneously detecting a wide range of tick-borne haemoparasites by combining PCR with hybridisation of amplified products to one or several small oligonucleotide probes on a membrane. The method has been in use since the late 1990s and it is now used widely for detection of *Anaplasma*, *Babesia*, *Ehrlichia* and *Theileria* species in different hosts and vectors (Gubbels et al., 1999; Bekker et al., 2002; Criado-Fornelio, 2007). Although this assay is important for detection of mixed infections and identifying novel genotypes of the above-mentioned genera within various hosts, as well as vectors, it has been reported to be less sensitive in detecting various haemoparasites than qPCR assays (Nijhof et al. 2005; Bhoora et al., 2009; Bhoora et al., 2010; Chaisi et al. 2011).

1.8.3.3. Quantitative real-time PCR

Quantitative real-time PCR has been reported to result in accurate detection and quantification of specific DNA in various biological samples. This type of an assay is based on the detection of the fluorescence produced by a reporter molecule, which increases as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules can be sequence specific probes (e.g. TaqMan® probes) or dyes (e.g. SYBR® Green) that bind to specific regions of the double stranded DNA. Real-time PCR facilitates the monitoring of the reaction as it progresses. The use of quantitative real-time PCR assays is recommended because of their higher reproducibility and time saving nature as there is no need for gel-electrophoresis (Criado-Fornelio, 2007).

A quantitative real-time PCR for detection of *A. marginale* and *A. centrale* has been developed and extensively validated (Carelli et al., 2007; Decaro et al., 2008; Chaisi et al., 2017). This test targets the *msp1β* gene of *A. marginale* and *groEL* gene *A. centrale*. Buling et al. (2007) previously developed SYBR Green-based qPCR assay that targets the cytochrome *b* gene of *B.*

bovis and *B. bigemina*. In the same year, Kim et al. (2007) developed Taqman-based real-time PCR assays that targets the *18S rRNA* gene for detection of two protozoan parasites, *B. bovis* and *B. bigemina*. The two assays developed by Kim et al. (2007) are currently being validated for detection of *B. bovis* and *B. bigemina* in cattle and ticks from different geographical areas in Africa (Byaruhanga, unpublished). Two qPCR assays based on the pCS20 sequence to detect and quantify *E. ruminantium* in different hosts and vectors have been developed and validated (Steyn et al., 2008; Cangi et al., 2017). Sibeko et al. (2008) developed a sensitive and specific qPCR assay based on the 18S rRNA gene for the detection of *T. parva* in both cattle and buffalo.

1.9. Problem statement and hypothesis

Comprehensive disease surveillance measures are implemented in the Mnisi communal area by State Veterinary Services as well as the Mnisi One Health Platform. The farmers regularly dip their cattle to control the tick burden, but due to the presence of tick vectors of important diseases such as Corridor disease, bovine babesiosis, heartwater and bovine anaplasmosis in the area, tick-borne diseases (TBDs) are likely to be a problem in the area.

Through the on-going Health and Demographic Surveillance System in Livestock (HDSS-Live) in the study area of the Mnisi One Health Platform within the Mnisi communal area, a great deal of information is being collected on the cattle in the area. More than 15 000 cattle have been identified and blood samples taken for serological analysis and parasite identification, and tick burdens are being assessed (Hein Stoltsz, personal communication). However, little is known about when the cattle in the Mnisi communal area become infected with tick-borne haemoparasites.

We hypothesized that, because of the proximity of wildlife reservoir hosts in private and public game parks, the incidence of several important pathogenic and non-pathogenic haemoparasites is higher in cattle at the wildlife/livestock interface than in peri-urban areas of the Mnisi communal area.

Therefore the main aims of this study were to compare the dynamics and time-course of TBD infections in calves and the presence of pathogenic and non-pathogenic haemoparasites in tick vectors in a peri-urban area and at the wildlife/livestock interface in the Mnisi study area (MSA) of the Mnisi Community Programme (MCP) given the regular acaride dipping schedule.

1.10. Objectives

The objectives of this study were therefore to:

i. Collect blood samples and adult ticks from new-born calves grazing at the wildlife/livestock interface and calves grazing distal to the wildlife/livestock interface in a peri-urban area of the MSA for a period of 1 year.

ii. Determine the seasonal tick burden of calves grazing at the wildlife/livestock interface and calves grazing in the peri-urban area, as well as the prevalence of ticks in the field in these areas.

iii. Detect various pathogenic and non-pathogenic haemoparasites in DNA extracts from calves and adult ticks using pathogen-specific RLB and qPCR assays

iv. Compare the presence of pathogenic and non-pathogenic haemoparasites detected in calves grazing at the wildlife/livestock interface and calves grazing in the peri-urban area, and follow the time-course of infection of the calves.

CHAPTER 2

MATERIALS AND METHODS

2.1. Study area

The project was conducted in the study area of the Mnisi Community Programme. The Mnisi community is situated in the Bushbuckridge Local Municipality, Mpumalanga Province, South Africa. Over 40,000 people live in the area and it is a mainly a Shangaan-speaking community.

The study area falls within the savannah ecosystem and is situated at a wildlife/livestock interface, as it borders the Andover and Manyeleti provincial game reserves, and two private game reserves, Timbavati and Sabi Sand. Manyeleti Game Reserve and other private game reserves in the area were incorporated into the Greater Kruger National Park in 1993 through the removal of a game fence, and now form part of the important conservation initiative of the Great Limpopo Transfrontier Conservation Area (GLTFCA). As a result thereof, wildlife is able to roam freely between the different game reserves.

The study area is within the foot and mouth disease (FMD) protection zone where FMD control measures are implemented by a FMD Veterinary Procedural Notice (VPN) and the local veterinary services. Comprehensive disease surveillance measures are implemented in the area by local veterinary services, mainly in the form of cattle dip tanks built throughout the region, which every cattle herd must visit for dipping and inspection once a week. The dip consists of the Delete® X5 acaricide which is used on cattle, sheep and goats, for the prevention and treatment of ectoparasite infestation.

Three villages in the area were chosen to participate in the study. These include Eglington, Utah 1 and Dixie. Eglington village (with a total of 1009 cattle), is located in a peri-urban area. Eglington is situated approximately 11.5 km away from the Manyeleti Game Reserve, 12.1 km from the Andover game reserve and 15.1 km from the Timbavati game reserve (Figure 2.1). The fully fenced cattle grazing camp of the Eglington village, where the chosen calves spent all of their time, is located located approximately 16 km away from the Manyeleti game reserve, 13 km from

the Andover game reserve and 15.1 km from the Timbavati game reserve. Utah 1 and Dixie are located close to the wildlife/livestock interface. Utah 1 has a total of 715 cattle and Dixie has a total of 137 cattle and they are located, respectively, approximately 2.4 km and 0.5 km away from the Manyeleti Game Reserve. In this area, cattle grazing camps are located adjacent to the Manyeleti Game Reserve and cattle are often seen grazing alongside wildlife separated only by the game fence. The two areas (the peri-urban area and wildlife/livestock interface) differ in the methods of cattle dipping by the local veterinary services. They use the plunge method of dipping cattle in the peri-urban area and the pour-on method of dipping cattle at the wildlife/livestock interface (Dixie village).

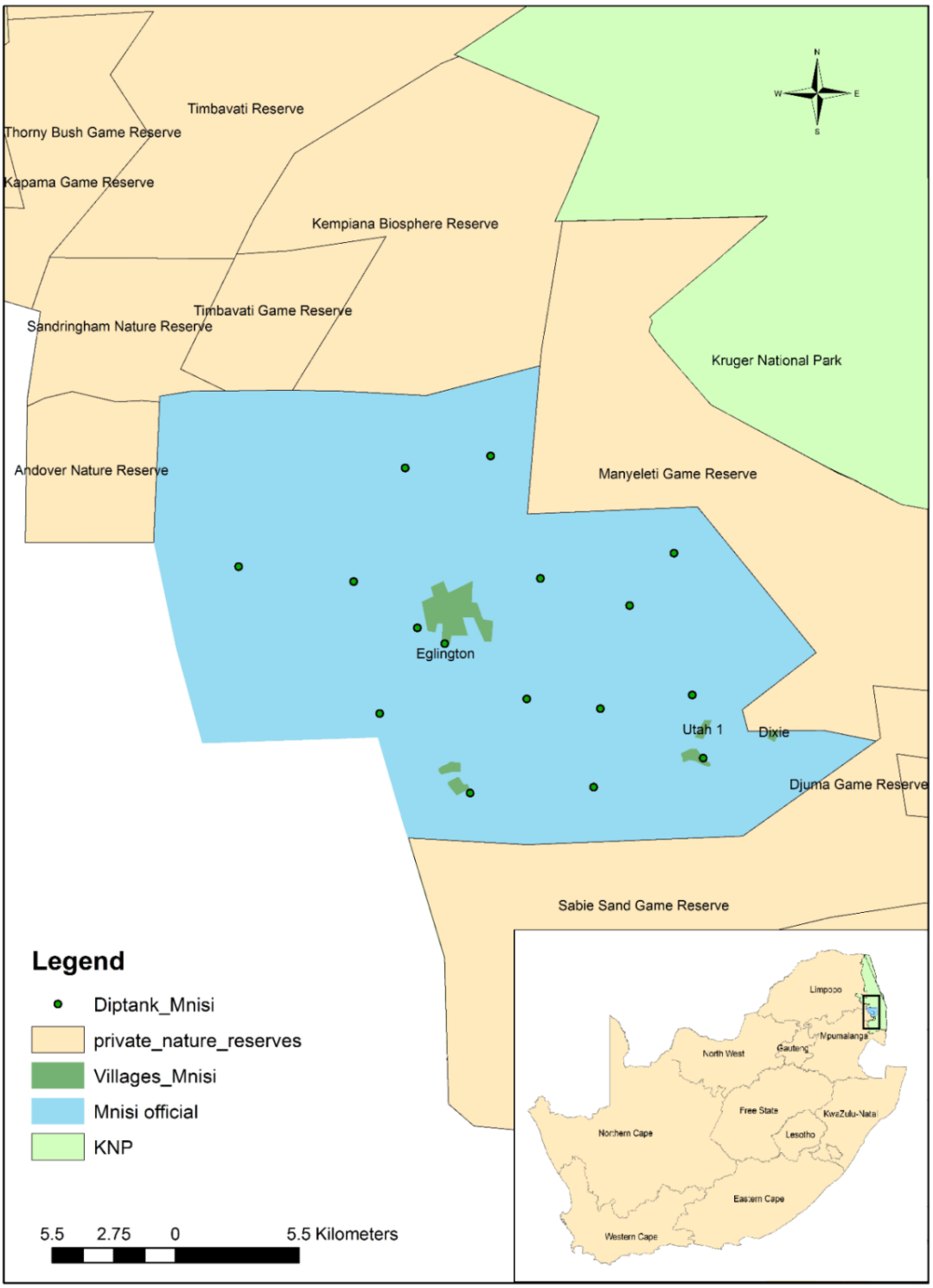


Figure 2.1: The chosen villages in the study area of the Mnisi community, Eglington, Utah 1 and Dixie, relative to various wildlife reserves and the Kruger National Park.

2.2. Animals

This research study was conducted in accordance with the guidelines of the Animal Ethics Committee of the University of Pretoria and permission was granted by the Department of Agriculture Fishery and Forestry (DAFF) to do the research in terms of section 20 of the Animal Disease Act, 1984 (ACT NO.35 of 1984). The research protocol was approved by the Animal Ethics Committee of the University of Pretoria certificate number V041-16 (Appendix 1.1, 1.2 and 1.3) and DAFF with reference number 12/11/1/1/6 (Appendix 2) prior to commencement of the study.

Ten mixed breed *Bos taurus* calves (0-1 months of age, 6 males and 4 females) were monitored for a period of one year. Five of the ten calves were situated in Utah 1 and Dixie villages, close to the wildlife/livestock interface, and the other five were based in Eglington village, a peri-urban area, located distal to the wildlife/livestock interface in the area. Owners and calves in the abovementioned villages were chosen in consultation with the Mnisi Community Programme (MCP) Research Committee. The study required farmers with a relatively small herd of cattle who do not dip their cattle privately.

2.3. Sample collection

2.3.1. Consent form

Permission to do research in the chosen villages was granted by the village leaders. A consent form (Appendix 3) was presented to the farmers before the study team collected samples from the identified calves and their mothers. The consent form was translated from English to the local language (Xitsonga) with the help of the University of Pretoria Environmental Monitors (EM's) to help farmers who did not have a good command of English to understand the purpose of the study. The nature and purpose of the study were clearly communicated to the cattle owners.

2.3.2. Collection of blood samples

The chosen calves were monitored monthly and blood samples were collected from November 2016 (at the age of 0-1 month old) to October 2017 according to a 12 time point experimental timeline (Figure 2.2) with the help of the EMs and the Animal Health Technicians (AHTs) of the Local Veterinary Services. Blood samples were also collected from the mothers of the calves at T1. Whole blood samples were collected in 10 ml Vacutainer® ethylenediaminetetraacetic acid (EDTA) tubes from the ten calves using 18G needles. All equipment and consumables used for collecting blood samples from the calves were sprayed with F10® (Health and Hygiene (Pty) Ltd, South Africa) disinfectant immediately before moving samples to the Hans Hoheisen Wildlife Research Station (HHWRS). A movement permit was obtained (Appendix 4) to allow the blood samples to be moved from the MCP area (an FMD control area with vaccination) to the HHWRS for DNA extraction.

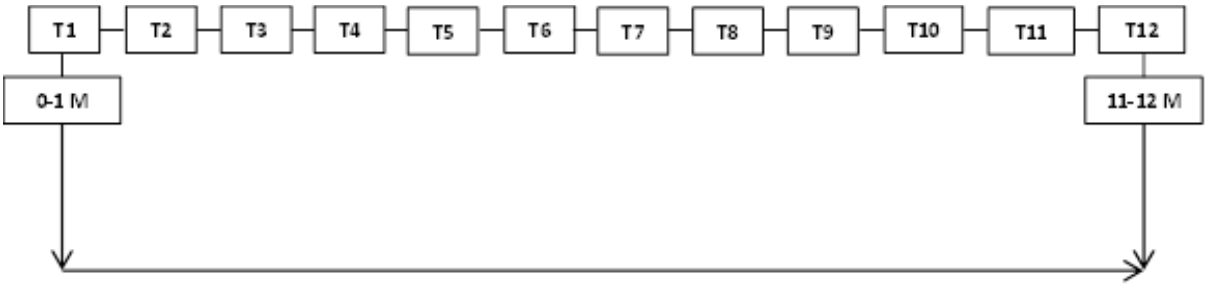


Figure 2.2: Sample collection timeline for the study. Samples were collected monthly from the five calves for a period of a year (T1- November 2016, T2- December 2016 and T12- October 2017), from the ages of between 0-1 months old (0-1M) to 11-12 months old (11-12M). T(x) = time point (month number).

2.3.3. Extraction of DNA from blood samples

Genomic DNA (gDNA) was extracted from all blood samples using the Qiagen® Blood and Tissue extraction protocol (Qiagen, Whitehead Scientific, South Africa) with modifications because samples were collected in an FMD control area. Prior to DNA extraction, all materials and consumables used for the blood collection were disinfected using F10® (Health and Hygiene (Pty) Ltd, South Africa) and 10% Corox (Ecolab, South Africa). Each DNA extraction procedure from blood samples and adult ticks was documented and signed off as proof of performance (Appendix 5) to ensure biosafety in the control of FMD outbreaks in the Mnisi communal area. The extraction

method was done according to the manufacturer's instructions as follows: QIAGEN Protease (or proteinase K) (20 µl of a 600 mAU/ml stock solution) was pipetted into a 1.5 ml microcentrifuge tube and 200 µl sample added to it. Thereafter 200 µl of Buffer AL was added to the sample and mixed by pulse-vortexing for 15 seconds. The reaction mixture was incubated at 56°C for 30 min and then briefly centrifuged. Ethanol (96–100%) (200 µl) was added to the sample, pulse-vortexed for 15 s and then briefly centrifuged. The mixture was carefully applied to the QIAamp Mini spin column and centrifuged for 1 min at 6 000 x g (8 000 rpm) using an Eppendorf centrifuge (5417R), after which the lysate was discarded. The spin column was placed in a clean 2 ml collection tube and centrifugation performed for 1 min at 6 000 x g (8 000 rpm) to remove the remaining lysate, which was also discarded. Buffer AW1 (500 µl) was added and centrifuged at 6 000 x g (8 000 rpm) for 1 min. The filtrate was discarded. The next step was to add 500 µl Buffer AW2 and centrifuge at full speed 20 000 x g (14,000 rpm) for 3 min. Finally the spin column was placed in a clean 1.5 ml microcentrifuge tube, 100 µl Buffer AE was added and it was incubated at room temperature (15–25°C) for 1 min, and then centrifuged at 6 000 x g (8 000 rpm) for 1 min to elute the DNA. The DNA was stored in the -80°C freezer in HHWRS until the time the DNA was moved to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria for analysis. Transport permits were issued for transport of the DNA samples to Pretoria (Appendix 6).

2.3.4. Adult tick collection

Live adult ticks were collected from the sampled calves manually using forceps in conjunction with blood collection from T1 to T12 as shown in Figure 2.2. Different body parts were targeted for adult tick collection such as the ears, dewlap, axillae, udder, groin, shoulders, dewlap, belly and the anus. Live ticks were placed in specimen bottles with cotton wool and stored in a coolbox until transported back to the laboratory at HHWRS. Ticks were then placed in a humidifier at a controlled temperature (22°C) for a week to allow digestion of their blood meal. All adult ticks were then counted, and identified to species level under a dissecting microscope, according to a standard morphological identification guide (Walker et al., 2003). Information on the tick infestation of the sampled calves and the rest of the herds at the dip tanks were captured adequately with the help of the state veterinary services in the area.

2.3.5. Extraction of DNA from tick tissues

Prior to individual tick dissection, all identified ticks were washed with 5% Corox (Liquid chlorine bleach, Ecolab, South Africa) and 5% ethanol (Lasec, South Africa) for 3 min followed by two rinses in ddH_20 for 3 min (Scoles et al., 2005). Ticks were dissected by removing the exoskeleton and extracting all internal organs from each tick and then pooled according to species, monthly collection and location. Between each tick dissection, all dissection tools were twice sterilized with 70% ethanol and Corox. The pooled tick tissues were stored in 200 µl PBS (Lasec, South Africa) at -20°C awaiting DNA extraction using a Qiagen® Blood and Tissue kit (Qiagen, Whitehead Scientific, South Africa) according to Crowder et al. (2010), with modification. The tick DNA extraction method was done as follows: tubes containing tick tissues and 200 µl PBS (Lasec, South Africa) were filled with 450 µl of lysis buffer made up of 425 µl of ATL buffer (Qiagen, Whitehead Scientific, South Africa) and 25 µl of 600 mAU/ml (equivalent to 20 mg/ml) proteinase K solution (Qiagen, Whitehead Scientific, South Africa). Samples were vortexed and briefly centrifuged for 2 min at 6 000 x g (8 000 rpm) and incubated overnight at 56°C on a shaker for lysis. The following day samples were vortexed and briefly centrifuged at 6 000 x g (8 000 rpm). Four hundred µl of recoverable supernatant was transferred into a fresh 2 ml Eppendorf tube. Four hundred µl of AL buffer (Qiagen, Whitehead Scientific, South Africa) was added to the supernatant and the mixture was vortexed thoroughly and incubated at 37°C for 10 min. Four hundred and eighty µl of 100% ethanol was subsequently added to each tube and mixed by vortexing for 30 s and briefly centrifuged. Samples were then transferred into marked QIAamp mini spin columns in two parts: first, 750 µl of sample was transferred and centrifuged at 6 000 x g (8 000 rpm) for 1 min. The flow-through was discarded and the remaining sample was loaded onto the same mini spin column which was again centrifuged at 6 000 x g (8 000 rpm) for 1 min and the flow-through was again discarded. Five hundred µl of AW1 buffer (Qiagen, Whitehead Scientific, South Africa) was added to the columns followed by centrifugation at 6 000 x g (8 000 rpm) for 1 min and the flow-through was discarded. Five hundred µl of AW2 buffer (Qiagen, Whitehead Scientific, South Africa) was added to the columns followed by centrifugation at 12 000 x g (14 000 rpm) for 3 min and the flow-through was discarded. The columns were then placed in a new collection tube and centrifuged at 12 000 x g (14 000 rpm) for 1 min to remove residual AW buffer (Qiagen, Whitehead Scientific, South Africa). The columns were placed in a marked 1.5 ml Eppendorf tube, 100 µl buffer AE was added to the each column and they were incubated at room temperature (15–25°C) for 1 min, and then centrifuged at 6,000 x g (8,000 rpm) for 1 min to elute the DNA. The tick DNA was stored in the -80°C freezer in HHWRS until it was moved to the

Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria for analysis. Transport permits were issued for transport of the tick DNA samples to Pretoria.

2.4. Molecular identification of *Rhipicephalus* adult ticks to confirm identification by morphological key

Adult ticks that were identified morphologically as belonging to the genus *Rhipicephalus*, were further identified using the polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) test developed and validated by Lempereur et al. (2010) with modifications on the PCR amplification. The test is based on sequence variability of the internal transcribed spacer (ITS2) between *R. decoloratus* and *R. microplus*.

Standard PCR amplification was performed in a 20 µl final reaction volume containing 10 µl 2X Phusion Flash Mastermix (ThermoFisher Scientific, South Africa), 0.5 µM of ITS2-specific forward and reverse primers, Boophits2-F and Boophits2-R (Table 2.1), and 2 µl of template DNA. Positive controls for the ITS2 amplification assay were DNA extracted from known *R. microplus* and *R. decoloratus* ticks provided by Dr Hein Stoltsz and Dr Jannie Crafford (Faculty of Veterinary Sciences, University of Pretoria, South Africa). Nuclease-free water was used as a negative control for the assay. The PCR thermal cycler (Gene Amp®, Applied BioSystems Veriti PCR System 9902, Life Technologies™, South Africa) was used with cycling conditions shown in Table 2.2. PCR products were analysed by gel-electrophoresis through loading 5 µl of each PCR mixture mixed with 2 µl of loading dye (2X) on an ethidium bromide stained 1.5% agarose gel (Lasec, South Africa). A 1 kb DNA ladder (Thermo Scientific, South Africa) was loaded on each gel. Samples were run for 90 min at 100 V and viewed using a ChemiDoc™ + Image Lab™ Software (BioRad, South Africa).

Table 2.1: Primers and probes for the ITS2 PCR assay

Primer	Sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
Boophits2 F	GCCGTCGACTCGTTTTGA	ITS2	765-832	Lempereur et al., (2010)
Boophits2 R	TCCGAACAGTTGCGTGATAAA		-	-

Table 2.2: Thermal cycling conditions for the amplification of the ITS2 from adult tick DNA using the 2X Phusion Flash PCR master mix

Cycle	Time	Temperature	Purpose
1 cycle	10 sec	98°C	Initial denaturation
30 cycles	1 sec	98°C	Denaturation
	5 sec	66°C	Annealing
	15 sec	72°C	Extension
1 cycle	1 min	72°C	Final extension
	Hold	4°C	

For some samples, amplification using the 2X Phusion Flash Mastermix resulted in amplification of a low molecular weight smear. These samples were amplified using DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, South Africa). The reaction was performed in a 50 µl final reaction volume containing 25 µl of the DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, South Africa), 0.1 µM of primers, Boophits2-F and Boophits2-R (Table 2.1), and 2 µl of template DNA. The same controls were used as for the Phusion Flash amplifications. The PCR thermal cycler (Gene Amp®, Applied BioSystems PCR System 2700, Life Technologies™, South Africa) with conditions shown in Table 2.3. PCR products were analysed by gel-electrophoresis as described above.

Table 2.3: Thermal cycling conditions for the amplification of the ITS2 from adult tick DNA using the 2X DreamTaq Green PCR Master Mix

Cycle	Time	Temperature	Purpose
1 cycle	3 min	95°C	Initial denaturation
40 cycles	30 sec	95°C	Denaturation
	30 sec	61°C	Annealing
	1 min	72°C	Extension
1 cycle	15 min	72°C	Final extension
	Hold	4°C	

Positive PCR products were digested using the restriction enzyme, *Msp1* (6U) (Inqaba Biotec™, South Africa), with 2.5 µl of PCR products in a 15 µl total volume. The reaction was incubated at 37°C for 15 min, and 5 µl of each digested sample was mixed with 1 µl of loading dye and loaded onto a polyacrylamide gel (BioRad, South Africa) with a 100 bp DNA ladder (ThermoFisher Scientific, South Africa) to determine the size of all the fragments. Vertical electrophoresis was used to separate the DNA fragments in TBE buffer for 80 min at 100 V. Restriction sites for *R*. *decoloratus* are located at 107 bp, 241 bp, 311 bp, 391 bp, 407 bp, 442 bp and 765 bp resulting in RFLP profiles with bands at around 323 bp, 134 bp, 107 bp, 80 bp, 70 bp, 56 bp, 35 bp and 16 bp. Restriction sites for *R*. *microplus* are located at 241 bp, 311 bp, and 408 bp resulting in RFLP profiles with bands around 421 bp, 241 bp, 197 bp and 70 bp (Lempereur et al., 2010). In the case of mixed species of *Rhipicephalus* ticks in a pool, brighter RFLP profiles would be generated from the species present at a higher concentration, and fainter profiles from the ones present at a lower concentration.

Amplicons from selected positive samples including those having extra bands were purified using the Qiagen® Purification kit (Qiagen, Whitehead Scientific, South Africa). Purified PCR products were sequenced bi-directionally using the Boophits2 F and Boophits2 R primers (Table 2.1) on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) at Inqaba Biotechnical Industries. Sequences were assembled and aligned using CLC Main Workbench 7.5.1. BLASTn (Altschul et al., 1990) was used to identify sequences by comparison with published sequences in public databases**.**

2.5. Pathogen detection

2.5.1. Pathogen-specific reverse line blot (RLB) hybridisation assay

The reverse line blot (RLB) hybridisation assay was used to detect the presence of the eukaryotic (*Babesia* and *Theileria*) and prokaryotic (*Anaplasma* and *Ehrlichia*) pathogens in DNA extracted from both blood samples and adult ticks as described previously (Gubbels et al., 1999; Bekker et al., 2002; Nijhof et al., 2003; Nijhof et al., 2005). Two sets of primers were used, one to amplify the V4 hypervariable loop of the protozoan 18S rRNA gene for the detectondetection of *Babesia* and *Theileria* spp. and the other to amplify the V1 variable region of the 16S rRNA gene for the detection of *Ehrlichia* and *Anaplasma* spp.

The RLB involves a number of steps including covalent linking of genus- and species-specific probes to a biodyne® C membrane (Separation Scientific, South Africa), polymerase chain reaction (PCR) amplification of target sequences from DNA samples, hybridisation of PCR products to the probes, and detection of hybridised products.

2.5.1.1. Membrane preparation

A biodyne® C membrane (Separation Scientific, South Africa) was prepared by measuring a piece of membrane according to the size of the miniblotter apparatus support cushion. It was then activated by incubating in 10 ml freshly prepared 16% EDAC (1-ethyl-3-(-3-dimethylamininopropyl) carbodiimide) (DNA Thunder™, Separation Scientific, South Africa) for 10 min. Each probe was diluted in 0.5 M NaHCO (Sigma, South Africa), pH 8.4 to a final concentration of 2 pmol/µl and 200 µl was loaded onto the membrane using a miniblotter apparatus (Immunetics, Cambridge). *Anaplasma, Ehrlichia, Theileria* and *Babesia* genus- and species-specific probes included on the membrane are shown in Table 2.4. Genus-specific probes were included to serve as positive controls for troubleshooting purposes. The membrane was incubated for 2 min at room temperature and inactivated with 100 mM freshly made NaOH (Sigma, South Africa) for 8 min at room temperature on a shaker. The membrane was then washed in 100 ml 2 X SSPE (ThermoFisher Scientific, South Africa)/0.1% SDS (Sigma, South Africa) at 60°C for 5 min.

Table 2.4: Genus and species-specific RLB oligonucleotide probes used in this study.

Pathogen	Sequence (5'-3')
Ehrlichia/*Anaplasma* genus-specific probe	GGG GGA AAG ATT TAT CGC TA
Anaplasma bovis	GTA GCT TGC TAT GR[a]G AAC A
Anaplasma centrale	TCG AAC GGA CCA TAC GC
Anaplasma marginale	GAC CGT ATA CGC AGC TTG
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG
Anaplasma (formerly *Ehrlichia*) sp. *Omatjenne*	CGG ATT TTT ATC ATA GCT TGC
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG
Theileria / *Babesia* group-specific probe	TAA TGG TTA ATA GGA RCR GTT G
Babesia genus-specific probe 1	ATT AGA GTG TTT CAA GCA GAC
Babesia genus-specific probe 2	ACT AGA GTG TTT CAA ACA GGC
Babesia bicornis	TTG GTA AAT CGC CTT GGT C
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT
Babesia canis	TGC GTT GAC CGT TTG AC
Babesia divergens	ACT RAT GTC GAG ATT GCA C
Babesia felis	TTA TGC GTT TTC CGA CTG GC
Babesia gibsoni Japan	TAC TTG CCT TGT CTG GTT T
Babesia gibsoni USA	CAT CCC TCT GGT TAA TTT G
Babesia leo	ATC TTG TTG CTT GCA GCT T
Babesia major	TCC GAC TTT GGT TGG TGT
Babesia microti	GRC TTG GCA TCW[b] TCT GGA
Babesia rossi	CGG TTT GTT GCC TTT GTG
Babesia vogeli	AGC GTG TTC GAG TTT GCC
Theileria genus-specific probe	ATT AGA GTG CTC AAA GCA GGC
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG
Theileria annulata	CCT CTG GGG TCT GTG CA
Theileria bicornis	GCG TTG TGG CTT TTT TCT G
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT
Theileria equi	TTC GTT GAC TGC GY[c]T TGG
Theileria lestoquardi	CTT GTG TCC CTC CGG G
Theileria mutans	CTT GCG TCT CCG AAT GTT
Theileria parva	GGA CGG AGT TCG CTT TG
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C
Theileria taurotragi	TCT TGG CAC GTG GCT TTT
Theileria velifera	CCT ATT CTC CTT TAC GAG T

[a] The degenerate position R denotes either A or G
[b] The degenerate position W denotes either A or T
[c] The degenerate position Y denotes either C or T

2.5.1.2. Polymerase chain reaction (PCR)

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'), specific for *Theileria* and *Babesia* species (Nijhof, et al., 2003), and primers EHR-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') and EHR-R (5'-biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3'), specific for *Anaplasma* and *Ehrlichia* species (Bekker et al., 2002), were used to amplify, respectively, the V4 and V1 hypervariable region of parasite small subunit rRNA genes. In each case, the reverse primer was labelled with biotin to allow for detection of the PCR product during the hybridisation process. The PCR mixture was prepared using Platinum® Quantitative PCR SuperMix-UDG (Life Technologies™, South Africa). The reaction was performed in a 25 µl final reaction volume containing 12.5 µl of Platinum® Quantitative SuperMix-UDG (which contained 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), 0.2 µM of each primer, 2.5 µl of template DNA (containing between 50 and 100 ng DNA) and 9.5 µl water (PCR grade).

Separate PCR master mix reactions were prepared for specific amplification of *Theileria* and *Babesia* species, and *Ehrlichia* and *Anaplasma* species. The primers RLB-F2 and RLB-R2 were used for the *Theileria*/*Babesia* PCR master mix (18S rRNA), while the EHR-F and EHR-R primers were used for the *Ehrlichia*/*Anaplasma* PCR master mix (16SrRNA). A PCR thermal cycler (Gene Amp®, PCR System 9700, Life Technologies™, South Africa) was used, with the thermocycling programme shown in Table 2.5 (Nijhof et al., 2005).

Two positive control samples (DNA extracted from the *A. centrale* and *B. bovis* blood vaccines produced by (Onderstepoort Biological Products (OBP), Pretoria, South Africa) and a negative control (no DNA template) were included on each blot to monitor the occurrence of false positive or false negative results.

Table 2.5: Thermocycling programme for the *Babesia*/*Theileria* and *Ehrlichia*/*Anaplasma* touchdown PCR

Cycle	Time	Temperature	Purpose
1 cycle	3 min	37ºC	Activate UDG
1 cycle	10 min	94ºC	Inactivate UDG & activate *Taq*
	20 sec	94ºC	Denature double stranded DNA template
2 cycles	30 sec	67ºC	Anneal primers
	30 sec	72ºC	Extension of PCR products by *Taq* polymerase
	20 sec	94ºC	
2 cycles	30 sec	65ºC	
	30 sec	72ºC	
	20 sec	94ºC	
2 cycles	30 sec	63ºC	
	30 sec	72ºC	
	20 sec	94ºC	
2 cycles	30 sec	61ºC	
	30 sec	72ºC	
	20 sec	94ºC	
2 cycles	30 sec	59ºC	
	30 sec	72ºC	
40 cycles	20 sec	94ºC	
	30 sec	57ºC	
	30 sec	72ºC	
1 cycle	7 min	72ºC	Final extension

2.5.1.3. Hybridisation

Hybridisation was performed as previously described (Nijhof et al., 2005). The biodyne® C membrane (Separation Scientific, South Africa) containing genus- and species-specific oligonucleotide probes was activated in approximately 50 ml 2 X SSPE/0.1% SDS in a plastic container at room temperature for 5 min.

The PCR products were prepared for hybridisation by adding 130 µl of 2 X SSPE/0.1% SDS to a 25 µl aliquot of each PCR product. The PCR products were then denatured for 10 min at 99.9°C on the thermal cycler and cooled on ice immediately. Denatured PCR products were then hybridised to genus- and species-specific oligonucleotide probes covalently linked to the activated membrane using the miniblotter apparatus (Immunetics, Cambridge). The membrane was placed on the blotter so that the lines of probes were perpendicular to the slots in the miniblotter. Hybridisation was done at 42°C for 60 min, subsequently samples were removed by aspiration and the membrane removed from the miniblotter. The membrane was then washed twice in preheated 2 X SSPE/0.5% SDS for 10 min at 50°C, then incubated with 10 ml 2 X SSPE/0.5% SDS and 12.5 µl streptavidin-POD (peroxidase labelled) conjugate (Roche Diagnostics, South Africa) (1.25 U) for 30 min at 42°C. The membrane was further washed twice in preheated 2 X

SSPE/0.5% SDS for 10 min at 42°C and twice with 2 X SSPE for 5 min at room temperature. All incubations and washes were performed in the incubator with gentle shaking. Hybridised PCR products were detected by enhanced chemiluminiscence (ECL) (DNA Thunder™, Separation Scientific, South Africa); 6 ml of ECL (3 ml ECL1 + 3 ml ECL2) was added onto the membrane and mixed by shaking the container gently and keeping the membrane covered with ECL for 1 min at room temperature. The membrane was exposed to an X-ray film (X-OMAT™ Blue XB-1, Kodak, Separation Scientific, South Africa). Once the X-ray film was developed, hybridisation was evident by dark spots on the film as a result of the chemiluminescence reaction (Gubbels et al., 1999).

2.5.1.4. Stripping of the membrane

After the blotting process, the membrane was stripped, by washing twice with 1% SDS preheated to 80°C for 30 min with gentle shaking. This was followed by one wash with 20 mM EDTA (pH8) at room temperature for 15 minutes with gentle shaking. After stripping, the membrane was stored at 4°C in 20 mM EDTA (pH8) in a plastic container.

2.5.2. Duplex real-time quantitative PCR for specific detection of *A. marginale* and *A. centrale*

Blood and tick DNA samples collected at the 12 time points were subjected to the *A. marginale* and *A. centrale* duplex qPCR assay described by Chaisi et al. (2017). The assay targets the *msp1β* gene of *A. marginale* and the *groEL* gene of *A. centrale*. Samples were run in duplicate. The efficiency of this assay has previously been reported to be 104% and 101% for *A. marginale* and *A. centrale* respectively, with a detection threshold of 36 cycles detecting 250 copies of *A. marginale* and 25 copies of *A. centrale* per reaction.

The assay was performed in a 20 µl final reaction volume consisting of: 4 µl FreshStart Taqman mix (Roche Diagnostics, South Africa), 0.6 µM of *A. marginale*-specific primers AM-For and AM-Rev (Table 2.6), 0.5 µl UDG, 0.9 µM of *A. centrale*-specific primers AC-For and AC-Rev (Table 2.6), 0.2 µM of *A. marginale*-specific and *A. centrale*-specific probes, AM-Pb and AC-Pb, respectively (Table 2.6) and 2.5 µl of template DNA (approximately 200 ng). Positive control for

the *A. centrale* assay was DNA extracted from the *A. centrale* vaccine strain obtained from Onderstepoort Biological Products (OBP), Pretoria, South Africa. Sample Am Soutpan 1:10 (field sample from a clinically sick bovine from Soutpan farm) was used as the positive control for the *A. marginale* assay. This sample was confirmed to be *A. marginale*-positive by amplification and sequence analysis of the *msp1b* gene. Negative control for the assay was nuclease-free water.

Table 2.6: Primers and probes for the duplex *A. marginale* and *A. centrale* qPCR assay

Assay	Primer/ Probe	Sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
Anaplasma marginale	AM-For	TTG GCA AGG CAG CAG CTT	*msp1β*	95	Carelli et al. (2007)
	AM-Rev	TTC CGC GAG CAT GTG CAT		-	-
	AM-Pb	6-FAM-TCG GTC TAA CAT CTC CAG GCT TTC AT-BHQ1		-	-
Anaplasma centrale	AC-For	CTA TAC ACG CTT GCA TCT C	*groEL*	77	Decaro et al. (2008)
	AC-Rev	CGC TTT ATG ATG TTG ATG C		-	-
	AC-Pb	LC610-ATC ATC ATT CTT CCC CTT TAC CTC GT-BHQ2		-	Chaisi et al. (2010)

The duplex assay was performed in a LightCycler v2 (Roche Diagnostics, Mannheim, Germany). The thermal cycling conditions (Table 2.7) were used as described by Chaisi et al. (2017). Results were analysed using the LightCycler Software version 4.0 (Roche Diagnostics, Mannheim, Germany) where positive results were indicated by a quantification cycle (Cq), which is equivalent to the crossing point (Cp) value, given by the Lightcycler instrument), which is the cycle at which emission of fluorescence from amplification exceeds the threshold fluorescence. A high starting concentration of target DNA in a sample is indicated by a lower Cq value. FAM fluorescence generated in *A. marginale* positive-samples was measured at 530 nm and LC-610 signals generated in *A. centrale*-positive samples were measured at 610 nm.

Table 2.7: Thermocycling conditions for the *A. marginale* and *A. centrale* duplex qPCR assay

Cycle	Time	Temperature	Purpose
1 cycle	10 min	40°C	UDG activation
1 cycle	10 min	95°C	Pre-incubation
40 cycles	1 min	95°C	Denaturation
	1 min	60°C	Annealing-extension
1 cycle	30 sec	40°C	Final cooling step

2.5.3. Quantitative real-time PCR for specific detection of *Ehrlichia ruminatium*

A newly developed and validated real-time PCR assay protocol was performed for detection and analysis of *E. ruminantium* positive samples collected from calves and ticks in the Mnisi community (Cangi et al., 2017). The assay targets a conserved region of *E. ruminantium*, pCS20. Samples were run in duplicate. This assay is 94% efficient with a detection threshold of 37 cycles detecting three copies of *E. ruminantium* per reaction.

The pCS20 Sol1$^{TaqMan\ (TqM)}$ qPCR assay was performed in a 25 µl final reaction volume consisting of: 12 µl of the TaqMan® Universal PCR Master Mix (LTC Tech South Africa (Pty) Ltd) (containing AmpliTaq Gold® DNA Polymerase LD, dNTPs with dUTP/dTTP blend and optimised buffer components), 0.25 µM of the pCS20 Sol1^{TqM} forward and reverse primers, Sol1F and Sol1R (Table 2.8), 0.2 µM of the probe, Sol1P (Table 2.8), 2.0 µl of template DNA (approximately 200 ng) and 9.25 µl of nuclease-free water. Positive control for the pCS20 Sol1^{TqM} assay was DNA extracted from the *E. ruminantium* vaccine strain obtained from Onderstepoort Biological Products (OBP), Pretoria, South Africa. Negative control for the assay was nuclease-free water.

Table 2.8: Primers and probe for the pCS20 Sol1^{TqM} qPCR assay

Primer/ Probe	Sequence (5'-3')	Target	Amplicon Size (bp)	Reference
Sol1 F	ACA AAT CTG GYC CAG ATC AC	pCS20 region of the *E. ruminatium* genome	110	Cangi et al. 2017
Sol1 R	CAG CTT TCT GTT CAG CTA GT			
Sol1^{TqM} Probe	6-FAM-ATC AAT TCA CAT GAA ACA TTA CAT GAA G-BHQ1			

The pCS20 Sol1^{TqM} qPCR assay was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies, South Africa). The thermal cycling conditions shown in Table 2.9 were used as described by Cangi et al. (2017). Results were analysed using Applied Biosystem StepOnePlus^{TM} Real-time PCR System Software version 2.3 (Life Technologies, South Africa) where positive results were indicated by a Cq value, which is equivalent to the cycle threshold (Ct) value given by the StepOnePlus^{TM} Real-time PCR System, which is the number of cycles required for the fluorescence signal to reach the threshold fluorescence value. A high starting concentration of target DNA in a sample is indicated by a lower Cq value.

Table 2.9: Thermocycling conditions for the *pCS20* Sol1^{TqM} qPCR assay

Cycle	Time	Temperature	Purpose
1 cycle	2 min	50°C	UNG incubation
1 cycle	10 min	95°C	AmpliTaq Gold pre-activation
40 cycles	15 sec	95°C	Denaturation
	1 min	55°C	Annealing-extension

2.5.4. Quantitative real-time PCR for specific detection of *Babesia bigemina*

A newly developed real-time PCR assay protocol for detection of *Babesia bigemina* was used (Byaruhanga, unpublished) which was modified from Kim et al. (2007). The assay targets a conserved region of the *B. bigemina* 18S rRNA gene. Samples were run in duplicate. This assay is 93% efficient with a detection threshold of 39.7 cycles detecting 6.9 x 10^{-1} copies of *B. bigemina* per reaction (Dr Charles Byaruhanga, University of Pretoria, personal communication).

The *Babesia bigemina*^{TqM} qPCR assay was performed in a 20 µl final reaction volume made up of: 8 µl of the TaqMan® Universal PCR Master Mix (LTC Tech South Africa (Pty) Ltd) (containing AmpliTaq Gold® DNA Polymerase LD, dNTPs with dUTP/dTTP blend and optimised buffer components), 0.5 µM of the *B. bigemina*-specific forward and reverse primers, BiF and BiR (Table 2.10), 0.25 µM of the probe BiP (Table 2.10), 2.0 µl of template DNA (approximately 200 ng) and 8.5 µl of nuclease-free water. Positive control for the *B*. *bigemina* qPCR assay was DNA extracted from the *B*. *bigemina* vaccine strain obtained from Onderstepoort Biological Products (OBP), Pretoria, South Africa. Negative control for the assay was nuclease-free water.

Table 2.10: Primers and probe for the *Babesia bigemina* qPCR assay

Primer/ Probe	Sequence (5'-3')	Target Gene	Amplicon Size (bp)	Reference
BiF	AA TAA CAA TAC AGG GCT TTC GTC T	18S rRNA	174	Kim et al. (2007)
BiR	AAC GCG AGG CTG AAA TAC AAC T			
Bi Probe	VIC-TTG GAA TGA TGG TGA TGT ACA ACC TCA-TAMRA			

The *Babesia bigemina* qPCR assay was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies, South Africa) using the thermal cycling conditions shown in Table 2.11. Results were analysed using a StepOnePlus™ Real-time PCR System Software version 2.3 and StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Life Technologies, South Africa).

Table 2.11: Thermocycling conditions for the *Babesia bigemina* qPCR assay

Cycle	Time	Temperature	Purpose
1 cycle	2 min	50°C	UNG incubation
1 cycle	10 min	95°C	AmpliTaq Gold pre-activation
45 cycles	20 sec	95°C	Denaturation
	1 min	57°C	Annealing-extension

2.6. Statistical analysis

Descriptive statistics (proportions, frequencies and graphs) were used to demonstrate the temporal (12-month period, November 2016 to October 2017) and spatial (the peri-urban area and wildlife/livestock interface of the Minisi communal area) variations in *A*. *marginale*, *B*. *bigemina*, *B*. *bovis* and *E*. *ruminantium* infections and tick vector infestation among the calves.

The independent samples T-Test was used to determine the statistical significance of the difference in the average number calves found positive for *T. mutans*, *T. velifera*, *B. bigemina*, *A. marginale* and *A. centrale*, as well as differences in tick infestation, between the two locations over a 12-month period. The Levene's test for equality of variance was done to determine if the variances for both areas of the Mnisi communal area are equal at a 5% level of significance.

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 25 (IBM SPSS, 2018), at 5% level of significance.

CHAPTER 3

RESULTS

3.1. Pathogen detection in cattle from two sites in the Mnisi communal area

A total of 129 whole blood EDTA samples collected monthly from November 2016 to October 2017 over the 12 time point experimental timeline (time points indicated hereafter as T1 to signify time point 1, T2 to signify time point 2, etc.). Of these, 119 blood samples were collected from ten calves; five from Eglington village, a peri-urban area, and five from the wildlife/livestock interface (two from Utah 1 and three from Dixie village) (Table 3.1). The total number of blood samples collected from the ten calves amounted to 119 instead of 120 because one calf (calf 4) in the peri-urban area died at T11 of the 12 time point experimental timeline. Blood samples were also collected from the dams of the ten calves at the time of first collection (T1). Genomic DNA extracted from the blood samples was subjected to the RLB hybridisation assay and pathogen-specific qPCR assays were used to detect haemoparasites.

Table Error! No text of specified style in document.**1:** Number of blood samples collected from cattle in the Mnisi communal area.

Village	Area	Number of calves	Number of dams	Number of samples from calves (+ dams)
Eglington	Peri-urban area	5	5	59* (+5)
Utah 1	Wildlife/livestock Interface	2	2	24 (+2)
Dixie	Wildlife/livestock Interface	3	3	36 (+3)
Total		**10**	**10**	**119 (+10)**

* Calf 4 from the peri-urban area died at T11.

3.1.1. Detection of pathogenic and non-pathogenic tick-borne haemoparasites in the calves using the RLB hybridisation assay

The RLB results (Tables 3.2 and 3.3) indicated the presence of various pathogenic and non-pathogenic tick-borne haemoparasites in the calves at the peri-urban area and at the wildlife/livestock interface, respectively, at different timepoints over the 12 month study period. All calves tested RLB-positive for one or more haemoparasite (*Theileria*, *Babesia*, *Anaplasma* and/or *Ehrlichia* species) at some point during their first year of life.

Table 3.2: RLB results for calves from the peri-urban area.

Time Point	Calf 1	Calf 2	Calf 3	Calf 4	Calf 5
T1	-[a]	-	-	-	-
T2	*E/A* catch-all[b] *A. marginale* (f)[d] *T/B* catch-all *T* catch-all *T. mutans* *T. velifera*	*T/B* catch-all[c] *T* catch-all[e] *B* catch-all 1[f] *T. mutans* *T. velifera*	*E/A* catch-all *A. marginale* *T/B* catch-all *T* catch-all *T. mutans* *T. velifera*	*E/A* catch-all *A. marginale* *T/B* catch-all *T* catch-all *B* catch-all 1 *T. mutans* T. velifera	*E/A* catch-all *A. marginale* *T/B* catch-all *T* catch-all *B* catch-all 1 *T. mutans* *T. velifera*
T3	*E/A* catch-all *A. marginale* *T/B* catch-all *T* catch-all *B* catch-all 1 (f) *B. bovis* (f) *T. velifera*	*E/A* catch-all *A. marginale* (f) *T/B* catch-all *T* catch-all *T. mutans* (f) *T. velifera*	*E/A* catch-all *A.* sp. (Omatjenne) (vf)[g]	*E/A* catch-all *A. marginale* *T/B* catch-all *T* catch-all *T. mutans* (f) *T. velifera*	*E/A* catch-all *A. marginale*
T4	*T/B* catch-all *T* catch- all *B* catch-all 1 (f) *B. bovis* (f) *T. velifera*	*E/A* catch-all *A. marginale* (vf) *T/B* catch-all *T* catch-all *B* catch- all 1 *B. bovis* *T. mutans* (vf) *T. velifera*	*E/A* catch-all *A. marginale* *T/B* catch-all *T* catch-all *B* catch all-1 *B* catch all-2[h] (vf) *T. mutans* (f) *T. velifera*	*E/A* catch-all *A. marginale* *T/B* catch-all *T. mutans* (f) *T. velifera*	*E/A* catch-all *A. marginale* (f) *A. platys* *A.* sp. (Omatjenne) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch- all 1 *B* catch- all 2 (f) *B. bovis*
T5	*E/A* catch-all *A. marginale* *A. platys* (vf) *A.* sp. (Omatjenne) *T/B* catch-all *T* catch-all *T. mutans* *T. taurotragi*	*E/A* catch-all *A. marginale* *T/B* catch-all *T* catch-all *B* catch-all 1 *B. bovis* *T. mutans* (f) *T.velifera*	-	*E/A* catch-all *A. marginale* *A.* sp. (Omatjenne) (vf) *T/B* catch-all *T* catch-all *T. mutans* (f) *T. velifera*	-

T6	*E/A* catch-all *A. marginale* (f) *T/B* catch-all *T* catch-all *T. mutans* (vf) *T. velifera*	*E/A* catch-all *A. marginale* (vf) *T/B* catch-all *B* catch-all 1	-	*E/A* catch-all *A. marginale* (f) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch all-1 (f) *B. bovis* (f)	-
T7	-	*E/A* catch-all (vf)	-	*E/A* catch-all *A. marginale* (f) *A.* sp. (Omatjenne) (vf) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch all-1 *B. bovis*	-
T8	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T.velifera* *B* catch-1 all *B* catch-2 all (vf) *B. bovis*	*T/B* catch-all *T* catch-all *T. mutans* (vf) *T. velifera*	*E/A* catch-all (vf) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch all-1 *B. bovis*	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* (f) *T. velifera* *B* catch all-1 *B* catch all-2 (vf) *B. bovis*	*E/A* catch-all *A. marginale* (vf) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*
T9	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T.velifera* *B* catch-1 all *B* catch-2 (vf) *B. bovis*	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* (f) *T. velifera*	*T/B* catch-all *T* catch-all *T. mutans* (vf) *T. velifera* *B* catch-all 1 *B. bovis*	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 (vf**)**	*E/A* catch-all *A. marginale* (vf) *A.* sp. (Omatjenne) (vf) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*
T10	*T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*	*E/A* catch- all *T/B* catch-all *T* catch-all *T. velifera*	*T/B* catch-all *T. velifera* *B* catch-all 1 *B. bovis*	*T/B* catch-all *T* catch-all *T. mutans* (vf) *T velifera*	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*
T11	*E/A* catch-all *A.* sp. (Omatjenne) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera*	*E/A* catch-all *T/B* catch-all *T* catch-all *T* velifera *B* catch all-1 (vf)	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B* catch-all 2 (vf) *B. bovis*	*E/A* catch-all (vf) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T.velifera* *B* catch-all 1 *B* catch-all 2 *B. bovis*
T12	*E/A* catch-all *A. platys* (vf)	*E/A* catch-all *T/B* catch-all *T* catch-all	*E/A* catch-all *A. marginale* (vf) *A. platys* (vf)	*[i]	*E/A* catch-all *A. marginale* (vf) *A. platys* (vf)

A. sp. (Omatjenne) (vf) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*	*T. mutans* (vf) *T. velifera* *B* catch-all 1 *B. bovis*	*A.* sp. (Omatjenne)(vf) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*	*A.* sp. (Omatjenne)(vf) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*

[a] indicates a negative result
[b] indicates hybridisation with the *Ehrlichia/Anaplasma* group-specific probe
[c] indicates hybridisation with the *Theileria/Babesia* group-specific probe
[d] indicates a faint hybridisation signal
[e] indicates hybridisation with the *Theileria* genus-specific probe
[f] indicates hybridisation with the *Babesia* genus-specific probe 1
[g] indicates a very faint hybridization signal
[h] indicates hybridisation with the *Babesia* genus-specific probe 2
[i] animal died

Table 3.3: RLB results for calves from the wildlife/livestock interface.

Time Point	Calf 6	Calf 7	Calf 8	Calf 9	Calf 10
T1	*T/B* catch-all[a] *T* catch-all[e] *T. mutans* (f)[f] *T. velifera*	*T/B* catch-all *T* catch-all *T. mutans*	*T/B* catch-all *T* catch-all *T. velifera*	-[b]	*E/A* catch-all[c] (vf)[d] *T/B* catch-all *T* catch-all *T. mutans* *T. velifera*
T2	*T/B* catch-all *T* catch-all *T. mutans* (f) *T. velifera*	*E/A* catch-all	*T/B* catch-all *T* catch-all *B* catch-all 1[g] *B* catch-all 2[h] (vf) *T. velifera*	-	*E/A* catch-all *E. ruminantium* *T/B* catch-all *T* catch-all *T. mutans* *T. velifera*
T3	*T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*	*E/A* catch-all *A. marginale* (f) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B* catch-all 2 (vf) *B. bovis*	*E/A* catch-all (vf) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera*	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* (vf) *T. velifera*	*E/A* catch-all
T4	*E/A* catch-all (vf) *T/B* catch-all *T* catch-all *T. velifera*	*T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 (f) *B. bovis* (f)	*E/A* catch-all *A. marginale* (vf) *T/B* catch-all *T* catch-all *T. mutans* (f) *T. velifera* *B* catch-all 1	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T. velifera*	-
T5	-	*T/B* catch-all *T* catch-all (f) *T. velifera* (f) *B* catch-all 1 *B* catch-all 2 (vf) *B. bovis*	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 (vf)	-	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T. velifera*
T6	*E/A* catch-all *T/B* catch-all *T* catch-all *T. velifera* *B* catch-all 1 (f) *B. bovis* (f)	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* (f) *T. velifera*	*T/B* catch-all *T* catch-all *T. velifera*	*T/B* catch-all *T* catch-all *T. velifera* (f)
T7	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* (vf) *T. velifera*	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* (vf) *T. velifera* (f) *B* catch-all 1 B. bovis	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T. velifera*	-	*E/A* catch-all *A.* sp. (Omatjenne) (f) *A. platys* (f) *T/B* catch-all *T* catch-all *T. mutans* (vf) *T. velifera*
T8	*T/B* catch-all *T* catch-all	*E/A* catch-all *T/B* catch-all	*T/B* catch-all *T* catch-all	*T/B* catch-all *T* catch-all	*E/A* catch-all (vf) *T/B* catch-all

	T. mutans *T. velifera*	*T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B* catch-all 2 *B. bovis*	*T. mutans* *T. velifera*	*T. mutans* *T. velifera*	*T* catch-all *T. mutans* *T. velifera* *B* catch-all 1
T9	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*	*T/B* catch-all *B* catch-all 1 *B* catch-all 2 *B. bovis*	*T/B* catch-all *T* catch-all *T. mutans* *T. velifera*	*E/A* catch-all (vf) *T/B* catch-all *T* catch-all *T. mutans* (vf) *T. velifera* *B* catch-all 1 (vf)	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* (vf) *T. velifera* *B* catch-all 1 (f)
T10	*T/B* catch-all *T* catch-all (f) *T. velifera* (f)	*T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*	*T/B* catch-all *T* catch-all *T. mutans* *T. velifera*	T/B catch-all *T* catch-all (vf) *T. velifera* (vf) *B* catch-all 1 (vf)	*E/A* catch-all (vf) *T/B* catch-all *T* catch-all *T. mutans* (f) *T. velifera* *B* catch-all 1(f) *B. bovis* (f)
T11	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* *T. velifera*	*T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1	*T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 (f)	*E/A* catch-all (f) *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*
T12	*E/A* catch-all *A. marginale* *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*	*E/A* catch-all *A. marginale* *T/B* catch-all *T* catch-all *T. mutans* *T. velifera* *B* catch-all 1 *B. bovis*	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* T. velifera	*E/A* catch-all *T/B* catch-all *T* catch-all *T. mutans* (f) *T. velifera* *B* catch-all 1	*E/A* catch-all *A. platys* *A.* sp. (Omatjenne) *T/B* catch-all *T* catch-all *T. mutans* (f) *T. velifera*

[a] indicates hybridisation with the *Theileria/Babesia* group-specific probe
[b] indicates a negative result
[c] indicates hybridisation with the *Ehrlichia/Anaplasma* group-specific probe
[d] indicates a very faint hybridisation signal
[e] indicates hybridisation with the *Theileria* genus-specific probe
[f] indicates a faint hybridisation signal
[g] indicates hybridisation with the *Babesia* genus-specific probe 1

Table 3.4 shows the frequency of tick-borne haemoparasite detection in the calves during the first year of life as determined by the RLB hybridisation assay. Only four of the ten calves tested positive for various pathogenic and non-pathogenic tick-borne haemoparasites at the first time point (T1) at the age of 0-1 month old. All four calves that tested positive at the first time point were located at the wildlife/livestock interface. The five calves located in the peri-urban area that tested negative, or were below the limit of detection for the RLB hybridisation assay, only started testing positive for the various tick-borne haemoparasites at T2. Calf 9, at the wildlife/livestock interface, was negative for two months and parasites could be detected for the first time at T3.

Pathogenic tick-borne haemoparasites detected in the calves by the RLB assay in the first 12 months of life were *A. marginale*, *B. bovis* and *E. ruminantium*, while mildly or non-pathogenic tick-borne species detected were *Anaplasma* sp. (Omatjenne), *A. platys*, *T. mutans*, *T. taurotragi* and *T. velifera*.

The haemoparasite with the highest frequency of occurrence over the first 12 months of life in the ten calves was *T. velifera* (76.7%), followed by *T. mutans* (67.5%), *B. bovis* (33.3%), and *A. marginale* (20.8%). There was no significant difference in the frequency of *B. bovis*, *T. velifera* and *T. mutans* (p-value > 0.05) detection in the two areas over the 12 months of the study. However, *A. marginale* was detected in calves from the peri-urban area more frequently than in calves at the wildlife/livestock interface ($p < 0.05$).

Although there was a significant difference between the frequency of *A. marginale* in calves in the peri-urban area and calves at the wildlife/livestock interface, analysis of the overall RLB results of the 12 time points (Figure 3.1) revealed no significant difference (p-value > 0.05) between the presence of all haemoparasites in the calves during their first year of life in the two sites of the Mnisi communal area.

Table 3.4: Frequency of detection of different pathogenic and non-pathogenic tick-borne haemoparasites by RLB hybridisation in the five calves at the peri-urban area (1-5) and five calves at the wildlife/livestock interface (6-10) during their first year of life.

Area	Calf	Time point of initial detection	Frequency of tick-borne haemoparasite detection Number of times detected in 12 months (%)												
			***E/A* catch-all**[a]	***A. marginale***	***Anaplasma* sp. (Omatjenne)**	***A. platys***	***E. ruminanium***	***T/B* catch-all**[b]	***T.* catch all**[c]	***T. mutans***	***T. taurotragi***	***T. velifera***	***B* catch-all 1**[d]	***B* catch-all 2**[e]	***B. bovis***
Peri-urban area	1	T2	8 (66.7)	4 (33.3)	3 (25.0)	2 (16.7)	0[f]	10 (83.3)	10 (83.3)	9 (75.0)	1 (8.3)	9 (75.0)	5 (41.7)	2 (16.7)	5 (41.7)
	2	T2	9 (75.0)	4 (33.3)	0	0	0	10 (83.3)	9 (75.0)	7 (58.3)	0	9 (75.0)	6 (50.0)	0	3 (25.0)
	3	T2	6 (50.0)	3 (25.0)	2 (16.7)	1 (8.3)	0	7 (58.3)	6 (50.0)	6 (50.0)	0	7 (58.3)	6 (50.0)	2 (16.7)	5 (41.7)
	4	T2	9 (75.0)	6 (50.0)	2 (16.7)	0	0	10 (83.3)	10 (83.3)	10 (83.3)	0	10 (83.3)	6 (50.0)	1 (8.3)	4 (33.3)
	5	T2	8 (66.7)	6 (50.0)	3 (25.0)	2 (16.7)	0	7 (58.3)	7 (58.3)	7 (58.3)	0	7 (58.3)	7 (58.3)	2 (16.7)	6 (50.0)
Wildlife/ livestock interface	6	T1	6 (50.0)	1 (8.3)	0	0	0	11 (91.7)	11 (91.7)	8 (66.7)	0	11 (91.7)	5 (41.7)	0	5 (41.7)
	7	T1	7 (58.3)	1 (8.3)	0	0	0	11 (91.7)	10 (83.3)	9 (75.0)	0	9 (75.0)	9 (75.0)	4 (33.3)	9 (75.0)
	8	T1	6 (50.0)	0	0	0	0	12 (100)	12 (100)	10 (83.3)	0	12 (100)	4 (33.3)	1 (8.3)	0
	9	T3	4 (33.3)	0	0	0	0	8 (66.7)	8 (66.7)	6 (50.0)	0	8 (66.7)	3 (25.0)	0	0
	10	T1	10 (83.3)	0	2 (16.7)	2 (16.7)	1 (8.3)	10 (83.3)	10 (83.3)	9 (75.0)	0	10 (83.3)	5 (41.7)	0	3 (25.0)
Mean percentage of detection (%)			**60.8**	**20.8**	**10.0**	**5.8**	**0.8**	**80.0**	**77.5**	**67.5**	**0.8**	**76.7**	**46.7**	**10.0**	**33.3**

[a] indicates hybridisation with the *Ehrlichia/Anaplasma* group-specific probe
[b] indicates hybridisation with the *Theileria/Babesia* group-specific probe
[c] indicates hybridisation with the *Theileria* genus-specific probe
[d] indicates hybridisation with the *Babesia* genus-specific probe 1
[e] indicates hybridisation with the *Babesia* genus-specific probe 2
[f] indicates a negative result

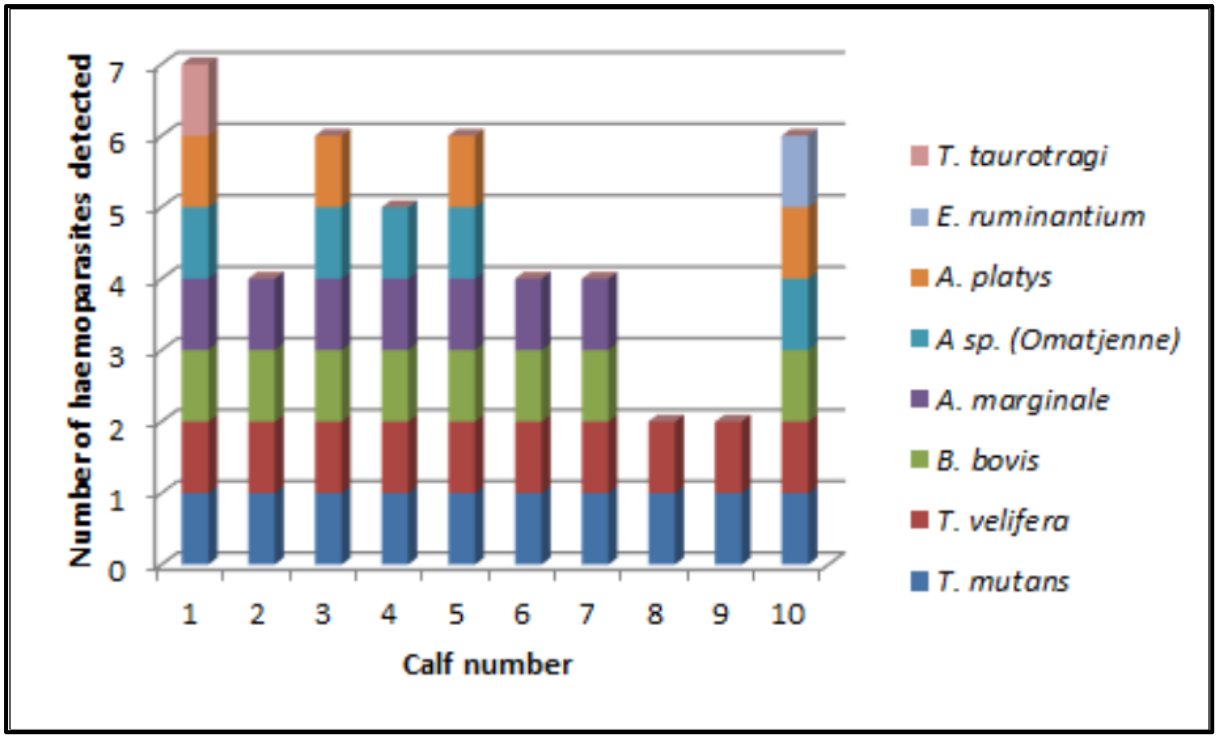


Figure 3.1: Haemoparasites detected by RLB. The stacked bar graph shows the number of haemoparasites detected in the 10 calves in the study. Calves 1-5 were from the peri-urban area, while calves 6-10 were from the wildlife/livestock interface.

3.1.2. Detection of tick-borne haemoparasites in the dams of the calves using the RLB hybridisation assay

DNA samples extracted from the dams of the sampled calves were also screened for the presence of various pathogenic and non-pathogenic tick-borne haemoparasites using the RLB hybridisation assay (Table 3.5). Only one cow in the peri-urban area of the Mnisi communal area tested positive (dam 4). All five dams (dams 6-10) located at the wildlife/livestock interface tested positive for various tick-borne haemopararsites.

The only haemoparasite detected in the one positive cow from the peri-urban area was *A. bovis*, while pathogenic haemoparasites detected at the wildlife/livestock interface were *A. marginale* and *B. bovis*, and non-pathogenic haemoparasites were *Anaplasma* sp. (Omatjenne), *A. platys*, *T. mutans*, *T. taurotragi* and *T. velifera*.

Table 3.5: Tick-borne haemoparasites detected in dams by RLB.

Area	Dam	RLB result
Peri-urban area	M1	-[a]
	M2	-
	M3	-
	M4	*E/A* catch-all[b], *A. bovis*
	M5	-
Wildlife/livestock interface	M6	*T/B* catch-all[c], *B* catch-all 1[d], *B. bovis*
	M7	*E/A* catch-all, *Anaplasma* sp. (Omatjenne), *A. platys*, *T/B* catch-all, *T* catch-all[e], *T. mutans*, *T. velifera*, *B* catch all-1, *B* catch all-2[f], *B. bovis*
	M8	*E/A* catch-all, *A. marginale*
	M9	*E/A* catch-all, *A. marginale,* *T/B* catch-all, *T* catch-all, *T. mutans, T. taurotragi, T. velifera*
	M10	*E/A* catch-all, *A. marginale,* *T/B* catch-all, *T* catch-all, *T. mutans, T. taurotragi, T. velifera*

[a] indicates a negative result
[b] indicates the *Ehrlichia/Anaplasma* group-specific probe
[c] indicates the *Theileria/Babesia* group-specific probe
[d] indicates the *Babesia* genus-specific probe 1
[e] indicates the *Theileria* genus-specific probe
[f] indicates the second *Babesia* genus-specific signals

3.1.3. Quantitative real-time PCR (qPCR) detection of *A. marginale* and *A. centrale*

The 119 DNA samples extracted from blood samples collected from the ten calves were subjected to the duplex *A. marginale*- and *A. centrale*-specific quantitative real-time PCR (qPCR). *Anaplasma marginale* was identified in 59 (49.6%) of the samples with Cq values ranging from 18.1 to 28.8 (Table 3.6). Results were further analysed using the *A. marginale* and *A. centrale* amplification curves (Figure 3.2), with a cut-off threshold of 36 cycles detecting 250 copies of *A. marginale*. *Anaplasma centrale* was not detected in any of the calves in their first year of life.

The duplex *A. marginale* and *A. centrale* qPCR assay showed that three of the five calves (Calves 1, 4 and 5) located in Eglington village in the peri-urban area tested positive for *A. marginale* at T1 (0-1 month old). All five calves in the peri-urban area tested positive for *A. marginale* by T2 with relatively low Cq values. Only two calves (Calves 6 and 7) located at the wildlife/livestock interface tested positive for *A. marginale*, Calf 7 at T7 and Calf 8 at T8 (Table 3.6). Three calves (Calves 8, 9 and 10) never tested positive by qPCR. These calves were either infected at levels below the detection limit (250 copies per reaction), of our assay or they were not exposed to *A. marginale* at all in their first year of life.

Table 3.6: Detection of *A. marginale* by qPCR.

Area	Calf	Cq value											
		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
Peri-urban area	1	25.4	24.6	24.5	24.0	26.6	24.0	-	-	27.0	24.9	28.8	26.7
	2	- [a]	24.9	22.5	27.3	25.7	26.0	-	-	23.7	24.2	24.8	26.2
	3	-	19.8	27.0	24.2	28.0	26.0	-	-	26.8	25.7	27.7	26.9
	4	18.1	26.2	22.2	25.2	25.4	23.5	23.0	24.6	0	28.0	27.6	*
	5	24.1	25.2	25.9	27.7	23.1	26.0	-	23.6	26.0	24.6	27.7	27.9
Wildlife/ livestock interface	6	-	-	-	-	-	-	-	23.2	23.1	24.9	27.5	26.7
	7	-	-	-	-	-	-	24.8	22.0	-	25.6	25.7	26.0
	8	-	-	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-

[a] indicates a negative result

* animal died

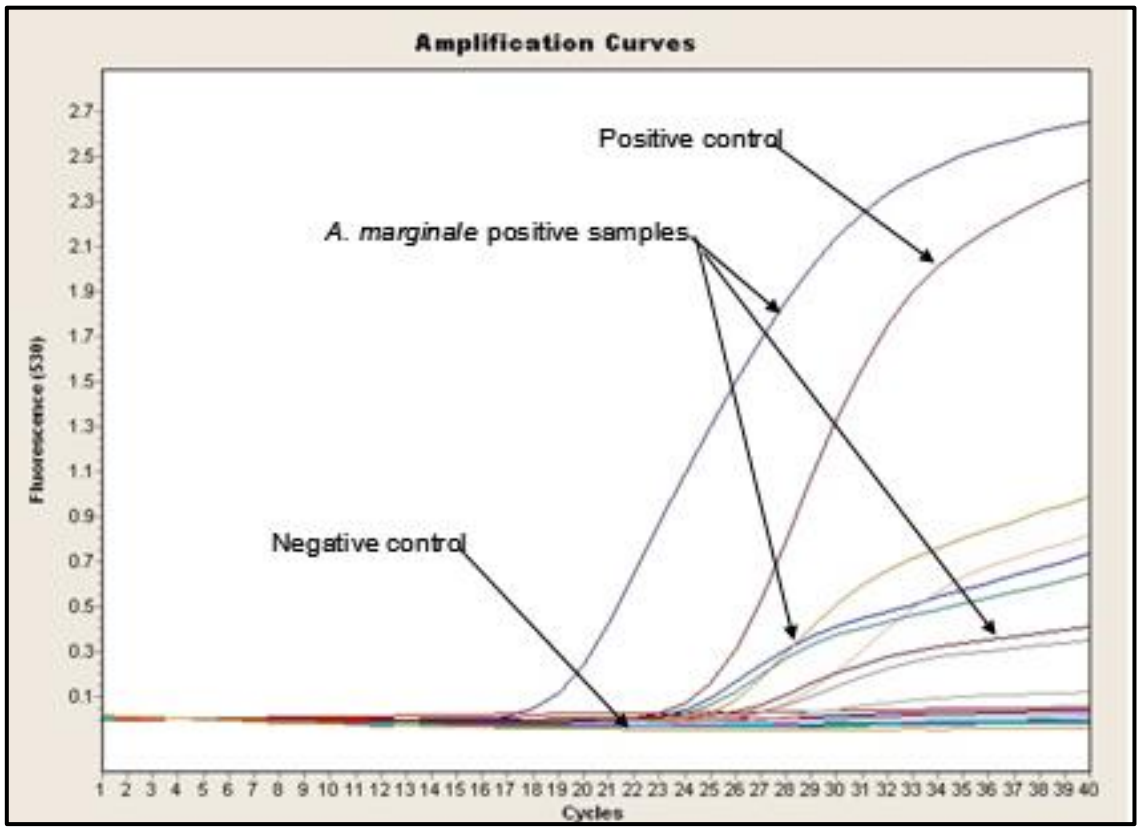


Figure 3.2: Examples of *A. marginale* qPCR data. Representative qPCR amplification curves for *A. marginale* are shown for positive control and test DNA samples. FAM fluorescence (530 nm) was generated in *A. marginale* positive samples.

The frequency of detection of *A. marginale* in calves located at the peri-urban area over the 12 month study period was found to be higher than in calves located at the wildlife/livestock interface (Table 3.7). The frequency of detection of *A. marginale* ranged from 75% to 91.7% at the peri-urban area and from 0% to 41.7% at the wildlife/livestock interface. The mean qPCR Cq values ranged from 21.9 to 25.8 in the peri-urban area and from 24.8 to 25.1 in the wildlife/livestock interface over the 12 month study period (Table 3.7).

Table 3.7: Frequency of *A. marginale* detection by qPCR.

Area	Calf	Time point of initial detection	Frequency of *A. marginale* detection		
			Number of time points at which *A. marginale* was detected	Percentage of time points at which *A. marginale* was detected	Mean Cq Value
Peri-urban area	1	T1	10	83.3	25.6
	2	T2	9	75.0	25.0
	3	T2	9	75.0	25.8
	4	T1	10	83.3	21.9
	5	T1	11	91.7	25.6
Wildlife/ livestock Interface	6	T8	5	41.7	25.1
	7	T7	5	41.7	24.8
	8	ND[a]	0	0	0
	9	ND	0	0	0
	10	ND	0	0	0

[a] ND: not detected

3.1.4. Detection of *A. marginale* and *A. centrale* in dams by qPCR

Only six of the dams tested positive for *A. marginale* using the duplex qPCR assay. Two of the dams were located at the peri-urban area while four were located at the wildlife/livestock interface (Table 3.8). *Anaplasma centrale* was not detected in any of the dams.

Table 3.8: Detection of *A. marginale* in dams by qPCR.

Area	Mother	Cq value
Peri-urban area	M1	-[a]
	M 2	24.7
	M 3	-
	M 4	-
	M 5	24.5
Wildlife/livestock interface	M 6	26.5
	M 7	-
	M 8	24.0
	M 9	22.8
	M 10	24.7

[a] indicates a negative result

3.1.5. Detection of *E. ruminantium* in calves by qPCR

Ehrlichia ruminantium was identified in 17 (14.3 %) of the calf samples using the *E. ruminantium*-specific qPCR assay, with Cq values ranging from 31.8 to 36.9 (Table 3.9). Results were analysed using the amplification curve, with a cut-off threshold of 37 cycles detecting three copies of *E. ruminantium* per reaction (Figure 3.3) and samples testing above the cut-off value of 37 cycles per reaction were considered false-positive.

Table 3.9 shows the initial time point of detection and temporal dynamics of *E. ruminantium* infection in the five calves at the peri-urban area and five calves at the wildlife/livestock interface over the 12 month study period. Four calves (Calves 1, 2, 6 and 9) tested positive using the *E. ruminantium* qPCR assay at T1 (0-1 month old). The number of calves testing positive for *E. ruminantium* was very low throughout the 12 time point, with calf 7 testing negative to *E. ruminantium* in the first year of life (although positive Ct values were obtained, they were below the limit of detection of the assay). All calves tested negative or below the limit of detection of the assay for *E. ruminatium* at T12. The levels of rickettsaemia in all the calves that tested positive were very low as shown by the high Cq values in Table 3.9.

Table 3.9: Detection of *E. ruminantium* by qPCR.

Area	Calf	Cq values											
		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
Peri-urban area	1	31.8	- [a]	-	-	-	(39.3)[b]	-	(39.8)	(38.3)	-	-	-
	2	35.6	(39.7)	35.7	-	-	-	-	-	-	-	-	-
	3	-	36.9	36.6	-	-	-	36.6	-	-	-	-	-
	4	-	36.1	-	34.6	-	(38.1)	-	-	-	-	-	*
	5	-	(37.2)	(37.5)	-	-	-	-	-	35.1	(37.7)	(37.3)	-
Wildlife/livestock interface	6	32.2	-	(37.5)	-	-	-	(37.7)	-	-	-	-	-
	7	-	-	-	(39.4)	-	-	(38)	-	(39.4)	(38.1)	-	-
	8	-	35.3	36.2	-	-	-	36.2	(37.2)	-	(37.7)	-	-
	9	32.2	-	36.5	-	-	-	-	(37.7)	-	35.8	-	-
	10	-	36.5	-	-	-	-	-	-	-	-	-	-

[a] indicates a negative result

[b] Cq values shown in brackets and in red font were below the cut-off point of 37 cycles and were considered to be false-positives.

* animal died

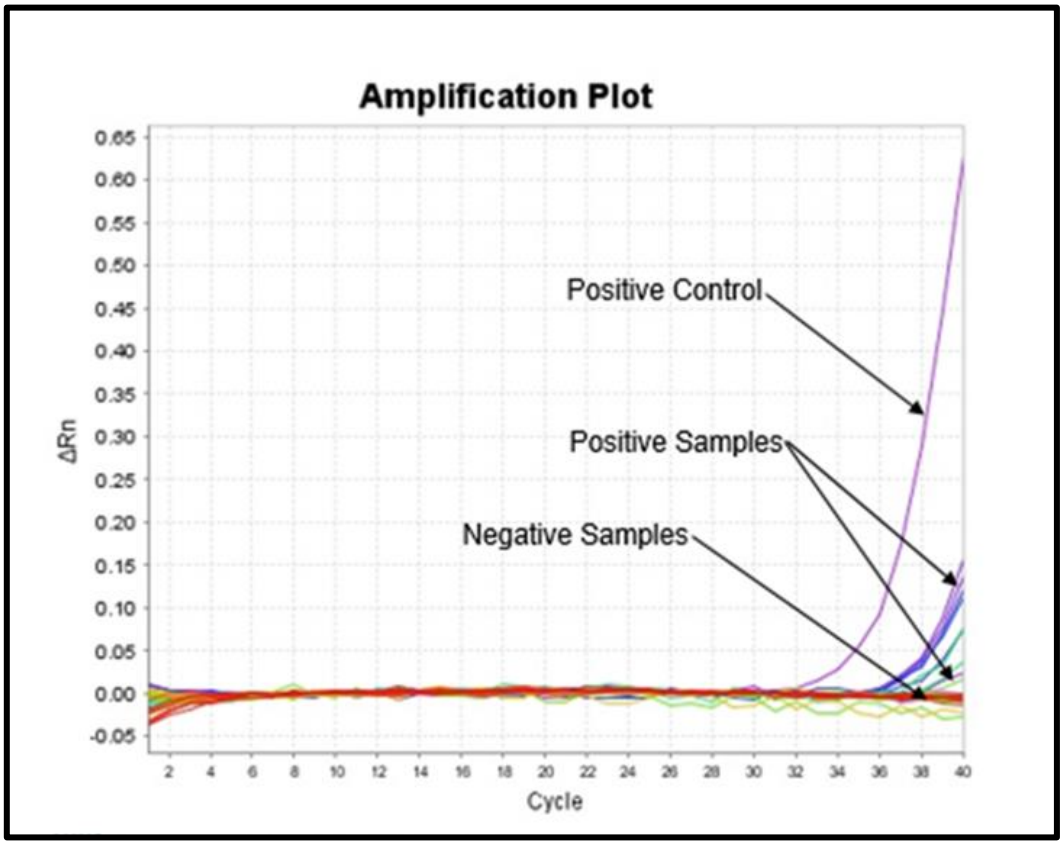


Figure 3.3: Examples of *E. ruminantium* qPCR data. Representative qPCR amplification curves for *E. ruminantium* are shown for positive control and test samples, confirming the presence of the parasite in positive DNA samples. ΔRn represents the measure of fluorescence emitted throughout the qPCR assay.

The frequency of detection of *E. ruminantium* in the calves located at both the peri-urban area and the wildlife/livestock interface was low, ranging from 8.1% to 25% with the mean Cq values ranging from 31.8 to 36.7 over the 12 month study period (Table 3.10).

Table 3.10: Frequency of detection of *E. ruminantium* by qPCR.

Area	Calf	Time point of initial detection	Number of time points at which *E. ruminantium* was detected	Percentage of time points at which *E. ruminantium* was detected	Mean Cq Value
Peri-urban area	1	T1	1	8.3	31.8
	2	T1	2	16.7	35.7
	3	T2	3	25.0	36.7
	4	T2	2	16.7	35.4
	5	T9	1	8.3	35.1
Wildlife/livestock Interface	6	T1	1	8.3	32.2
	7	ND[a]	0	0	0
	8	T2	3	25	35.9
	9	T1	3	25	34.8
	10	T2	1	8.3	36.5

[a] not detected

3.1.6. Detection of *E. ruminantium* in dams by qPCR

All the dams in both the peri-urban area and at the wildlife/livestock interface tested negative or were infected at levels below the detection limit (3 copies per reaction) of the *E. ruminantium* assay.

3.1.7. Detection of *B. bigemina* in the calves by qPCR

Babesia bigemina was identified in 65 (54.6%) of the calf samples using the *B. bigemina* qPCR assay with Cq values ranging from 28.1 to 39.7 (Table 3.11) which was confirmed by amplification curve analysis (Figure 3.4). This assay is 93% efficient with a detection threshold of 39.7 cycles detecting 6.9 x 10^{-1} copies of *B. bigemina* per reaction (Byaruhanga, unpublished). Samples testing above the cut-off value of 39.7 cycles per reaction were considered false-positive.

Table 3.11 shows the initial time point of detection and temporal dynamics of *B. bigemina* infection in the calves from the peri-urban and wildlife/livestock interface areas over the 12 month study period. Only two calves in the peri-urban area (calves 4 and 5) tested positive using the *B. bigemina* real-time assay at T1 (0-1 month old). A third calf tested positive for the first time in the second month (T2). All calves in the peri-urban area had tested positive for *B. bigemina* by T4 and most were positive until T12. Four of the five calves located at the wildlife/livestock interface (calves 6, 7, 9 and 10) first tested positive for *B. bigemina* late in the year, with calf 6 testing positive at T6, calves 7 and 10 at T7 and calf 9 at T9 (Table 3.11).

Table 3.11: Detection of *B. bigemina* by qPCR.

Area	Calf	Cq value											
		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
Peri-urban area	1	-[a]	-	-	37.7	38.5	-	(40.6)[b]	35.3	35.8	35.5	38.2	38.5
	2	-	34.0	-	35.9	37.1	35.5	33.3	(40.8)	36.9	(40.5)	35.6	34.3
	3	-	-	-	29.8	-	-	38.4	33.9	33.8	39.4	36.1	38.2
	4	37.7	32.0	38.2	36.4	35.5	(40.0)	36.4	37.9	34.1	34.7	35.6	*
	5	31.8	35.0	34.5	-	39.3	(44.7)	(43.2)	36.4	37.0	37.0	38.6	38.8
Wildlife/livestock interface	6	-	-	-	-	-	37.4	38.2	39.2	35.1	36.9	39.1	38.2
	7	-	-	-	-	-	-	39.6	35.9	(42.9)	39.7	(40.4)	38.1
	8	-	28.1	-	33.8	35.8	-	-	-	-	37.1	37.6	37.3
	9	-	-	-	-	-	-	-	-	32.3	31.4	35.9	32.4
	10	-	-	-	-	-	-	35.5	35.3	35.3	-	-	-

[a] indicates a negative result

[b] Cq values shown in brackets and in red font were below the cut-off point of 39.7 cycles and were considered to be false-positives.

* animal died

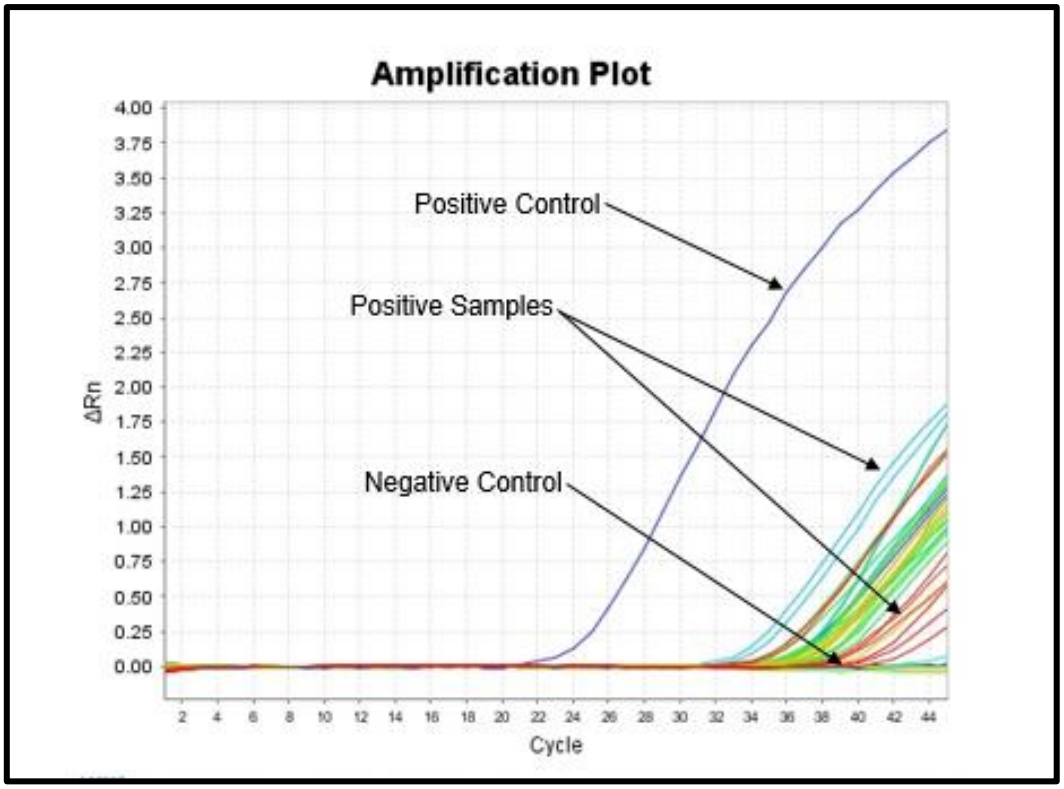


Figure 3.4: Representative qPCR amplification curves for *B. bigemina*, confirming the presence of the parasite in positive DNA samples. ΔRn represents the measure of fluorescence emitted throughout the qPCR assay.

The frequency of detection of *B. bigemina* in calves located at the peri-urban area was found to be higher than in calves located at the wildlife/livestock interface. The frequency of detection of *B. bigemina* in the calves over the 12 month study period ranged from 58.3% to 83.3% in the peri-urban area and from 25% to 58.3% at the wildlife/livestock interface. The mean qPCR Cq values ranged from 35.7 to 37.0 in the peri-urban area and from 34.9 to 38.3 in the wildlife/livestock interface over the 12 month study period (Table 3.12).

Table 3.12: Frequency of detection of *B. bigemina* by qPCR.

Area	Calf	Time point of initial detection	Number of time points at which *B. bigemina* was detected	Percentage of time points at which *B. bigemina* was detected	Mean Cq Value
Peri-urban area	1	T4	7	58.3	37.0
	2	T2	8	66.7	35.3
	3	T4	7	58.3	35.7
	4	T1	10	83.3	35.9
	5	T1	9	75.0	36.4
Wildlife/livestock Interface	6	T6	7	58.3	37.8
	7	T7	4	33.3	38.39.4
	8	T2	6	50.0	34.9
	9	T9	4	33.3	35.0
	10	T7	3	25.0	35.4

3.1.8. Detection of *B. bigemina* in dams by qPCR

All the mothers of the calves in both the peri-urban area and the wildlife/livestock interface tested negative or were infected at levels below the detection limit of the *B. bigemina* assay.

3.1.9. Comparison of pathogenic haemoparasite occurrence in calves at the two areas of the Mnisi communal area

The pathogen-specific qPCR assays showed that the five calves in the peri-urban area were exposed to all three pathogenic haemoparasites between their first and fourth months of life (Figure 3.5A). The calves at the wildlife/livestock interface generally became positive for pathogenic haemoparasites later in their first year of life or not at all (Figure 3.5B). Eight of the calves became positive for *E. ruminantium* between the first and fourth month of life (four in the peri-urban area and four at the wildlife/livestock interface), one in the peri-urban area became positive only in the ninth month, while one at the wildlife/livestock interface was considered negative in the first year of life (although a number of false-positive results may indicate that it was infected at levels below the detection limit of our assay). Although *B. bigemina* was detected in all of the calves, only one calf became positive in the second month; the other four calves became positive between the sixth and ninth months. Two of the calves became positive for *A. marginale* in the seventh and eighth months, but three of the five calves

at the wildlife/livestock interface were either infected at levels below the detection limit of our assays, or they were not infected by *A. marginale* at all in their first year of life.

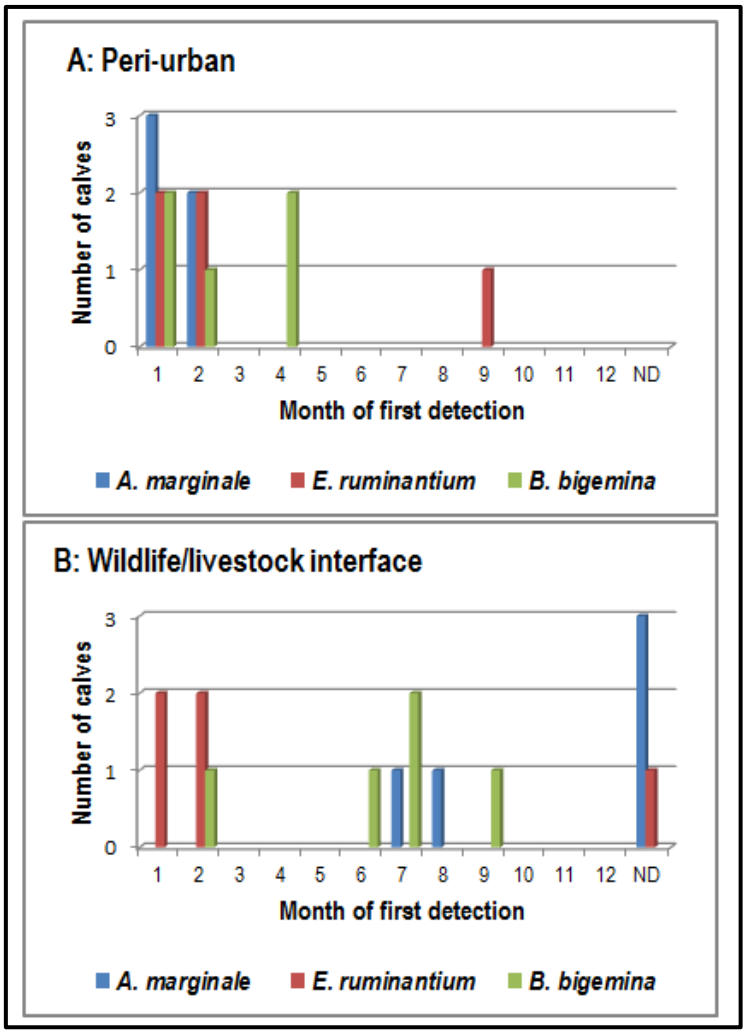


Figure 3.5: Initial time point of pathogen detection by pathogen-specific qPCR assays. Panel A shows results for calves from the peri-urban area, and panel B shows results for calves at the wildlife/livestock interface. ND: not detected

The pathogen-specific qPCR assays showed that detectable levels of infection in individual calves varied from time of infection to one year old and at some time points dropped below the detection limit (Figure 3.6). Calves 8, 9 and 10 had detectable levels of *E. ruminantium* and *B. bigemina*, but *A. marginale* was not detected in these three calves in their first year of life. Pathogenic haemoparasites were more frequently detected in calves in the peri-urban area than calves at the wildlife/livestock interface.

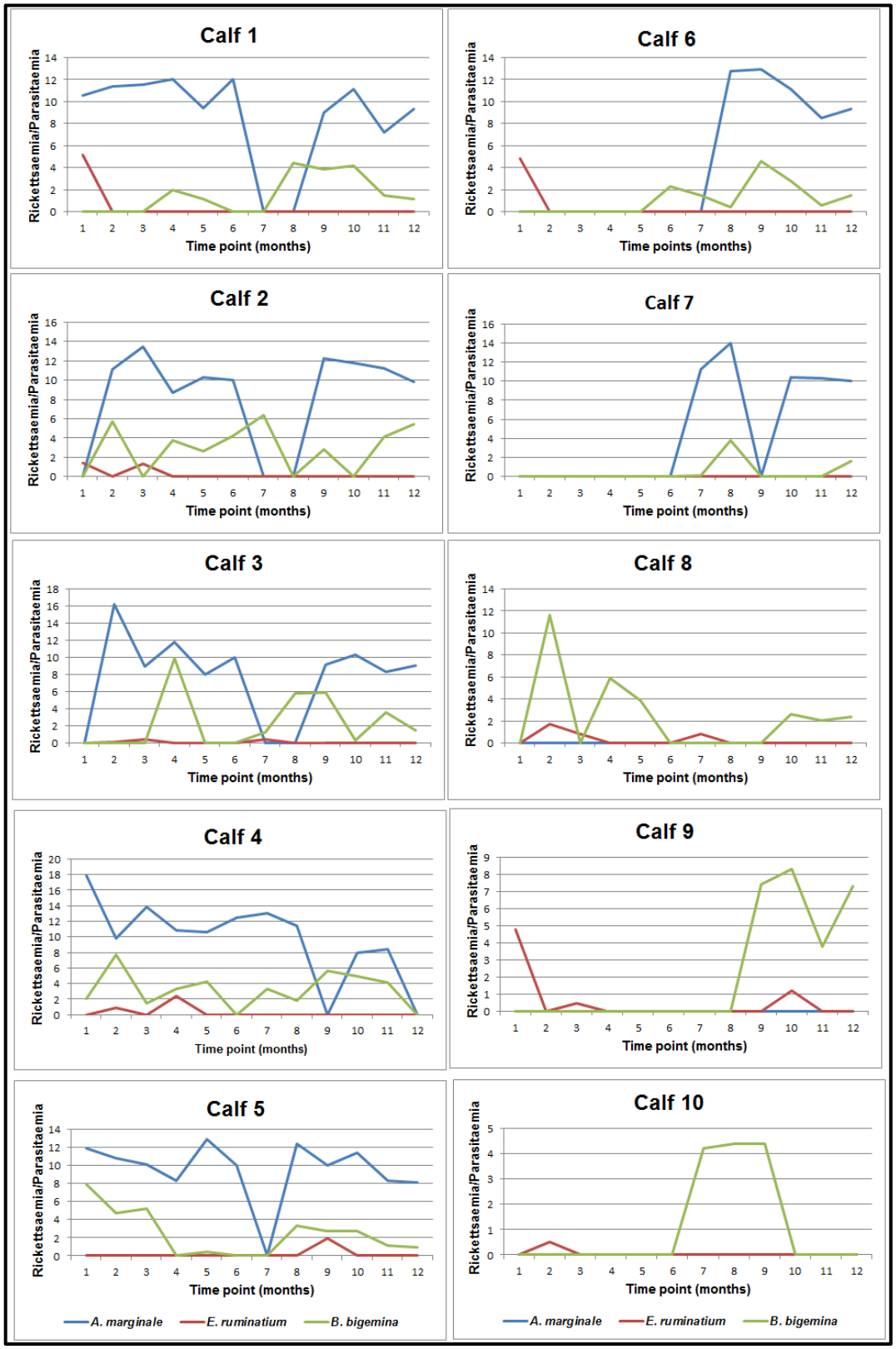


Figure 3.6: Relative pathogen infection levels in calves over the 12-month study period**.** Infection levels were determined by pathogen-specific qPCR assays in calves at the peri-urban area (calves 1-5) and at the wildlife/livestock interface (calves 6-10). Since the Cq value is inversely proportional to the rickettsaemia/parasitaemia, and standard curves were not available for all of the qPCR assays, levels of infection were expressed as qPCR Cq value cut-off point less the measured Cq value.

Figure 3.7 shows the frequency and standard error of the mean ($\sigma\bar{x}$) of pathogen detection by pathogen-specific qPCR assays, classified by the different habitat areas. There was a significant difference in the frequency of detection of *A. marginale* and *B. bigemina* between the peri-urban area and the wildlife/livestock interface (p-value ≤ 0.05) with 0.82 and 0.62 effect sizes of independent-samples test respectively, suggesting a very large effect of the differences observed in the two areas. There was no significant difference between the frequency of detection of *E. ruminantium* in the two areas in the 12 month study period (p-value > 0.05).

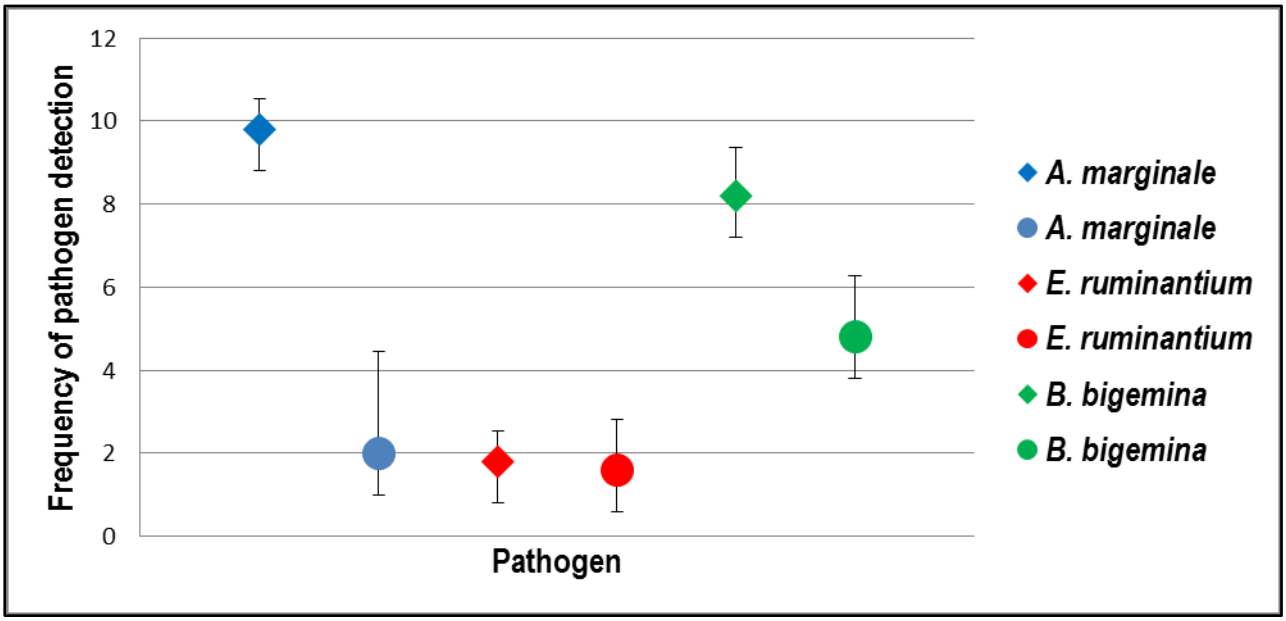


Figure 3.7: Frequency of pathogen detection by pathogen-specific qPCR assays in different habitat areas. The table shows the frequency of number of time points in which the qPCR test was positive in five calves in each area during the year (expressed as standard error of the mean ($\sigma\bar{x}$)). Diamonds: calves from the peri-urban area; Circles: calves from the wildlife/livestock interface.

3.1.10. Adult tick collection

A total of 805 live adult ticks were collected from the ten calves at the three dip tanks in the Mnisi communal area from November 2016 to October 2017. A total of 420 (52.2%) adult ticks were collected from the five calves at the peri-urban area, Eglington village, and 385 (47.8%) adult ticks were collected from the other five calves at the wildlife/livestock interface, Utah 1 and Dixie villages.

3.2. Identification of adult ticks to species level using morphological keys

A morphological guide (Walker et al., 2003) was used to identify the 805 ticks, and only two species were identified: *Amblyomma hebraeum* and *Rhipicephalus microplus*.

3.2.1. Molecular identification of *Rhipicephalus* adult ticks to confirm identity

As it is difficult to distinguish between adult tick species in the genus *Rhipicephalus*, a molecular method was used to confirm the adult tick identifications.

The adult ticks collected over the 12 time points were pooled per dip tank for DNA extraction (five to ten ticks per pool). A total of 29 pools of DNA extracted from the adult ticks that were identified to species level as *R. microplus* were subjected to a PCR-RFLP test that identifies African *Rhipicephalus* ticks (Lempereur et al., 2010).

The ITS2 sequence was amplified from the pools of *Rhipicephalus* tick DNA resulting in PCR products between 765 bp and 832 bp (Figure 3.8)

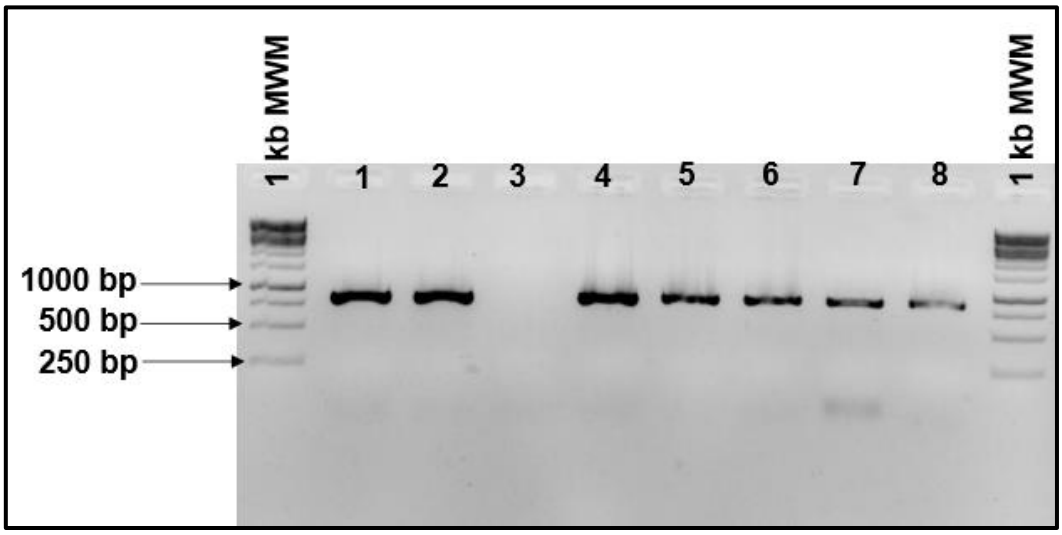


Figure 3.8: PCR products for the ITS2 sequence amplification. 1 kb Molecular weight ladder on the left and right, Lane1-2: *R. microplus* positive controls, lane 3: negative control, lane 4-8: pools of *Rhipicephalus* tick DNA identified by morphological keys.

The RFLP assay according to Lempereur et al. (2010) confirmed that all pools of *Rhipicephalus* adult ticks collected in both areas of the Mnisi communal area were indeed *R. microplus*. The profiles of the pools of adult *Rhipicephalus* ticks collected from the calves generated by *Msp*I digestion revealed several profiles for *R. microplus* ticks (Figure 3.9). The first *R. microplus* profile resulted in five bands on the gel as expected (around 400 bp, 250 bp, 180 bp, 100 bp and 70 bp) as shown in Lane 1 in Figure 3.9, confirmed by sequence analysis. The second *R. microplus* profile resulted in three bands on the gel (around 400 bp, 300 bp, and 100 bp) as shown in Lane 7 in Figure 3.9. BLAST results revealed that, the sequence obtained from the pool of ticks collected from calves in the peri-urban area (lane 7) was indeed *R. microplus* with a 99% identity, but the nucleotide differences are such that there are only two *Msp*1 restriction sites in this sequence, and digestion of this ITS2 PCR product results in a different profile with just three bands. Some profiles appeared to contain a mixture of these two profiles (e.g. lanes 4, 5 and 6 in Figure 3.9). Although sequence analysis showed that the sequences obtained from these PCR products were identical to the *R. microplus* control ITS2 sequence (lane 1), a background signal could be seen in the sequence traces, indicating the presence of a second, less dominant sequence.

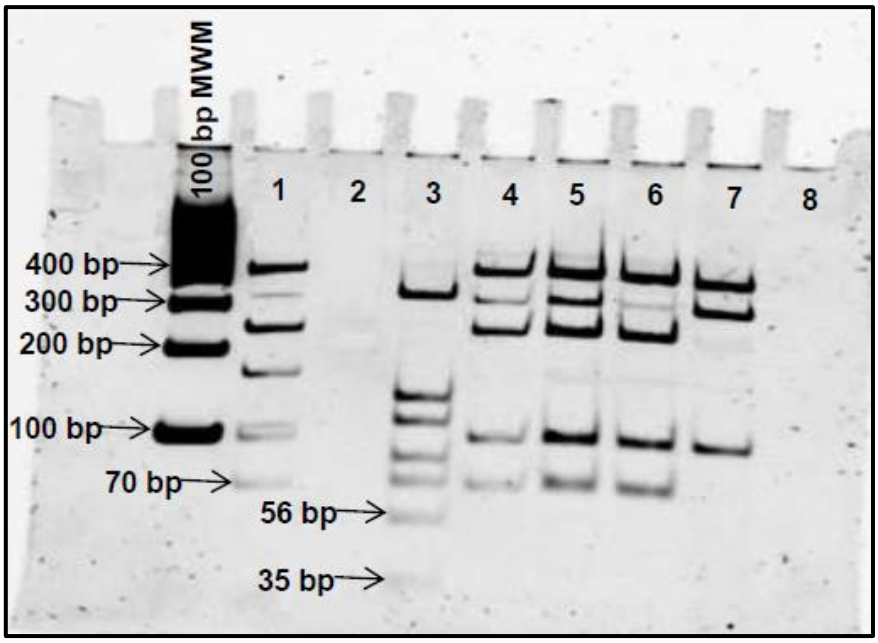


Figure 3.9: PCR-RFLP profiles of tick pools. PCR-RFLP profiles were generated by *Msp*I digestion of the ITS2 PCR products, and resolved on a polyacrylamide gel (BioRad, South Africa). 100 bp molecular weight marker (ThermoFisher Scientific, South Africa) on the left. Lane 1: *R. microplus* positive control. Lane 2: negative control. Lane 3: *R. decoloratus* positive control. Lanes 4-7: Pools of *Rhipicephalus* tick DNA collected from calves. Lane 8: empty lane.

Thus, using a combination of morphological guides and molecular confirmation, 533 adult ticks (66.2%) were identified to species level as *A. hebraeum* and 272 (33.8%) were identified to species level as *R. microplus* (Figure 3.10). Of the 533 *A. hebraeum* adult ticks, 280 were collected from the five calves at the peri-urban area (Eglington village) and 253 were collected from the five calves at the wildlife/livestock interface. Of the 272 *R. microplus* adult ticks, 140 were collected from the five calves at the peri-urban area (Eglington village) and 132 (48.5%) were collected from the five calves at the wildlife/livestock interface (Figure 3.10).

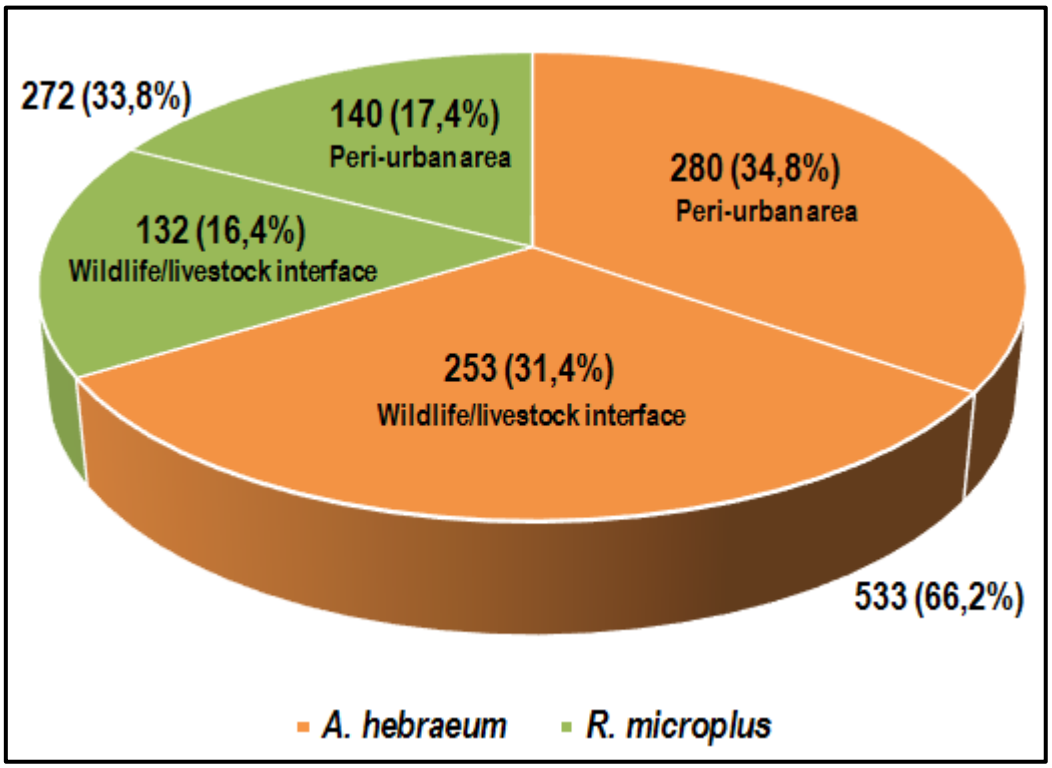


Figure 3.10: Proportion of tick species collected from the peri-urban area and the wildlife/livestock interface.

Amblyomma hebraeum adult ticks were collected at all 12 time points in the peri-urban area and at ten of the 12 time points at the wildlife/livestock interface (Figure 3.11 A and B). *R. microplus* adult ticks only became evident at T8 after the rainy season (Figure 3.11 A and B). There was no significant difference between the number of *A. hebraeum* adult ticks collected from calves in the peri-urban area and the wildlife/livestock interface (p-value > 0.05). The number of *R. microplus* adult ticks collected in the two areas of the Mnisi communal area after the rainfall also showed no significant difference (p-value > 0.05).

Tick infestation was also assessed weekly with the help of the local veterinary services and environmental monitors by observing all the sampled calves and the other cattle in the herds visiting the three chosen dip tanks for weekly inspection and acaricide dipping, and the results are shown in Table 3.13, 3.14, 3.15.

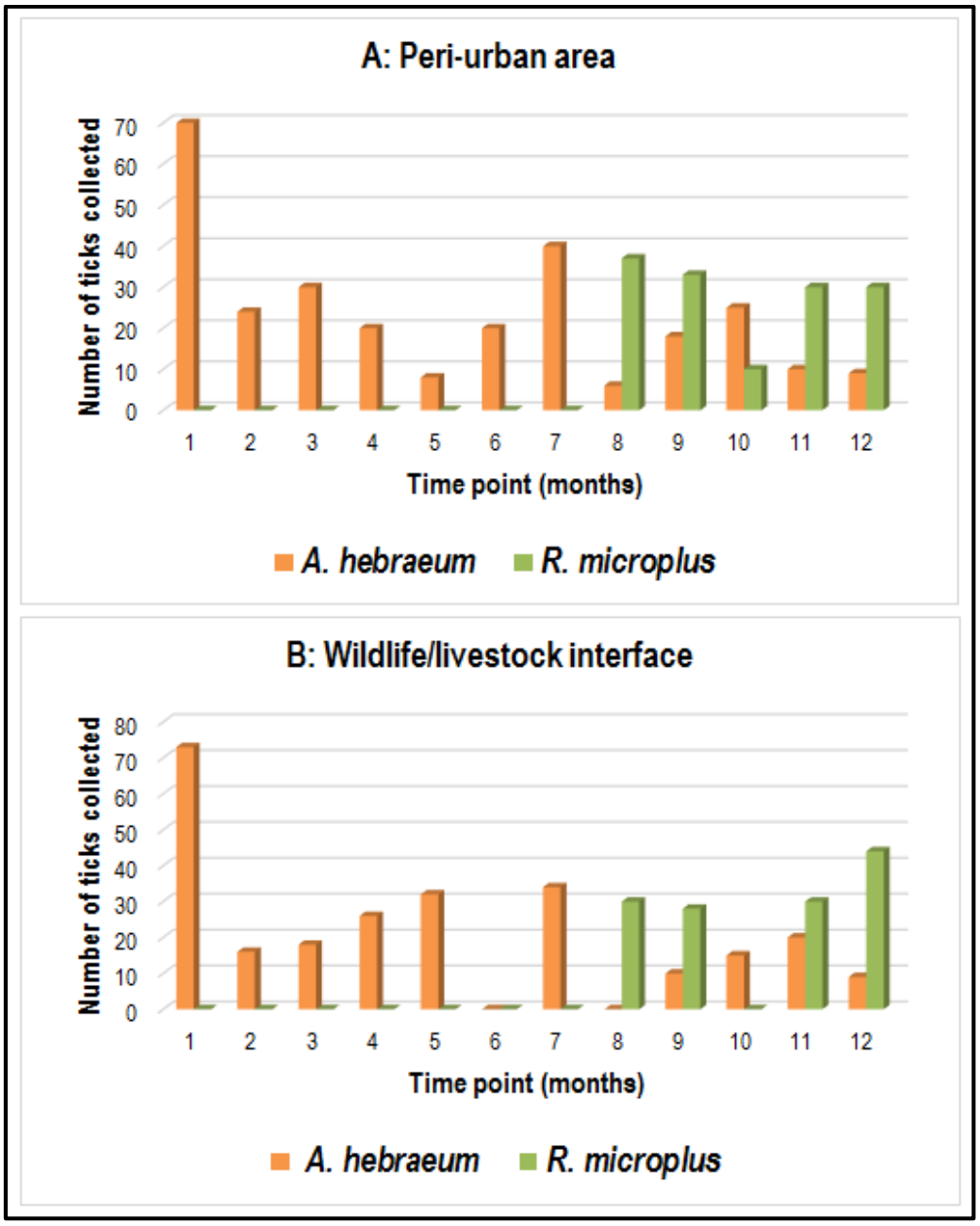


Figure 3.11: Numbers of *A. hebraeum* and *R. microplus* ticks collected. Panel A) reports the adult ticks collected from the five calves at the peri-urban area and panel B) reports the ticks collected from the five calves at the wildlife/livestock interface of the Mnisi communal area at each time point over the 12-month study period.

Table 3.13: Tick infestation during the study period at Eglington dip tank.

Time Point	Number of ticks collected	Tick species identified	Tick infestation on study calves	Tick infestation on other cattle	Tick population on other cattle	Monthly weather and rainfall
T1	70	*A. hebraeum*-70	Low	Low	Adult *Amblyomma*	Hot, dry with little rain (30 mm)
T2	24	*A. hebraeum*-24	Low	Low	Adult *Amblyomma*	Hot with rain (120 mm)
T3	30	*A. hebraeum*-30	Low	Low	Adult *Amblyomma*	Cool with rain (73 mm)
T4	20	*A. hebraeum*-20	Low	Low	Adult *Amblyomma*	Hot, dry with very little rain (4 mm)
T5	8	*A. hebraeum*-8	Low	Moderate	Adult *Amblyomma* Immature *Rhipicephalus*	Hot with rain (107 mm)
T6	20	*A. hebraeum*-20	Low	Moderate	Adult *Amblyomma* Immature *Rhipicephalus*	Hot with little rain (44.5 mm)
T7	40	*A. hebraeum*-40	Low	Moderate	Adult *Amblyomma* Immature *Rhipicephalus*	Very hot with very little rain (8 mm)
T8	43	*A. hebraeum*-6 *R. microplus*-37	Low	Moderate	Adult *Amblyomma* Adult *Rhipicephalus*	Very hot with no rain (0 mm)
T9	51	*A. hebraeum*-18 *R. microplus*-33	Low	Moderate	Adult *Amblyomma* Adult *Rhipicephalus*	Very hot and dry with no rain (0 mm)
T10	35	*A. hebraeum*-25 *R. microplus*-10	Low	Moderate	Adult *Amblyomma* Adult *Rhipicephalus*	Very hot and dry with no rain (0 mm)
T11	40	*A. hebraeum*-10 *R. microplus*-30	Low	Moderate	Adult *Amblyomma* Adult *Rhipicephalus*	Very hot and dry with no rain (0 mm)
T12	39	*A. hebraeum*-9 *R. microplus*-30	Low	Moderate	Adult *Amblyomma* Adult *Rhipicephalus*	Very hot and dry with little rain (30mm)

Table 3.14: Tick infestation during the study period at the Utah 1 dip tank.

Time Point	Number of ticks collected	Tick species identified	Tick infestation on study calves	Tick infestation on other cattle	Tick population on other cattle	Monthly weather and rainfall
T1	21	*A. hebraeum*-21	Low	Low	Adult *Amblyomma*	Hot, dry with little rain (30 mm)
T2	2	*A. hebraeum*-2	Low	Low	Adult *Amblyomma*	Hot with rain (120 mm)
T3	10	*A. hebraeum*-10	Low	Low	Adult *Amblyomma*	Cool with rain (73 mm)
T4	18	*A. hebraeum*-18	Low	Low	Adult *Amblyomma*	Hot, dry with very little rain (4 mm)
T5	20	*A. hebraeum*-20	Low	Low	Adult *Amblyomma* Immature *Rhipicephalus*	Hot with rain (107 mm)
T6	0					Hot with little rain (44.5 mm)
T7	15	*A. hebraeum*-15	Low	Low	Adult *Amblyomma* Immature *Rhipicephalus*	Very hot with very little rain (8 mm)
T8	19	*R. microplus*-19	Low	Low	Adult *Rhipicephalus*	Very hot with no rain (0 mm)
T9	18	*R. microplus*-18	Low	Low	Adult *Rhipicephalus*	Very hot and dry with no rain (0 mm)
T10	10	*A. hebraeum*-10	Low	Low	Adult *Amblyomma* Adult *Rhipicephalus*	Very hot and dry with no rain (0 mm)
T11	29	*A. hebraeum*-9 *R. microplus*-20	Low	Low	Adult *Amblyomma* Adult *Rhipicephalus*	Very hot and dry with no rain (0 mm)
T12	32	*A. hebraeum*-9 *R. microplus*-23	Low	Low	Adult *Amblyomma* Adult *Rhipicephalus*	Very hot and dry with little rain (30mm)

Table 3.15: Tick infestation during the study period at the Dixie dip tank.

Time Point	Number of ticks collected	Tick species identified	Tick infestation on study calves	Tick infestation on other cattle	Tick population on other cattle	Monthly weather and rainfall
T1	53	*A. hebraeum*-53	Low	Low	Adult *Amblyomma*	Hot, dry with little rain (30 mm)
T2	14	*A. hebraeum*-14	Low	Low	Adult *Amblyomma*	Hot with rain (120 mm)
T3	8	*A. hebraeum*-8	Low	Low	Adult *Amblyomma*	Cool with (73 mm)
T4	8	*A. hebraeum*-8	Low	Low	Adult *Amblyomma*	Hot, dry with very little rain (4 mm)
T5	12	*A. hebraeum*-12	Low	Low	Adult *Amblyomma* Immature *Rhipicephalus*	Hot with rain (107 mm)
T6	0					Hot with little rain (44.5 mm)
T7	19	*A. hebraeum*-19	Low	Low	Adult *Amblyomma* Immature *Rhipicephalus*	Very hot with very little rain (8 mm)
T8	11	*R. microplus*-11	Low	Low	Adult *Rhipicephalus*	Very hot with no rain (0 mm)
T9	20	*A. hebraeum*-10 *R. microplus*-10	Low	Low	Adult *Amblyomma* Adult *Rhipicephalus*	Very hot and dry with no rain (0 mm)
T10	5	*A. hebraeum*-5	Low	Low	Adult *Amblyomma*	Very hot and dry with no rain (0 mm)
T11	21	*A. hebraeum*-11 *R. microplus*-10	Low	Low	Adult *Amblyomma* Adult *Rhipicephalus*	Very hot and dry with no rain (0 mm)
T12	21	*R. microplus*-21	Low	Low	Adult *Rhipicephalus*	Very hot and dry with little rain (30mm)

3.3. Pathogen detection in ticks collected from the study calves

The adult ticks collected over the 12 time points were pooled per dip tank, according to the species, into pools of five to ten ticks for DNA extraction. A total of 97 pools of tick DNA (68 pools of *A. hebraeum* comprising 36 pools from the peri-urban area and 32 pools from the wildlife/livestock interface, and 29 pools of *R. microplus* comprising 14 pools from the peri-urban area and 15 pools from the wildlife/livestock interface) were screened for the presence of pathogenic and non-pathogenic haemoparasites in the genus *Anaplasma*, *Babesia*, *Ehrlichia* and *Theileria* using the RLB hybridisation assay and pathogen-specific qPCR assays.

3.3.1. RLB hybridisation assay for the detection of pathogenic and non-pathogenic haemoparasites in adult tick DNA

Of the 97 pools of adult ticks tested using the RLB hybridisation assay, 61 (62.9%) [42 (61.8%) *A. hebraeum* and 19 (65.5%) *R. microplus*] were found to be positive for *Babesia*, *Ehrlichia* and *Theileria* spp. A total of 26 (38.2%) *A. hebraeum* pools and 10 (34.5%) *R. microplus* pools tested negative or were infected at levels below the limit of detection for the RLB assay (Table 3.16).

The most frequently detected haemoparasite in the *A. hebraeum* pools collected in the peri-urban area, as determined by RLB, was *E. ruminantium* which was detected in 11 pools, followed by *T. velifera* in four pools, *T. mutans* in one pool and *B. bovis* in one pool. A total of 18 pools were positive for the *Ehrlichia/Anaplasma* group-specific probe (*E/A* catch-all), while a *Theileria/Babesia* group-specific signal (*T/B* catch-all) was detected in 10 pools. Genus-specific signals were detected as follows: *Theileria* catch-all in 9 pools, *Babesia* catch all-1 in 2 pools. The most frequently detected haemoparasite in the *A. hebraeum* pools collected at the wildlife/livestock interface was *E. ruminantium* in eight pools, followed by *T. mutans* and *T. velifera* in one pool each. A total of 15 pools were positive for the *Ehrlichia/Anaplasma* group-specific probe (*E/A* catch-all), while a *Theileria/Babesia* group-specific signal (*T/B* catch-all) was detected in four pools. Genus-specific signals were detected as follows: *Theileria* catch-all in five pools, *Babesia* catch all-1 in 3 pools and *B*abesia catch all-2 in one pool (Table 3.16).

The most frequently detected haemoparasites in the *R. microplus* pools collected in the peri-urban area, as determined by RLB, were *T. mutans* and *T. velifera* in two pools each. A total

of 11 pools were positive for the *Ehrlichia/Anaplasma* group-specific probe (*E/A* catch-all), while a *Theileria/Babesia* group-specific signal (*T/B* catch-all) was detected in six pools. Genus-specific signals were detected as follows: Theileria catch-all in eight pools, *B*. catch all-1 in four pools (24.1%). The only haemoparasite detected in the *R. microplus* pools collected at the wildlife/livestock interface was *T. buffeli* in one pool. A total of four pools were positive for the *Ehrlichia/Anaplasma* group-specific probe (*E/A* catch-all), while a *Theileria/Babesia* group-specific signal (*T/B* catch-all) was only detected in one pool. Genus-specific signals were detected as follows: *Theileria* catch-all in four pools, *Babesia* catch all-1 in three pools (Table 3.16).

Although many *E/A* catch-all signals were detected in both tick species, no *Anaplasma* species-specific signals were detected in either tick species by RLB hybridisation assay.

Table 3.16: Detection of haemoparasites in pools of adult ticks by RLB.

Tick species	Area	Number of ticks collected	Number of pools tested	Negative	*E/A* catch-all[a]	*E. ruminantium*	*T/B* catch-all[b]	*T.* catch-all[c]	*T. equi*	*T. mutans*	*T. velifera*	*T. buffeli*	*B.* catch all-1[d]	*B.* catch all-2[e]	*B. bovis*
A. hebraeum	Peri-urban	280	36	11	18	11	10	9	0	1	4	0	2	0	1
	Wildlife/ livestock interface	253	32	15	15	8	4	5	0	1	1	0	3	1	0
	Total	**533**	**68**	**26 (38.2%)**	**33 (48.5%)**	**19 (27.9%)**	**14 (20.6%)**	**14 (20.6%)**	**0**	**2 (2.9%)**	**5 (7.4%)**	**0**	**5 (8.8%)**	**1 (1.4%)**	**1 (1.4%)**
R. microplus	Peri-urban	140	14	2	11	0	6	8	1	2	2	0	4	0	0
	Wildlife/ livestock interface	132	15	8	4	0	1	4	0	0	0	1	3	0	0
	Total	**272**	**29**	**10 (34.5%)**	**15 (51.7%)**	**0**	**7 (24.1%)**	**12 (41.4%)**	**1 (3.4%)**	**2 (6.9%)**	**2 (6.9%)**	**1 (3.4%)**	**7 (24.1%)**	**0**	**0**

[a] indicates hybridisation with the *Ehrlichia/Anaplasma* group-specific probe
[b] indicates hybridisation with the *Theileria/Babesia* group-specific probe
[c] indicates hybridisation with the *Theileria* genus-specific signals probe
[d] indicates hybridisation with the *Babesia* genus-specific probe 1
[e] indicates hybridisation with the *Babesia* genus-specific probe 2

3.3.2. Detection of pathogenic haemoparasites in adult tick DNA by qPCR

Of the 68 *A. hebraeum* pools, 54 (79.4%) tested positive using the *E. ruminantium*-specific qPCR assay (Table 3.17). All of the *A. hebraeum* pools tested negative for *A. marginale, A. centrale* and *B. bigemina*. 77.8% of the *A. hebraeum* pools from the peri-urban area tested positive for *E. ruminantium*, while 81.3% of the *A. hebraeum* pools from the wildlife/livestock interface tested positive for *E. ruminantium*.

The pathogen species-specific qPCR assay revealed that *R. microplus* pools were positive for *A. marginale* (55.2%), *A. centrale* (3.5%), *E. ruminantium* (20.7%) and *B. bigemina* (2.1%) (Table 3.17). A higher percentage (71.4%) of the *R. microplus* pools from the peri-urban area tested positive for *A. marginale*, compared to *R. microplus* pools from the wildlife/livestock interface (40%). One of the *R. microplus* pools collected at the peri-urban area also tested positive for *A. centrale*. A few *R. microplus* pools collected from both the peri-urban area and the wildlife/livestock interface tested positive for the presence of *E. ruminantium* and *B. bigemina* (Table 3.17).

Table 3.17: Detection of haemoparasites by qPCR in pools of adult ticks.

Tick species	Area	Number of ticks collected	Number of pools tested	Number (Percentage) of positive pools			
				A. marginale	*A. centrale*	*B. bigemina*	*E. ruminantium*
A. hebraeum	Peri-urban area	280	36	0	0	0	28 (77.8%)
	Wildlife/livestock interface	253	32	0	0	0	26 (81.3%)
	Total	**533**	**68**	0	0	0	54 (79.4%)
R. microplus	Peri-urban area	140	14	10 (71.4%)	1 (7.1%)	1 (7.1%)	3 (21.4%)
	Wildlife/livestock interface	132	15	6 (40%)	0	1 (6.7%)	3 (20%)
	Total	**272**	**29**	16 (55.2%)	1 (3.5%)	2 (6.9%)	6 (20.7%)

Figure 3.12 shows the percentage of *A. hebraeum* pools that tested positive for pathogenic haemoparasites at each time point, while Figure 3.13 shows the percentage of *R. microplus* pools that tested positive at each time point. *Amblyomma hebraeum* pools tested positive for *E. ruminantium* throughout the year in both areas, while adult *R. microplus* pools tested positive for *A. centrale*, *A. marginale* and *B. bigemina*. *Anaplasma marginale* was detected more frequently in *R. microplus* pools collected from calves in the peri-urban area than from pools from calves at the wildlife/livestock interface. Surprisingly, *R. microplus* pools in the peri-urban area and at the wildlife/livestock interface also tested positive for *E. ruminantium.*

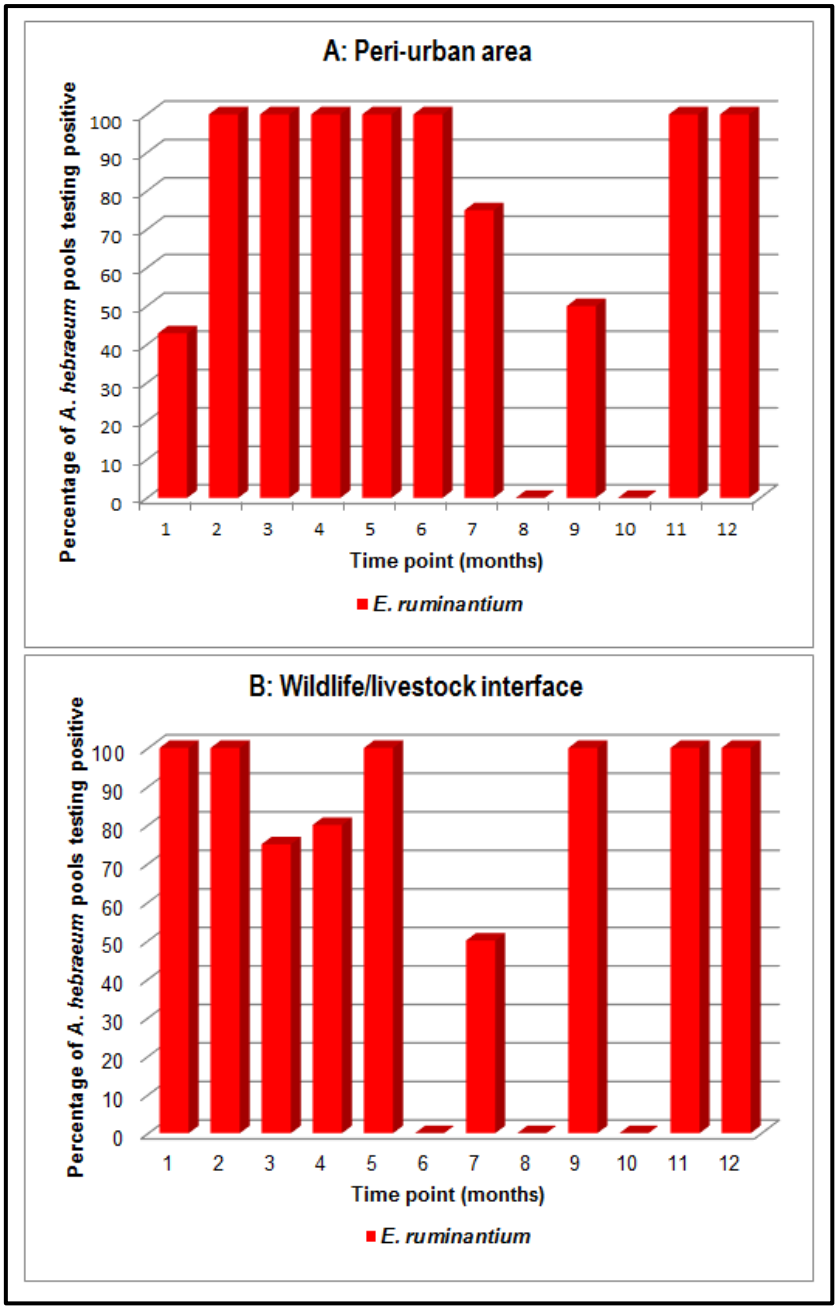


Figure 3.12: Percentage of pools of *Ablyomma hebraeum* ticks that tested positive for *Ehrlichia ruminantium* at each time point; ticks were collected from calves in the peri-urban area (A) and at the wildlife/livestock interface (B).

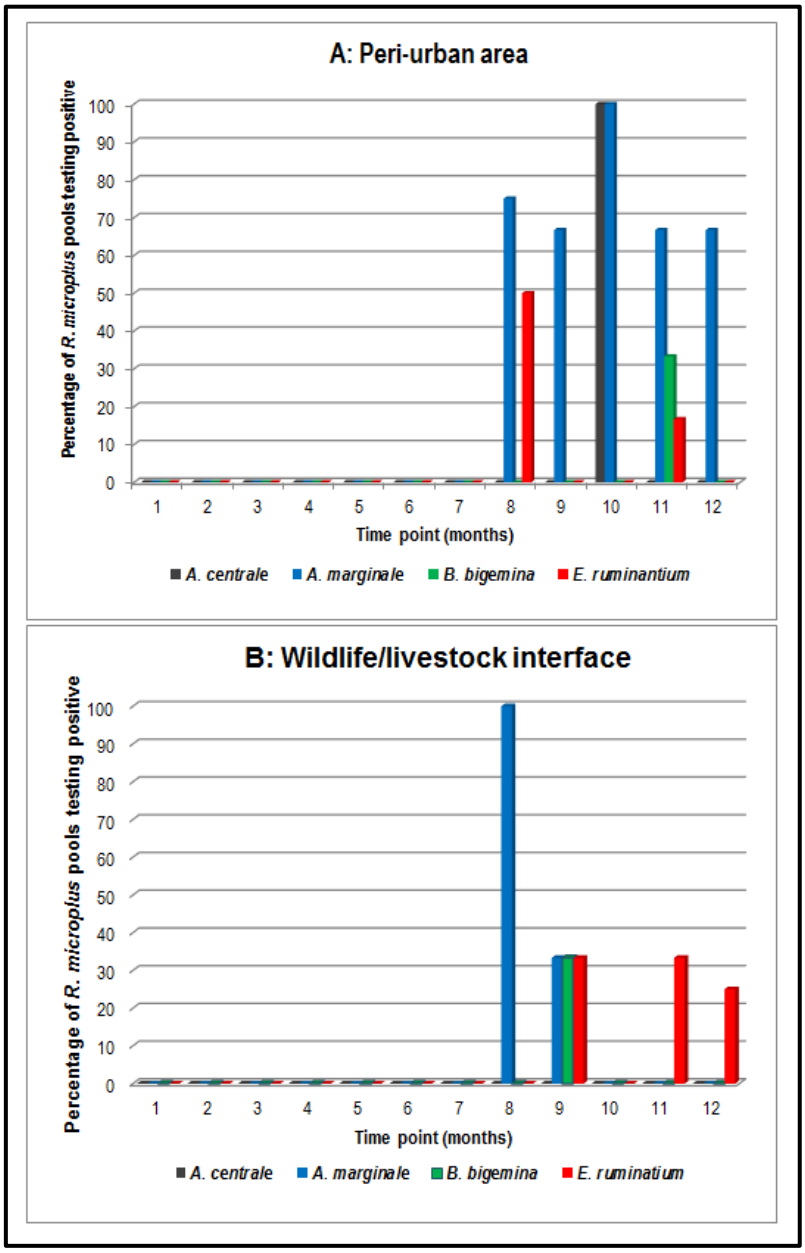


Figure 3.13: Percentage of pools of *Rhipicephalus microplus* ticks that tested positive for *Anaplasma centrale*, *A. marginale*, *Babesia bigemina* and *Ehrlichia ruminantium* at each time point; ticks were collected from calves in the peri-urban area (A) and at the wildlife/livestock interface (B). It should be noted that *R. microplus* was not collected until month 8 of the study.

CHAPTER 4

DISCUSSION

In this longitudinal pilot study, we examined the temporal dynamics of tick-borne infections in ten calves from birth to one year in two sites of the Mnisi communal area (a peri-urban area and the wildlife/livestock interface). Using the reverse-line blot (RLB) hybridisation assay and pathogen species-specific quantitative real-time polymerase chain reaction (qPCR) assays, several important pathogenic tick-borne haemoparasites of cattle, which could present a constraint to the health and well-being of livestock, were identified in monthly samples collected from the calves. These included *A. marginale*, *E. ruminantium*, *B. bigemina* and *B. bovis*.

Results of the study showed discrepancies between the two diagnostic assays used to determine the time-course of tick-borne infection in calves at the Mnisi communal area. These discrepancies were caused by differences in assay sensitivities between the RLB and the qPCR assays used in the study. This was expected as the RLB assay has been shown previously to be less sensitivity than qPCR assays (Bhoora et al., 2010; Chaisi et al., 2017). However, it remains a valuable and commonly used diagnostic assay for detecting a wide range of haemoparasites in livestock and wildlife, as well as in ticks (Gubbels et al., 1999; Bekker et al., 2002; Chaisi et al., 2017). Pathogenic and non-pathogenic haemoparasites detected by the RLB assay in the ten calves included *A. marginale*, *Anaplasma* sp. (Omatjenne), *A. platys, E. ruminantium*, *T. mutans*, *T. taurotragi*, *T. velifera* and *B. bovis*. The number of haemoparasites detected varied throughout the 12 time points of the study. RLB results revealed that *T. velifera*, *T*. *mutans*, *B. bovis* and *A. marginale* were the most prevalent haemoparasites in the ten calves throughout the 12 time points of the study with detection of *A. marginale* showing a significant difference between the two sites of the Mnisi communal area. The occurrence of a variety of pathogenic and non-pathogenic haemoparasites in calves at the Mnisi communal area is of no surprise as the area is surrounded by provincial and private game reserves with abundant wildlife species such as buffalo which are known to be asymptomatic carriers of *Anaplasma*, *Babesia*, *Theileria* and *Ehrlichia* species (Andrew & Norval, 1989; Allsopp, et al., 1993; Gubbels, et al., 1999; Oura, et al., 2004; Pienaar, et al., 2011; Chaisi, et al., 2011; Debeila, 2012; Eygelaar et al., 2015; Henrichs et al., 2016; Khumalo et al., 2016; Sisson et al., 2017). Blesbok, blue and black wildebeest, eland, gemsbok, giraffe,

impala, roan and sable antelope, nyala and waterbuck have also been confirmed to be carriers of various tick-borne haemoparasites in the genera *Anaplasma, Babesia, Ehrlichia* and *Theileria* (Tonetti et al., 2009: Pfitzer et al., 2011; Berggoetz et al., 2014; Khumalo et al., 2016). Exposure of cattle in the area to apathogenic haemoparasites might provide some cross-protection against the pathogenic species and thereby contribute to endemic stability (Woolhouse et al., 2015).

The presence of tick-borne haemoparasites causing bovine anaplasmosis, heartwater and babesiosis was further confirmed in the calves by species-specific qPCR assays. By four months of age, *A. marginale*, *B. bigemina* and *E. ruminantium* had been detected in all five calves in the peri-urban area. Similarly, *E. ruminantium* was only detected in four calves at the wildlife/livestock interface by four months of age, but in contrast, it took longer (up to nine months) to detect *A. marginale* and *B. bigemina* in the calves from this area. In fact, *A. marginale* was not detected at all in three of the calves at the wildlife/livestock interface. Furthermore, the frequency of occurrence of *A. marginale* in calves in the peri-urban area and calves at the wildlife/livestock interface was significantly different (p-value < 0.05).

Only two species of adult ticks, *Amblyomma hebraeum* and *Rhipicephalus microplus,* were observed and collected from the ten calves. *Amblyomma hebraeum* ticks are the known vectors of *E. ruminantium* in South Africa (Allsopp et al., 1999), while *R. microplus* can transmit *A. marginale* and both *B. bovis* and *B. bigemina* (de Vos, 1979). Adult *A. hebraeum* ticks were collected from the ten calves throughout the 12-month study period. The RLB and the *E. ruminantium*-specific qPCR indicated that *E. ruminantium* was present in most of the pools of adult *A. hebraeum* ticks from both areas. Adult *R. microplus* ticks were only observed and collected from the ten calves after the rainfall, from T8 (June 2017) to T12 (October 2017), although immature brown ticks were observed on the calves in the months prior to this. Since adult *R. microplus* ticks were not present when the calves became infected, the transmission of *A. marginale* and *B. bigemina* was probably effected by immature stages. The pathogen-specific qPCR assays showed that pools of adult *R. microplus* ticks were positive for *A. marginale* from T8 to T12 in the peri-urban area but only at T8 and T9 at the wildlife/livestock interface, which might partly explain the lower infection rates in calves at the wildlife/livestock interface, although we do not have data on infections in immature ticks. However, *B. bigemina* was not frequently detected in adult *R. microplus* ticks from either area, suggesting that the infection rate in adult *R. microplus* ticks does not explain differences in infection rates in calves in the different areas.

Our results suggest that calves in the peri-urban area were more likely to be exposed to and infected with *A. marginale* and *B. bigemina* than calves at the wildlife/livestock interface (significant difference, p-value < 0.05), whereas infection with *E. ruminantium* was similar at the two sites (no significant difference, p-value > 0.05). We expected calves at the wildlife/livestock interface to show higher infection rates than calves at the peri-urban area, due to their close proximity to wildlife in the neighboring game reserves. However, while the presence of these haemoparasites in wildlife in game reserves may present a constraint to domestic animals, it does not seem as though calves located at the livestock/wildlife interface are more at risk from tick-borne pathogens than calves further from the interface. Rather, other factors which differ between the two areas, such as method of acaride treatment, cattle density and shortage of water might be contributing to tick-borne disease transmission.

Since different methods of acaricide treatments are used in the two areas included in the study, this might have had an effect on disease transmission dynamics observed in the area. A study conducted by Sungirai et al. (2016) showed how several factors, such as the inability to clean and empty the dip tank resulting in a heavily silted dip tank, and incorrect mixing ratios of water and the acaricide, are the prime causes of tick control failure at communal plunge dip tanks, such as the one used at Eglington village (the peri-urban study site). The frequency of communal cattle dipping in the Mnisi area is greatly affected by water shortages. Thus, a hand spraying method, as is used at Dixie village (the study site at the wildlife/livestock interface) may be more effective in controlling tick infestation than the plunge method of cattle dipping where the concentration of the acaricide in the dip tank might not be as consistent as when the acaricide is applied by hand-spraying. However, the number of adult ticks collected at the two sites did not differ that much, suggesting that the different methods didn't make much difference in adult tick control.

Mason and Norval (1981) showed that *R. microplus* ticks (larvae and adult ticks) migrate between cattle, and Bennett (1975) showed that a proportion of the larvae and nymphs of *R. microplus* had the ability to detach from one host and attach to another. The higher the cattle density in an area, the easier it is for ticks to migrate from one animal to another, thus transmitting pathogens between hosts as they feed. Aguirre et al. (1994) showed that three *R. microplus* ticks that fed on a calf were enough to transmit *A. marginale*. Furthermore, transmission studies using *D. andersoni* tick vector as model, showed that transmission of *A. marginale* can be achieved efficiently by using as few as one and three ticks (Scoles et al., 2005; Scoles et al., 2008). Host to host transfers of *R. microplus* may also result in the field transmission of babesiosis, as it has been shown that *B. bigernina* can be transmitted from infected to susceptible bovines (Dalgleish et al., 1978). More cattle are kept at the peri-urban

study site (Eglington village) of the Mnisi communal area (there are 1009 cattle in an area of approximately 5.2 km^2, giving an average density of 194.04 cattle/km^2), and the cattle graze closely together in grazing camps. Fewer cattle are located at the study sites at the wildlife/livestock interface (there are 715 cattle in Utah 1, with an area of approximately 5.61 km^2, giving an average density of 127.5 cattle/km^2 and there are 135 cattle in Dixie, with an area of approximately 4.92 km^2, giving an average density of 27.4 cattle/km^2). It was observed that cattle located at the wildlife/livestock interface tend to be more spread out when grazing. This observation suggests that the difference between parasite transmission in calves in the two areas might be due to the density of the cattle in an area, as cattle that are more frequently in contact with each other provide more the opportunities for ticks to migrate from one host to another.

Anaplasma marginale was one of the most frequently detected pathogens in the ten calves during the 12-month study period according to both the qPCR and RLB assays. Bovine anaplasmosis is endemic in most of the cattle-farming areas in southern Africa (Krystynak, 1986). Recent studies (Mutshembele et al., 2014; Hove et al., 2018) have confirmed the presence of *A. marginale* in cattle in all South African provinces except for the Northern Cape, which is free of the tick vectors. The presence of *A. marginale* in young calves as revealed by our results suggest that the pathogen infects calves early in their lives or possibly also during intra-uterine development (Aubry & Geale, 2011), since three calves were already positive at T1. Although seven of the calves included in the study tested positive for *A. marginale*, they did not show any clinical symptoms. This is due to inverse age resistance (Bock et al. 2004; Jonsson et al. 2012), for which calves up to 12 months of age are not clinically affected by anaplasmosis despite being exposed to *A. marginale*. The level of rickettsaemia in calves at the peri-urban area ranged from $2.5x10^5$ to $2.5x10^7$ infected red blood cells/ml from time of infection to a year old. This is in agreement with the widely documented phenomenon of rickettsaemic cycles of *A. marginale* in persistently infected animals, which is expected to range from 10^2 to 10^6 or 10^7 infected red blood cells/ml at 5-week intervals (Coetzee et al., 2005). The three calves at the wildlife/livestock interface that tested negative for *A. marginale* in the 12-month period of the study raises concern because naïve adult cattle are susceptible to anaplasmosis. Although it is possible that the calves were infected at levels below the detection limit of either test, this seems unlikely given that the test has previously been shown to be highly sensitive and specific and was able to detect the presence *of A. marginale* in all of the other calves in this study. Although the small sample size might have skewed the results, the fact that three calves were apparently negative for *A. marginale* by one year of age might contribute to cases of anaplasmosis found in the area (Dr Louise Biggs, University of Pretoria- Hluvukani Animal Health Clinic, personal communication). This might present a

constraint to improvement of livestock production in the Mnisi communal area and requires further investigation.

Other *Anaplasma* species detected in the calves were *A. platys* and *Anaplasma* sp. (Omatjenne), while *A. centrale* was detected in one pool of *R. microplus* ticks and *A. bovis* was detected in one of the dams. It is possible that other *Anaplasma* spp. could contribute to endemic stability, since it is known that vaccination with *A. centrale* confers protection against *A. marginale* infection (Kocan et al., 2003).

Although the *A. centrale* qPCR assay is highly sensitive (PCR efficiency of 101%), with a detection limit of 25 copies per reaction, none of the calves were positive at any point during the year. *Anaplasma centrale* has previously been detected in cattle in the Mnisi communal area, but the rate of detection was very low (Choopa, 2015). Farmers in the Mnisi communal area do not practice vaccination against anaplasmosis, which could be part of the reason why *A. centrale* is not frequently detected in cattle in the area.

Anaplasma sp. (Omatjenne) was detected in five of the ten calves and one of the dams, suggesting that this haemoparasite is circulating in cattle in the Mnisi communal area. The presence of this haemoparasite in cattle requires further investigation to determine its significance and its role in the epidemiology of anaplasmosis in the South African cattle population. This haemoparasite has previously been detected in wildlife such as nyala (*Tragelaphus angasii*) in South Africa and Cape buffalo in Botswana and South Africa, and livestock such as cattle and goats in Mozambique, Ethopia, Uganda and Turkey (Bekker et al., 2002; Aktas et al., 2011; Debeila, 2011; Pfitzer et al., 2011; Aktas et al., 2012; Eygelaar et al., 2015; Byaruhanga et al., 2016; Hailemariam et al., 2017).

Anaplasma platys was detected by the RLB assay in three calves in the peri-urban area and one calf at the wildlife/livestock interface. Low detection of *A. platys* by the RLB assay could mean that it was present in other calves but at levels below the limit of detection for this assay; however, this could not be confirmed due to lack of a sensitive qPCR assay specific for this pathogen. This pathogen is a thrombocytotropic bacterium implicated in the cause of canine cyclic thrombocytopenia by infecting canine platelets (Dumler et al., 2001). The presence of this organism in cattle has been confirmed by recent molecular studies that detected it in cattle in Italy and Algeria (Zobba et al., 2014; Dahmani et al., 2015). However, an *A. platys*-like pathogen has also been recently reported in ruminants worldwide (Inokuma et al., 2005; De La Fuente et al., 2006; Liu et al., 2012; Yang et al., 2015; Zobba et al., 2015) suggesting that organisms similar to *A. platys* are present in a wide range of hosts. The main tick vector for *A.*

platys is *Rhipicephalus sanguineus,* which is commonly known as the brown dog tick because of its host preference (Inokuma et al., 2000). *Dermacentor auratus* and *Hyalomma truncatum* tick species have also been implicated in its transmission (Parola et al., 2003; Huang et al., 2005). According to Dantas-Torres (2010), *R. sanguineus* have also been found and collected on cattle in Nigeria. Although the presence of *R. sanguineus* has never been reported on cattle in South Africa, it has been reported to be prevalent on dogs in the Mnisi communal area (Kolo et al., 2016; Kolo, 2018, (unpublished)) and furthermore, farmers and herdsmen in the area are often accompanied by dogs to the various dip tanks for cattle dipping. The close interaction between cattle and dogs in the area might explain the presence of this pathogen in the cattle.

Although infection with *A. bovis* is usually asymptomatic, it can cause clinical symptoms such as fever, weight loss and in some cases results in death (Rar and Golovljova, 2011). This tick-borne pathogen has been recently reported in *R. sanguineus* and *R. turanicus* ticks collected in Israel (Harrus et al., 2011). African tick vectors such as *Hyalomma excavatum*, *R. appendiculatus* and *A. variegatum* have been implicated in the transmission of *A. bovis* (Dumler et al., 2001). This parasite has been detected previously in cattle in the Mnisi communal area (Choopa, 2015); in our study it was detected in one cow in the peri-urban area, but it was not detected in the ten calves during their first year of life.

The two causal agents of bovine babesiosis, *B. bovis and B. bigemina*, were both detected in the ten calves from the two sites of the Mnisi communal area. *Babesia bovis* was only detected using the RLB assay; a previously reported qPCR assay (Kim et al, 2017) did not detect *B. bovis* in RLB-positive samples and, although a modified qPCR assay is currently being developed, it is not yet ready for use (C. Byaruhanga, personal communication). None of the samples collected from the calves tested positive for *B. bigemina* using the RLB assay, but this pathogen was detected in the calves using a newly validated *B. bigemina*-specific qPCR assay modified from the test developed by Kim et al. (2017) (C. Byaruhanga, personal communication). Martins et al. (2010) and Byaruhanga et al. (2016) also noted that the existing *B*. *bigemina*-specific RLB probe did not detect all *B*. *bigemina* genotypes in cattle from Mozambique and Uganda. This is because the existing *B*. *bigemina*-specific RLB probe was designed using only three *B*. *bigemina* 18S rRNA sequences from Mexico (Gubbels et al., 1999); DNA sequence variation in the *B. bigemina* 18S rRNA gene has subsequently been reported in several countries. The presence of *B. bovis* as revealed by RLB could mean that more of the calves were also positive for this pathogen during their first year of life because the RLB assay has been reported to be less sensitive than qPCR assays for the detection of carrier animals (Bhoora et al., 2010; Chaisi et al., 2017).

The other economically important tick-borne haemoparasite detected in the calves was *E. ruminantium*. Heartwater only occurs where the tick vector, *A. hebraeum*, is present (Allsopp, 2010). Andrew and Norval (1989) showed that sheep, cattle and African buffalo remain carriers of heartwater for long periods and therefore serve as reservoirs of infection. Other African game species such as blesbok, black wildebeest, and eland have also been shown to be natural reservoirs of *E. ruminantium* (Neitz, 1935; Allsopp et al., 1999a; Allsopp et al., 1999b; Andrew and Norval, 1989; Wesonga et al., 2001). Barre & Camus (1987) suggest that recovered goats are possible carriers of *E. ruminantium*, however, it is still not clear if they are significant reservoirs of the infection. Goats are certainly susceptible to heartwater and over 800 outbreaks of heartwater in goats were reported by veterinarians in South Africa between 1993 and 2014 (South African National Department of Agriculture, Disease database, 2014). It is possible that the presence of cattle, sheep and goats in the Mnisi communal area, as well as buffalo and other African game species in the neighbouring game reserves could contribute to transmission of *E. ruminantium* and may explain why *E. ruminantium* is widespread in the area. *Ehrlichia ruminantium* was detected by the qPCR assay in nine calves at some time point in their first year of life, with one calf at the wildlife/livestock testing negative or below the limit of detection of the assay throughout the 12 time points of the study. Levels of rickettsaemia in the calves that tested positive were generally very low and this could be why this pathogen was not detected by the less-sensitive RLB assay. Even though this pathogen was detected at very low rickettsaemias, our qPCR results suggested that the sampled calves were exposed to the pathogen from an early age. Endemic stability to heartwater has previously been shown to be maintained by the age-related resistance to *E. ruminantium* possessed by newborn ruminants, a high infection rate in *Amblyomma* ticks and the long-term carrier state of the pathogen in ruminants (Neitz et al., 1947; Andrew and Norval, 1989; Norval et al., 1990, Deem et al., 1996).

Theileria parva, which causes Corridor disease, was not detected in the ten calves, although a few cases of Corridor disease were reported during the 12-month study period in Utah 1, which is located at the wildlife/livestock interface. Corridor disease has previously been reported in cattle in the Mnisi communal area (Choopa, 2015), and was previously shown to be transmitted to cattle at the wildlife/livestock interface where there is more likely to be contact between African buffalo and cattle. Although no *R. appendiculatus* ticks were collected from the calves during the 12-month study period, the few cases of Corridor disease that did occur suggest the presence of this tick vector in the area.

The RLB hybridisation assay revealed that the calves were infected with *T. mutans*, *T. velifera* and *T. taurotragi* in their first year of life. While *T. velifera* is reported to be apathogenic, *T.*

mutans and *T. taurotragi* have been reported to be mildly pathogenic in cattle (Mbassa et al., 1994). However, the three species are commonly detected in cattle in South Africa, and are thought to be of little economic consequence in most cases (Stoltsz, 1989). Our findings are in agreement with the study conducted by Neitz, (1957), who showed that these parasites are the most common parasites of cattle in many parts of Africa. *Theileria mutans* and *T. velifera* are transmitted by *A. hebraeum* ticks (Uilenberg et al., 1982). *Rhipicephalus appendiculatus* (Uilenberg et al., 1982) and possibly *R. zambeziensis* (Lawrence et al., 1983) are tick vectors of *T. taurotragi.* The presence of *T. taurotragi* in one calf and two dams thus suggests the presence of these tick vectors in the area or that other tick vectors are responsible for transmission of this pathogen to cattle in the Mnisi communal area.

The qPCR results obtained in our study show that the level of parasitaemia or rickettsaemia in calves infected by TBDs does not remain constant from point of infection to a year old. The level of parasitaemia and rickettsaemia in infected calves initially rises then fluctuates with time. At some time points of the 12 month study period, the level of parasitaemia and rickettsaemia dropped to levels undetectable by the qPCR assays. According to Calder et al. (1996) when recovered animals sustain subclinical infection, this is known as the carrier state. Although carrier animals are not clinically ill, they play a major role in disease transmission within a herd, as they are a reservoir for infection for tick vectors. Our results are in line with the findings of Calder et al. (1996), who showed that parasitaemia varies over time, differing between early and late infection. The study further showed that detection of pathogens (*B. bovis*) in a carrier animal does not remain constant throughout due to low parasitaemias. Thus, a negative result when the parasitaemia is very low is common. *Anaplasma marginale* negative results observed from three calves at T7 and T8 in the peri-urban area might suggest an error with the test or failure of the test to detect *A. marginale* at a higher rickettsaemia during the time point as the the three calves were persistently infected with *A. marginale* as revealed by the Cq values of the qPCR assay in the previous time points. According to Coetzee et al. (2005) rickettsaemic cycles of *A. marginale* in persistently infected animals ranges from 10^2 to 10^6 to 10^7 infected red blood cells/ml at 5-week intervals.

In contrast to the results in the calves, the RLB hybridisation assay revealed more infections in the dams of the calves situated at the wildlife/livestock interface than dams at the peri-urban area. Dams at the wildlife/livestock interface tested positive for pathogenic and non-pathogenic haemoparasites including: *A. marginale*, *Anaplasma* sp. (Omatjenne), *A. platys*, *B. bovis*, *T. mutans*, *T. taurotragi* and *T. velifera*, while only one of the dams in the peri-urban area tested positive for *A. bovis*. This suggests that mothers in the peri-urban area were either negative for other haemoparasites or that the levels of parasitaemia and rickettsaemia were

below the limit of detection of the RLB assay. The latter hypothesis is corroborated by the *A. marginale*-specific qPCR assay which revealed the presence of *A. marginale* in six of the ten dams: four located at the wildlife/livestock interface and two in the peri-urban area. On the other hand, all ten dams tested negative or were infected at levels below the detection limit of the *E. ruminantium* and *B. bigemina* qPCR assays. These two pathogens were detected in all ten calves, indicating the presence of these two pathogens in the Mnisi communal area. Low levels of *E. ruminatium* detection in adult cattle is in agreement with a previous study in the Mnisi communal area (Choopa, 2015), where *E. ruminantium* was only detected in 5.8% of sick cattle (n=137). In that study, no clinical cases of heartwater were observed even in the presence of a large number of *A. hebraeum* ticks. Cattle in heartwater-endemic areas are normally exposed to *E. ruminantium* from an early age (Allsopp et al., 1999a) and will therefore be immune to local strains.

Although our study focused on adult tick collection, immature ticks (nymphs and larvae) on the calves were also observed and recorded. Our results indicated the presence of *A. hebraeum* adult ticks on the ten calves throughout the 12-month time points of the study period in both the peri-urban area and the wildlife/livestock interface. *R. microplus* adult ticks were only observed and collected from the ten calves in both areas after the rainfall, from June 2017 to October 2017. According to Horak et al, (2015) the most important tick species common to both cattle and wild animals are *Amblyomma hebraeum*, *Ixodes rubicundus*, *Rhipicephalus appendiculatus*, *Rhipicephalus decoloratus* and *Rhipicephalus microplus*. *Amblyomma hebraeum* ticks are vectors for *E. ruminantium, T. mutans* and *T. velifera* in southern Africa, while *R. microplus* ticks transmit *B. bigemina* and *B. bovis* as well as *A. marginale* (De Vos & Roos, 1981; Allsopp et al., 1999b; Coetzer et al., 1994; Madder et al., 2012).

Amblyomma hebraeum was the most frequently collected tick species in both areas of the Mnisi communal area. Our findings are in line with studies done on cattle in the Eastern Cape Province, where *A. hebraeum* was the most prevalent tick species (Fourie & Horak, 1990; Horak, 1999). Similar results were also observed at the Kruger National Park, where *A. hebraeum* ticks were the most numerous tick species on kudus (Horak et al., 1992). *Amblyomma hebraeum* ticks are vectors for *E. ruminantium*, *T. mutans* and *T. velifera* (De Vos and Roos, 1981; Norval et al., 1992; Allsopp et al., 1999b). The RLB assay revealed that pools of *A. hebraeum* adult ticks from both areas of the Mnisi community were positive for *E. ruminantium* (27.9%), *T. velifera* (7.4%), *T. mutans* (2.9%) and *B. bovis* (1.4%). The species-specific qPCR assay revealed that most pools of *A. hebraeum* adult ticks were positive for *E. ruminanium*, 77.8% in the peri-urban area and 81.3% in the wildlife/livestock interface. High levels of *E. ruminantium* in most of the pools of adult *A. hebraeum* ticks does not correlate

with the qPCR assay results which detected only low levels of *E. ruminatium* in the calves throughout the 12 months of the study. With over 800 outbreaks of heartwater in goats in South Africa between 1993 and 2014 (South African National Department of Agriculture, Disease database, 2014), low levels of *E. ruminantium* in cattle might suggest that the pathogen is maintained in another species such as goats, this requires further investigation. On the other hand, the presence of apathogenic haemoparasites associated with theileriosis in pools of *A. hebraeum* adult ticks as determined by the RLB assay, correlates with the presence of these parasites in the ten calves in the Mnisi communal area. The presence of *B. bovis* as detected by RLB in pools of *A. hebraeum* could be an incidental finding due to ticks feeding on calves infected with *B. bovis*, as *A. hebraeum* has never been implicated in the transmission of *B. bovis* to cattle.

Rhipicephalus microplus ticks are widely distributed in South Africa and have been collected in the Eastern Cape, Gauteng, Limpopo, Mpumalanga, North West, and KwaZulu-Natal provinces (Baker et al., 1989; Spickett et al., 2011; Walker et al. 2003; Tønnesen et al., 2004; Nyangiwe et al., 2017). Although adult *R. microplus* ticks were only noted and collected after the rainfall, immature brown ticks were observed in the months before adult ticks were evident on the calves located in the peri-urban area and the wildlife/livestock interface. *Rhipicephalus* (*Boophilus*) spp. have been reported to peak during the spring and autumn seasons on cattle in South Africa (Rechav, 1982; Baker et al., 1989; Bryson et al., 2002; Rikhotso et al., 2005). In our study, adult *R. microplus* ticks were collected in the spring and summer months. The distribution of tick species is not constant and changes over time due to many factors, such as the movement of hosts, changes in local tick control procedures and selection for resistance to acaricides as well as variation in seasonal rainfall (Tønnesen et al., 2004). According to Leger et al. (2013) tick species of the genus *Rhipicephalus* are very sensitive and vulnerable to climate change because their local survival, maintenance and reproduction strongly depend on environmental factors such as temperature, sunlight and water or humidity. The Mnisi communal area has been experiencing drought for six years now, with rainfall being below area average for the last two years. This has even led to mortalities in bulk feeders such as buffalos due to poor veld conditions (personal communication with local clinician and Manyeleti Game Reserve Manager).

The RLB hybridisation assay showed that pools of adult *R. microplus* ticks were positive for *T. buffeli* (3.4%), *T. equi* (3.4*%), T. mutans* (6.9%), and *T. velifera* (6.9%). The species-specific qPCR, however, showed that pools of adult *R. microplus* ticks were also positive for *A. marginale* (55.2%), *A. centrale* (3.5%), *E. ruminantium* (20.7%) and *B. bigemina* (6.9%). It is possible that *A. marginale*, *A. centrale*, *B. bovis* and *B. bigemina* were not detected by RLB

because this assay is known to be less sensitive than qPCR assays (Bhoora et al., 2010; Chaisi et al., 2017). Some of the *A. marginale*-, *B. bovis*- and *B. bigemina*-positive samples might have been detected by the group/genus-specific probes but not by the species-specific probes (*E/A* catch-all and *B*. catch all-1 signals were detected in 51.7% and 24.1% of pools of *R. microplus* ticks, respectively). Furthermore, *B. bigemina*–positive samples might not have been detected by the species-specific RLB probe because of sequence variation in the probe region of local *B. bigemina* strains.

Adult *R. microplus* ticks in the peri-urban area were only observed and collected from T8 to T12 (June 2017 to October 2018), but the calves were all infected with *A. marginale* by T4. This then suggests that immature (larvae and nymphs) ticks were responsible for transmission of *A. marginale* to the calves from birth in the peri-urban area. *Rhipicephalus microplus* ticks have been shown to transmit *A. marginale* both intrastadially and transtadially (Connell & Hall, 1972; Connell, 1974; Samish et al., 1993),

The fact that *B. bovis* was not detected at all and *B. bigemina* was only detected in a few of the adult tick pools could also suggest that immature ticks in the area were responsible transmitting these two pathogens. According to Uilenberg, (2006) when the larva is infected with both *B. bovis* and *B. bigemina*, only the nymph is infective, and when the nymph is infected, only the adult tick becomes infective. Furthermore, de Vos, (1979) also demonstrated transovarial transmission of *B. bovis* between generations of ticks, where adult ticks become infected with the parasite and larvae of the next generation then transmit the pathogen to the host.

Theileria equi is an intra-erythrocytic protozoan that causes equine theileriosis (Mehlhorn & Schein, 1998). *Rhipicephalus microplus* is one of the ticks implicated in transmission of *T. equi* (Guimaraes et al., 1997; Guimaraes et al., 1998; Ueti et al., 2005). *Theileria equi* was detected in only one pool of *R. microplus* adult ticks collected in the peri-urban area of the Mnisi communal area. Although the prevalence of this pathogen is unknown, it is considered common in the donkey population in tropical and subtropical countries including South Africa (Kumar et al., 2009).

Theileria buffeli was also detected in one pool of *R. microplus* adult ticks collected at the wildlife/livestock interface. This parasite has been detected in the African buffalo populations in the Addo Elephant Game Park (AEGP), Kruger National Park (KNP) and Hluhluwe–iMfolozi Game Park (HIP) in South Africa (Allsopp et al., 1999a; Chaisi et al., 2013; Chaisi et al., 2014). This apathogenic *Theileria* parasite is transmitted by tick species of the genus *Haemaphysalis*

in Asia (Preston, 2001; de la Fuente et al., 2008). Although transmission of this pathogen in South Africa is poorly understood, *Haemaphysalis silacea* ticks are implicated as a possible vector of *T. buffeli*. This tick vector appears to have a wide host range and has been collected from nyalas in various game reserves in South Africa (Horak et al., 1983; Horak et al., 1992; Horak et al., 1995). *Theileria buffeli* has not been reported in cattle in South Africa. Therefore, the presence of this parasite in one pool of *R. microplus* adult ticks suggests that a small number of *R. microplus* adult ticks that were included in the pools might have fed on infected buffalo in the game reserves prior to feeding on calves at the wildlife/livestock interface.

The presence of *E. ruminantium* in pools of *R. microplus* ticks was unexpected since *R. microplus* ticks were kept alive for more than three days to digest the blood meal prior to dissection and DNA extraction. Furthermore, *R. microplus* ticks have never been implicated in the transmission of *E. ruminantium* to cattle. This observation may have been an incidental finding due to the presence of dead organisms that were still present in the blood meal due to ticks feeding on calves infected with *E. ruminantium*.

The effects of drought on the cattle in the Mnisi communal area included: weight loss at times resulting in death, increased number of dystocias in pregnant cattle, decreased immunity in animals, increased number of clinical anaplasmosis due to increased nutritional stress, movement of animals to areas with better vegetation and number of cattle presented at various dip tanks in the area drastically decreased to avoid injuries and in some cases death in compromised animals (Dr Louise Biggs, University of Pretoria - Hluvukani Animal Health Clinic, personal communication). Any of these factors could account for the death of one calf (Calf 4) in the peri-urban area of the Mnisi communal area, which died towards the end of the end of the 12 month-period of the study.

Our results demonstrate that young calves in the Mnisi communal area were exposed to and infected by various tick-borne haemoparasites early in their lives, and ticks collected from the calves were positive for various tick-borne haemoparasites. Jonsson et al. (2012) described endemic stability as the presence of high numbers of ticks infected with various tick-borne haemoparasites and lack of or low incidence of clinical cases. Norval et al. (1984) showed that, for establishment of endemic stability in a herd, 75% of calves less than nine months of age must be exposed to TBDs. Although we only examined ten calves (low sample size), our findings together with previous studies conducted in the area (Choopa, 2016; Dr Louise Biggs, University of Pretoria - Hluvukani Animal Health Clinic, personal communication) suggest that cattle in the area are indeed exposed to various pathogenic and non-pathogenic haemoparasites at a very young age. Further investigations with adequate sample size would

be required to conclusively determine the status of endemic stability in the Mnisi communal area.

Our results suggested a higher prevalence of tick-borne infections at the peri-urban area than the wildlife/livestock interface. This was unexpected, as higher tick-borne infection prevalence was expected at the wildlife/livestock interface due to the close proximity of wildlife reservoirs. However, factors such as method of acaride treatment, cattle density and water shortage which differ between the peri-urban area and wildlife/livestock interface of the Mnisi communal area might be reasons for the unexpected higher infection prevalence in the peri-urban area. These factors may have influenced the presence of tick vectors (adult and immature), thus leading to differences in tick infestation, particularly of immature tick vectors, on calves in the two different areas, which might have been responsible for some of the tick-borne pathogen transmission in the calves. Furthermore, our results indicate that calves at the wildlife/livestock interface are less likely to be infected early in life and correlate with findings of increased incidence of TBDs in this area following missed acaricide treatments (Louise Biggs, personal communication).This shows that weekly communal dip tank visitation by farmers is very important in order to reduce cattle mortality that could be attributed to TBDs. The free dipping regime as applied by the State Veterinary Services is effective enough to reduce the tick burden while still allowing the cattle to become infected, thus allowing the establishment of a state of endemic stability as shown by very few clinical cases that could be attributed to TBDs (with the exception of infection with *T. parva*). It is also possible, however, that these results may have been influenced by the small sample size that the study employed, and a larger sample size might have revealed different results.

CONCLUSION

Results of our study confirm that calves in the Mnisi communal area are exposed to important pathogenic haemoparasites, including *A. marginale*, *E. ruminantium*, *B. bigemina* and *B. bovis*. The study further illustrated the presence of non-pathogenic haemoparasites such as *Anaplasma* sp. (Omatjenne), *A. platys*, *T. mutans*, *T. taurotragi* and *T. velifera*, which might provide some cross-protection against the pathogenic species, thereby contributing to endemic stability in the Mnisi communal area. Haemoparasites could be detected in the calves from as early as 0-1 month, although some of the pathogens could not be detected in the calves until 6–7 months. *Anaplasma marginale* was not detected at all in three calves at the wildlife/livestock interface and significant differences in transmission of *A. marginale* and *B. bigemina* were observed between calves in the peri-urban area and at the wildlife/livestock interface. Although various game reserves surrounding the Mnisi communal area may present opportunities for disease transmission in the area, other factors such as cattle density and dipping methods (which differ at the two sites) seem to be more important in affecting the number of infected tick vectors, and thus time-course of infection in new born calves. It is clear that detailed information for cattle in different localities in the Mnisi communal area will be required in order to build accurate mathematical models to describe and predict infections. This study also illustrated a lack of species diversity in adult ticks collected from calves in two sites of the Mnisi communal area during the study period. Environmental factors such as drought may have contributed to poor veld conditions in the area, which were unfavourable for growth and maintenance of *Rhipicephalus* tick vectors, as well as cattle maintenance.

FUTURE RECOMMENDATIONS

While the results from this study suggest that there are differences in the time-course of infection in calves in different areas of the Mnisi community, it should be noted that only five calves were examined from each area. A future in-depth longitudinal study in more villages of the Mnisi communal area with a larger sample size is recommended to confirm and further analyse temporal dynamics of tick-borne haemoparasite infections of importance to the health of cattle in the Mnisi communal area.

Although the presence of African game species in game reserves surrounding the Mnisi communal area could be a source of pathogenic and non-pathogenic haemoparasites, our results suggest that other factors that differ between villages at the wildlife/livestock interface and the peri-urban area of the Mnisi communal area are more likely to be contributing to transmission of pathogens. Wildlife may rather contribute to the tick-borne haemoparasite strain composition in cattle grazing near the interface, and this might therefore differ between the two areas of the Mnisi communal area. Further work could be done to assess if there is a significant difference in strains transmitted in the two areas taking cattle species into account.

Babesia bovis infections in the calves in this study were not examined due to the lack of a specific qPCR assay. A highly sensitive qPCR assay is currently being developed, and the test should be used to examine the samples collected in this study to complete the data set.

With sufficient funding, future work on determining the endemic stability status and factors required for maintenance of endemic stability in the Mnisi communal area using both molecular and serological methods could be done. The collection of sufficient data could contribute to the eventual development of a mathematical model to further describe and predict disease transmission in the area.

We further recommend future studies to determine the role and significance of the occurence of non-pathogenic haemoparasites such as *Anasplma* sp. (Omatjenne), *A. platys*, *T. mutans*, *T. taurotragi* and *T. velifera* in cattle, wildlife and ticks.

CHAPTER 5

REFERENCES

AGUIRRE, D.H. GAIDO, A.B. VINABAL, A.E. DE ECHAIDE, S.T. & GUGLIELMONE, A.A. 1994. Transmission of *Anaplasma marginale* with adult *Boophilus microplus* ticks fed as nymphs on calves with different levels of rickettsaemia. *Parasite*, 1 (4): 405-407.

AKTAS, M. ALTAY, K. & DUMANLI, N. 2011. Molecular detection and identification of *Anaplasma* and *Ehrlichia* species in cattle from Turkey. *Ticks and Tick-Borne Diseases,* 2 (1): 62-65.

AKTAS, M. ALTAY, K. OZUBEK, S. & DUMANLI, N. 2012. A survey of ixodid ticks feeding on cattle and prevalence of tick-borne pathogens in the Black Sea region of Turkey. *Veterinary Parasitology,* 187 (3-4): 567-571.

ALLSOPP, B.A. 2010. Natural history of *Ehrlichia ruminantium*. *Veterinary Parasitology,* 167 (2): 123-135.

ALLSOPP, B.A. 2015. Heartwater-*Ehrlichia ruminantium* infection. *Revue Scientifique et Technique-Office International Des Epizooties*, 34 (2): 557-68.

ALLSOPP, B.A. BAYLIS, H.A. ALLSOPPI, M.T.E.P. CAVALIER-SMITH, T. BISHOP, R.P. CARRINGTON, D.M. SOHANPAL, B. & SPOONER, P. 1993. Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences. *Parasitology,* 107 (2): 157-165.

ALLSOPP, B.A., BEZUIDENHOUT, J.D. & PROZESKY, L. 2004. Heartwater, p507-535. In Infectious diseases of livestock, Volume One. Edited by Coetzer J.A.W. & Tustin R.C., Cape Town, Oxford University Press.

ALLSOPP, M.T., THERON, J., COETZEE, M.L., DUNSTERVILLE, M.T. & ALLSOPP, B.A., 1999b. The occurrence of *Theileria* and *Cowdria* parasites in African buffalo (*Syncerus caffer*) and their associated *Amblyomma hebraeum* ticks. *Onderstepoort Journal of Veterinary Research*, 66 (3): 245-9.

ALLSOPP, M.T.E.P. HATTINGH, C.M. VOGEL, S.W. & ALLSOPP, B.A. 1999a. Evaluation of 16S, *map1* and pCS20 probes for detection of *Cowdria* and *Ehrlichia* species. *Epidemiology and Infection*, 122 (2): 323-328.

ALTSCHUL, S.F. GISH, W. MILLER, W. MYERS, E.W. & LIPMAN, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology*, *215* (3): 403-410.

ANDERSON, K. EZENWA, V.O. & JOLLES, A.E. 2013. Tick infestation patterns in free ranging African buffalo (*Syncercus caffer*): Effects of host innate immunity and niche segregation among tick species. *International Journal for Parasitology: Parasites and Wildlife,* 2 1-9.

ANDERSON, R.M. & MAY, R.M. 1990. Immunisation and herd immunity. *The Lancet,* 335 (8690): 641-645.

ANDREW, H. & NORVAL, R. 1989. The carrier status of sheep, cattle and African buffalo recovered from heartwater. *Veterinary Parasitology,* 34 (3): 261-266.

AUBRY, P. & GEALE, D. 2011. A review of bovine anaplasmosis. *Transboundary and Emerging Diseases,* 58 (1): 1-30.

BAKER, G.F. 1989. The seasonal tick populations on traditional and commercial cattle grazed at four altitudes in Natal. *Journal of the South African Veterinary Association*, 60 (2): 95-101.

BARRÉ, N. & CAMUS, E. 1987. The reservoir status of goats recovered from heartwater. *Onderstepoort Journal of Veterinary Research*, 54 (3): 435-437.

BARRY, D.N. RODWELL, B.J. TIMMS, P. & MCGREGOR, W. 1982. A microplate enzyme immunoassay for detecting and measuring antibodies to *Babesia bovis* in cattle serum. *Australian Veterinary Journal*, 59 (5):136-140.

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

BEKKER, C.P. DE VOS, S. TAOUFIK, A. SPARAGANO, O.A. & JONGEJAN, F. 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. *Veterinary Microbiology,* 89 (2-3): 223-238.

BENGIS, R. KOCK, R. & FISCHER, J. 2002. Infectious animal diseases: the wildlife/livestock interface. *Revue Scientifique et Technique-Office International Des Epizooties,* 21 (1): 53-66.

BENNETT, G.F. 1975. *Boophilus microplus* (Canestrini) Acaridae: Ixodidae) on the bovine host. I. Mortality during the developmental cycle. *Acarologia*, 16 (4): 643-650.

BERGGOETZ, M. SCHMID, M. STON, D. WYSS, V. CHEVILLON, C. PRETORIUS, A. & GERN, L. 2014. Tick-borne pathogens in the blood of wild and domestic ungulates in South Africa: interplay of game and livestock. *Ticks and Tick-borne Diseases,* 5 (2): 166-175.

BEZUIDENHOUT, J.D. 1987. Natural transmission of heartwater. *The Onderstepoort Journal of Veterinary Research,* 54 (3): 349-351.

BHOORA, R. FRANSSEN, L. OOSTHUIZEN, M.C. GUTHRIE, A.J. ZWEYGARTH, E. PENZHORN, B.L. JONGEJAN, F. & COLLINS, N.E. 2009. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. *Veterinary Parasitology*, 159 (2): 112-120.

BHOORA, R. QUAN, M. FRANSSEN, L. BUTLER, C.M. VAN DER KOLK, J.H. GUTHRIE, A.J. ZWEYGARTH, E. JONGEJAN, F. & COLLINS, N.E. 2010. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. *Veterinary Parasitology*, 168 (3-4): 201-211.

BISHOP, R. MUSOKE, A. MORZARIA, S. GARDNER, M. & NENE, V. 2004. *Theileria*: intracellular protozoan parasites of wild and domestic ruminants transmitted by ixodid ticks. *Parasitology,* 129 (S1): S271-S283.

BISHOP, R. SOHANPAL, B. KARIUKI, D.P. YOUNG, A.S. NENE, V. BAYLIS, H. ALLSOPP, B.A. SPOONER, P.R. DOLAN, T.T. & MORZARIA, S.P. 1992. Detection of a carrier state in *Theileria parva*-infected cattle by the polymerase chain reaction. *Parasitology*, 104 (2): 215-232.

BLOUIN, E.F. KOCAN, K.M. DE LA FUENTE, J. & SALIKI, J.T. 2002. Effect of tetracycline on development of *Anaplasma marginale* in cultured *Ixodes scapularis* cells. *Veterinary Parasitology,* 107 (1-2): 115-126.

BOCK, R. JACKSON, L. DE VOS, A. & JORGENSEN, W. 2004. Babesiosis of cattle. *Parasitology,* 129 (S1): S247-S269.

BONNET, S. DE LA FUENTE, J. NICOLLET, P. LIU, X. MADANI, N. BLANCHARD, B. MAINGOURD, C. ALONGI, A. TORINA, A. & FERNÁNDEZ DE MERA, I. 2013. Prevalence of tick-borne pathogens in adult *Dermacentor* spp. ticks from nine collection sites in France. *Vector-Borne and Zoonotic Diseases,* 13 (4): 226-236.

BÖSE, R. JORGENSEN, W.K. DALGLIESH, R.J. FRIEDHOFF, K.T. & DE VOS, A.J. 1995. Current state and future trends in the diagnosis of babesiosis. *Veterinary Parasitology*, 57 (1-3): .61-74.

BRAHMBHATT, D.P. FOSGATE, G.T. DYASON, E. BUDKE, C.M. GUMMOW, B. JORI, F. WARD, M.P. & SRINIVASAN, R. 2012. Contacts between domestic livestock and wildlife at the Kruger National Park Interface of the Republic of South Africa. *Preventive Veterinary Medicine,* 103 (1): 16-21.

BROWN, W.C. NORIMINE, J. KNOWLES, D.P. & GOFF, W.L. 2006. Immune control of *Babesia bovis* infection. *Veterinary Parasitology,* 138 (1–2): 75-87.

BRYSON, N.R. TICE, G.A. HORAK, I.G. STEWART, C.G. & DU PLESSIS, B.J.A. 2002. Ixodid ticks on cattle belonging to small-scale farmers at 4 communal grazing areas in South Africa. *Journal of the South African Veterinary Association*, 73 (3): 98-103.

BUCZEK, A. BARTOSIK, K. & KUCZYNSKI, P. 2014. Comparison of the toxic effect of pyrethroids on Ixodes ricinus and Dermacentor reticulatus females. *Annals of Agricultural and Environmental Medicine*, 21 (2): 263–266.

BUCZEK, A. LACHOWSKA-KOTOWSKA, P. & BARTOSIK, K. 2015. The effect of synthetic pyrethroids on the attachment and host-feeding behaviour in *Dermacentor reticulatus* females (Ixodida: *Amblyommidae*). *Parasites & Vectors*, 8 (1): 366.

BULING, A. CRIADO-FORNELIO, A. ASENZO, G. BENITEZ, D. BARBA-CARRETERO, J. & FLORIN-CHRISTENSEN, M. 2007. A quantitative PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina*. *Veterinary Parasitology,* 147 (1): 16-25.

BURRIDGE, M. BROWN, C. & KIMBER, C. 1974. *Theileria annulata*: cross-reactions between a cell culture schizont antigen and antigens of East African species in the indirect fluorescent antibody test. *Experimental Parasitology,* 35 (3): 374-380.

BURRIDGE, M.J. & KIMBER, C.D. 1972. The indirect fluorescent antibody test for experimental East Coast fever (*Theileria parva* infection of cattle). Evaluation of a cell culture schizont antigen. *Research in Veterinary Science* 13: 4511–15.

BYARUHANGA, C. COLLINS, N.E. KNOBEL, D. CHAISI, M.E. VORSTER, I. STEYN, H.C. & OOSTHUIZEN, M.C. 2016. Molecular investigation of tick-borne haemoparasite infections among transhumant zebu cattle in Karamoja Region, Uganda. *Veterinary Parasitology: Regional Studies and Reports,* 3: 27-35.

CALDER, J.A. REDDY, G.R. CHIEVES, L. COURTNEY, C.H. LITTELL, R. LIVENGOOD, J.R. NORVAL, R.A. SMITH, C. & DAME, J.B. 1996. Monitoring *Babesia bovis* infections in cattle by using PCR-based tests. *Journal of Clinical Microbiology,* 34 (11): 2748-2755.

CANGI, N. PINARELLO, V. BOURNEZ, L. LEFRANÇOIS, T. ALBINA, E. NEVES, L. & VACHIÉRY, N. 2017. Efficient high-throughput molecular method to detect *Ehrlichia ruminantium* in ticks. *Parasites & Vectors*, 10 (1): 566.

CARELLI, G. DECARO, N. LORUSSO, A. ELIA, G. LORUSSO, E. MARI, V. CECI, L. & BUONAVOGLIA, C. 2007. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. *Veterinary Microbiology,* 124 (1-2): 107-114.

CARELLI, G. DECARO, N. LORUSSO, E. PARADIES, P. ELIA, G. MARTELLA, V. BUONAVOGLIA, C. & CECI, L. 2008. First report of bovine anaplasmosis caused by *Anaplasma centrale* in Europe. *Annals of the New York Academy of Sciences,* 1149 (1): 107-110.

CARON, A. MIGUEL, E. GOMO, C. MAKAYA, P. PFUKENYI, D.M. FOGGIN, C. HOVE, T. & DE GARINE-WICHATITSKY, M. 2013. Relationship between burden of infection in ungulate populations and wildlife/livestock interfaces. *Epidemiology and Infection,* 141 (07): 1522-1535.

CAWDERY, M.H., SIMMONS, D.J.C., MCANULTY, E.G. AND ROSS, H.M., 1968. *Theileria parva*: Possible serological test for East Coast fever. *Experimental Parasitology*, 23 (2): 234-237.

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

CHAISI, M.E. COLLINS, N.E. & OOSTHUIZEN, M.C. 2014. Phylogeny of *Theileria buffeli* genotypes identified in the South African buffalo (*Syncerus caffer*) population. *Veterinary Parasitology,* 204 (3): 87-95.

CHAISI, M.E. JANSSENS, M.E. VERMEIREN, L. OOSTHUIZEN, M.C. COLLINS, N.E. & GEYSEN, D. 2013. Evaluation of a real-time PCR test for the detection and discrimination of *Theileria* species in the African buffalo (*Syncerus caffer*). *PloS One,* 8 (10): e75827.

CHAISI, M.E. SIBEKO, K.P. COLLINS, N.E. POTGIETER, F.T. & OOSTHUIZEN, M.C. 2011. Identification of *Theileria parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequence variants in the African Buffalo (*Syncerus caffer*) in southern Africa. *Veterinary Parasitology,* 182 (2-4): 150-162.

CHOOPA, C.N. 2016. Diagnosis of tick-borne diseases in cattle in Bushbuckridge Mpumalanga South Africa and identification of *Theileria parva* carriers. MSc (Veterinary Science) Dissertation. University of Pretoria, Pretoria, South Africa.

COETZEE, J.F. APLEY, M.D. & KOCAN, K.M. 2006. Comparison of the efficacy of enrofloxacin, imidocarb, and oxytetracycline for clearance of persistent *Anaplasma marginale* infections in cattle. *Veterinary Therapeutics*, 7 (4): 347.

COETZEE, J.F. APLEY, M.D. KOCAN, K.M. RURANGIRWA, F.R. & VAN DONKERSGOED, J. 2005. Comparison of three oxytetracycline regimens for the treatment of persistent Anaplasma marginale infections in beef cattle. *Veterinary Parasitology*, 127 (1): 61-73.

COETZER, J.A.W. THOMSON, G.R. & TUSTIN, R.C. 1994. Infectious disease of livestock with special reference to Southern Africa, p460-475. In African horsesickness. Oxford University Press Oxford, United Kingdom.

COLEMAN, P.G. PERRY, B.D. & WOOLHOUSE, M.E.J. 2001. Endemic stability—a veterinary idea applied to human public health. *The Lancet*, 357 (9264): 1284-1286.

COLLINS, N. & ALLSOPP, B. 1999. *Theileria parva* ribosomal internal transcribed spacer sequences exhibit extensive polymorphism and mosaic evolution: application to the characterization of parasites from cattle and buffalo. *Parasitology,* 118 (06): 541-551.

COLLINS, N.E. ALLSOPP, M.T. & ALLSOPP, B.A. 2002. Molecular diagnosis of theileriosis and heartwater in bovines in Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 96 (Supplement 1): S217-S224.

COLLINS, N.E. LIEBENBERG, J. DE VILLIERS, E.P. BRAYTON, K.A. LOUW, E. PRETORIUS, A. FABER, F.E. VAN HEERDEN, H. JOSEMANS, A. VAN KLEEF, M. STEYN, H.C. VAN STRIJP, M.F. ZWEYGARTH, E. JONGEJAN, F. MAILLARD, J.C. BERTHIER, D. BOTHA, M. JOUBERT, F. CORTON, C.H. THOMSON, N.R. ALLSOPP, M.T. & ALLSOPP, B.A. 2005. The genome of the heartwater agent *Ehrlichia ruminantium* contains multiple tandem repeats of actively variable copy number. *Proceedings of the National Academy of Sciences of the United States of America,* 102 (3): 838-843.

CONNELL, M. & Hall, W.T.K. 1972. Transmission of *Anaplasma marginale* the cattle tick *Boophilus microplus*. *Australian Veterinary Journal*, 48 (8): 477-477.

CONNELL, M.L. 1974. Transmission of *Anaplasma marginale* by the cattle tick *Boophilus microplus*. *Queensland Journal of Agricultural and Animal Sciences*, 31 (3): 185-193.

COSTA, S.C.L. DE MAGALHÃES, V.C.S. DE OLIVEIRA, U.V. CARVALHO, F.S. DE ALMEIDA, C.P. MACHADO, R.Z. & MUNHOZ, A.D. 2016. Transplacental transmission of bovine tick-borne pathogens: Frequency, co-infections and fatal neonatal anaplasmosis in a region of enzootic stability in the northeast of Brazil. *Ticks and Tick-borne Diseases,* 7 (2): 270-275.

COWDRY, E.V. 1925a. Studies on the Etiology of Heartwater : I. Observation of a Rickettsia, Rickettsia Ruminantium (N. Sp.), in the Tissues of Infected Animals. *The Journal of experimental medicine,* 42 (2): 231-252.

COWDRY, E.V. 1925b. Studies on the Etiology of Heartwater : Ii. Rickettsia Ruminantium (N. Sp.) in the Tissues of Ticks Transmitting the Disease. *The Journal of Experimental Medicine,* 42 (2): 253-274.

CRIADO-FORNELIO, A. 2007. A review of nucleic acid-based diagnostic tests for *Babesia* and *Theileria*, with emphasis on bovine piroplasms. *Parassitologia*, 49: 39.

CROWDER, C.D. ROUNDS, M.A. PHILLIPSON, C.A. PICURI, J.M. MATTHEWS, H.E. HALVERSON, J. SCHUTZER, S.E. ECKER, D.J. & ESHOO, M.W. 2010. Extraction of total nucleic acids from ticks for the detection of bacterial and viral pathogens. *Journal of Medical Entomology,* 47 (1): 89-94.

DAHMANI, M. LOUDAHI, A. MEDIANNIKOV, O. FENOLLAR, F. RAOULT, D. & DAVOUST, B. 2015. Molecular detection of *Anaplasma platys* and *Ehrlichia canis* in dogs from Kabylie, Algeria. *Ticks and Tick-Borne Diseases*, 6 (2): 198-203.

DALGLEISH, R.J., STEWART, N.P. AND CALLOW, L.L., 1978. Transmission of Babesia bigemina by transfer of adult male *Boophilus microplus* [cattle tick]. Letter to the editor. *Australian Veterinary Journal*, 54: 205–206.

DANTAS-TORRES, F. 2010. Biology and ecology of the brown dog tick, *Rhipicephalus sanguineus*. *Parasites and Vectors*, *3* (1): 26.

DE CASTRO, J. JAMES, A. MINJAUW, B. DI GIULIO, G. PERMIN, A. PEGRAM, R. CHIZYUKA, G. & SINYANGWE, P. 1997. Long-term studies on the economic impact of ticks on Sanga cattle in Zambia. *Experimental and Applied Acarology,* 21 (1): 3-19.

DE GARINE-WICHATITSKY, M. MIGUEL, E. MUKAMURI, B. GARINE-WICHATITSKY, E. WENCELIUS, J. PFUKENYI, D.M. & CARON, A. 2013. Coexisting with wildlife in transfrontier conservation areas in Zimbabwe: Cattle owners' awareness of disease risks and perceptions of the role played by wildlife. *Comparative Immunology, Microbiology and Infectious Diseases,* 36 (3): 321-332.

DE LA FUENTE, J. TORINA, A. CARACAPPA, S. TUMINO, G. FURLÁ, R. ALMAZÁN, C. & KOCAN, K.M. 2005. Serologic and molecular characterization of *Anaplasma* species infection in farm animals and ticks from Sicily. *Veterinary Parasitology,* 133 (4): 357-362.

DE LA FUENTE, J. TORINA, A. NARANJO, V. NICOSIA, S. ALONGI, A. LA MANTIA, F. & KOCAN, K.M. 2006. Molecular characterization of *Anaplasma platys* strains from dogs in Sicily, Italy. *BMC Veterinary Research,* 2 (1): 24.

DE LA FUENTE, J. VAN DEN BUSSCHE, R.A. & KOCAN, K.M. 2001. Molecular phylogeny and biogeography of North American isolates of *Anaplasma marginale* (Rickettsiaceae: *Ehrlichieae*). *Veterinary Parasitology*, 97 (1): 65-76.

DE LA FUENTE, J., ESTRADA-PENA, A., VENZAL, J.M., KOCAN, K.M. AND SONENSHINE, D.E., 2008. Overview: ticks as vectors of pathogens that cause disease in humans and animals. *Front Biosci*, 13 (13): 6938-6946.

DE VOS, A. & POTGIETER, F. 1994. Bovine babesiosis, p412-422. In Manual of Standards for Diagnostic Tests and Vaccines, Fourth Edition, Paris, Office International des Epizootic.

De VOS, A.J. & BOCK, R.E. 2000. Vaccination against bovine babesiosis. *Annals of the New York Academy of Sciences,* 916 (1): 540-545.

DE VOS, A.J. & ROOS, J.A. 1981. The isolation of *Theileria*? *taurotragi* in South Africa. *Onderstepoort Journal of Veterinary Research*, 48: 149-153.

DE VOS, A.J. 1979. Epidemiology and control of bovine babesiosis in South Africa. *Journal of the South African Veterinary Association*, 50 (4): 357-362.

DE WAAL,D. 2000. Anaplasmosis Control and Diagnosis in South Africa. *Annals of the New York Academy of Sciences,* 916 (1): 474-483.

DEBEILA, E.M. 2012. Occurrence of *Anaplasma* and *Ehrlichia* species in African buffalo (Syncerus caffer) in Kruger National Park and Hluhluwe-iMfolozi Park in South Africa. MSc (Veterinary Science) Dissertation. University of Pretoria, Pretoria, South Africa.

DECARO, N. CARELLI, G. LORUSSO, E. LUCENTE, M.S. GRECO, G. LORUSSO, A. RADOGNA, A. CECI, L. & BUONAVOGLIA, C. 2008. Duplex real-time polymerase chain reaction for simultaneous detection and quantification of *Anaplasma marginale* and *Anaplasma centrale*. *Journal of Vveterinary Ddiagnostic Iinvestigation,* 20 (5): 606-611.

DEEM, S. NORVAL, R. DONACHIE, P. & MAHAN, S. 1996. Demonstration of vertical transmission of *Cowdria ruminantium*, the causative agent of heartwater, from cows to their calves. *Veterinary Parasitology,* 61 (1-2): 119-132.

DOLAN, T. 1989. Theileriosis: a comprehensive review. *Revue Scientifique et Technique, Office International des Epizooties*. 8: 11-36.

DOLAN, T.T. 1981. Progress in the chemotherapy of theileriosis, p186-208. In Advances in the Control of Theileriosis. Springer, Dordrecht.

DOLAN, T.T. 1986. Chemotherapy of East Coast fever. Treatment of infections induced by isolates of *Theileria parva* with halofuginone. *Acta Tropica*, 43 (2): 165-173.

DOLAN, T.T. 1999. Dogmas and misunderstandings in East Coast fever. *Tropical Medicine & International Health,* 4 (9): A3-A11

D'OLIVEIRA, C. VAN DER WEIDE, M. HABELA, M.A. JACQUIET, P. & JONGEJAN, F. 1995. Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. *Journal of Clinical Microbiology,* 33 (10): 2665-2669.

DUFFUS, W.P.H. & WAGNER, G.G. 1974. Immunochemical studies on East Coast fever: III. Development of an indirect hemagglutination assay using *Theileria parva* piroplasm antigen. *Parasitology*. 60: 860-865

DUMLER, J.S. BARBET, A.F. BEKKER, C.P. DASCH, G.A. PALMER, G.H. RAY, S.C. RIKIHISA, Y. & RURANGIRWA, F.R. 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *International Journal of Systematic and Evolutionary Microbiology,* 51 (Pt 6): 2145-2165.

DUMLER, J.S. CHOI, K.S. GARCIA-GARCIA, J.C. BARAT, N.S. SCORPIO, D.G. GARYU, J.W. GRAB, D.J. & BAKKEN, J.S. 2005. Human granulocytic anaplasmosis and *Anaplasma phagocytophilum*. *Emerging Infectious Diseases,* 11 (12): 1828-1834.

ERIKS, I.S. PALMER, G.H. MCGUIRE, T.C. ALLRED, D.R. & BARBET, A.F. 1989. Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. *Journal of Clinical Microbiology*, 27(2): 279-284.

ERIKS, I.S. STILLER, D. & PALMER, G.H. 1993. Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. *Journal of Clinical Microbiology,* 31 (8): 2091-2096.

EYGELAAR, D. JORI, F. MOKOPASETSO, M. SIBEKO, K.P. COLLINS, N.E. VORSTER, I. TROSKIE, M. & OOSTHUIZEN, M.C. 2015. Tick-borne haemoparasites in African buffalo (*Syncerus caffer*) from two wildlife areas in Northern Botswana. *Parasites and Vectors,* 8 (1): 26.

FAHRIMAL, Y. GOFF, W.L. & JASMER, D.P. 1992. Detection of *Babesia bovis* carrier cattle using polymerase chain reaction amplification of parasite DNA. *Journal of Clinical Microbiology*, 30:1374-1379.

FIGUEROA, J.V. & BUENING, G.M. 1995. Nucleic acid probes as a diagnostic method for tickborne haemoparasites of veterinary importance. *Veterinary Parasitology*, 57 (1-3): 75-92.

FOURIE, L.J. & HORAK, I.G. 1990. Parasites of cattle in the south western Orange Free State. *Journal of the South African Veterinary Association*, 61 (1): 27-28.

FUJINAGA, T. MINAMI, T. & ISHIHARA, T., 1980. Serological relationship between a large *Babesia* found in Japanese cattle and *Babesia* major, *B*. *bigemina* and *B*. *bovis*. *Research in Veterinary Science*, 29 (2): 230-234.

GAFF, H.D. & GROSS, L.J. 2007. Modeling tick-borne disease: a metapopulation model. *Bulletin of Mathematical Biology,* 69 (1): 265-288.

GALE, K.R. DIMMOCK, C.M. GARTSIDE, M. & LEATCH, G. 1996. *Anaplasma marginale*: detection of carrier cattle by PCR-ELISA. *International Journal for Parasitology*, 26 (10): 1103-1109.

GEORGES, K. LORIA, G. RIILI, S. GRECO, A. CARACAPPA, S. JONGEJAN, F. & SPARAGANO, O. 2001. Detection of haemoparasites in cattle by reverse line blot hybridisation with a note on the distribution of ticks in Sicily. *Veterinary Parasitology,* 99 (4): 273-286.

GHARBI, M. & DARGHOUTH, M.A. 2015. Control of tropical theileriosis (*Theileria annulata* infection in cattle) in North Africa. *Asian Pacific Journal of Tropical Disease,* 5 (7): 505-510.

GOFF, W.L. JOHNSON, W.C. PARISH, S.M. BARRINGTON, G.M. TUO, W. & VALDEZ, R.A. 2001. The age-related immunity in cattle to *Babesia bovis* infection involves the rapid induction of interleukin-12, interferon-γ and inducible nitric oxide synthase mRNA expression in the spleen. *Parasite Immunology,* 23 (9): 463-471.

GOFF, W.L. STILLER, D. ROEDER, R.A. JOHNSON, L.W. FALK, D. GORHAM, J.R. & MCGUIRE, T.C. 1990. Comparison of a DNA probe, complement-fixation and indirect immunofluorescence tests for diagnosing *Anaplasma marginale* in suspected carrier cattle. *Veterinary Microbiology*, 24 (3-4): 381-390.

GRAY, J.S. 2006. Identity of the causal agents of human babesiosis in Europe. *International Journal of Medical Microbiology,* 296: 131-136.

GUBBELS, J.M. DE VOS, A.P. VAN DER WEIDE, M. VISERAS, J. SCHOULS, L.M. DE VRIES, E. & JONGEJAN, F. 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. *Journal of Clinical Microbiology,* 37 (6): 1782-1789.

GUBBELS, M. HONG, Y. VAN DER WEIDE, M. QI, B. NIJMAN, I.J. GUANGYUAN, L. & JONGEJAN, F. 2000. Molecular characterisation of the *Theileria buffeli/orientalis* group. *International Journal for Parasitology,* 30 (8): 943-952.

GUIMARAES, A.M. LIMA, J.D. & RIBEIRO, M.F.B. 1998. Sporogony and experimental transmission of *Babesia equi* by *Boophilus microplus*. *Parasitology Research*, 84 (4): 323-327.

GUIMARAES, A.M. LIMA, J.D. RIBEIRO, M.F.B. CAMARGOS, E.R.S. & BOZZI, I.A. 1997. Ultrastructure of sporogony in *Babesia equi* in salivary glands of adult female *Boophilus microplus* ticks. *Parasitology Research*, 84 (1): 69-74.

GUL, N. AYAZ, S. GUL, I. ADNAN, M. SHAMS, S. & UL AKBAR, N. 2015. Tropical Theileriosis and East Coast Fever in Cattle: Present, Past and Future Perspective. *International Journal of Current Microbiology and Applied Sciences,* 4 (8): 1000-1018.

HAILEMARIAM, Z. AHMED, J.S. CLAUSEN, P. & NIJHOF, A.M. 2017. A comparison of DNA extraction protocols from blood spotted on FTA cards for the detection of tick-borne pathogens by Reverse Line Blot hybridization. *Ticks and Tick-borne Diseases,* 8 (1): 185-189.

HAILEMARIAM, Z. KRÃCKEN, J. BAUMANN, M. AHMED, J.S. CLAUSEN, P. & NIJHOF, A.M. 2017. Molecular detection of tick-borne pathogens in cattle from Southwestern Ethiopia. *Plos One,* 12 (11): e0188248.

HAMMAC, G.K. KU, P. GALLETTI, M.F. NOH, S.M. SCOLES, G.A. PALMER, G.H. & BRAYTON, K.A. 2013. Protective immunity induced by immunization with a live, cultured *Anaplasma marginale* strain. *Vaccine,* 31 (35): 3617-3622.

HARRISON, A. BASTOS, A.D. MEDGER, K. & BENNETT, N.C. 2013. Eastern rock sengis as reservoir hosts of *Anaplasma bovis* in South Africa. *Ticks and Tick-borne Diseases*, 4 (6): 503-505.

HARRUS, S. PERLMAN-AVRAHAMI, A. MUMCUOGLU, K. MORICK, D. EYAL, O. & BANETH, G. 2011. Molecular detection of *Ehrlichia canis*, *Anaplasma bovis*, *Anaplasma platys*, *Candidatus Midichloria mitochondrii* and *Babesia canis vogeli* in ticks from Israel. *Clinical Microbiology and Infection,* 17 (3): 459-463.

HAYASHIDA, K. HARA, Y. ABE, T. YAMASAKI, C. TOYODA, A. KOSUGE, T. SUZUKI, Y. SATO, Y. KAWASHIMA, S. KATAYAMA, T. WAKAGURI, H. INOUE, N. HOMMA, K. TADA-UMEZAKI, M. YAGI, Y. FUJII, Y. HABARA, T. KANEHISA, M. WATANABE, H. ITO, K. GOJOBORI, T. SUGAWARA, H. IMANISHI, T. WEIR, W. GARDNER, M. PAIN, A. SHIELS, B. HATTORI, M. NENE, V. & SUGIMOTO, C. 2012. Comparative genome analysis of three eukaryotic parasites with differing abilities to transform leukocytes reveals key mediators of *Theileria*-induced leukocyte transformation. *mBio,* 3 (5): e00204-12.

HENRICHS, B. OOSTHUIZEN, M.C. TROSKIE, M. GORSICH, E. GONDHALEKAR, C. BEECHLER, B.R. EZENWA, V.O. & JOLLES, A.E. 2016. Within guild co-infections influence parasite community membership: a longitudinal study in African Buffalo. *Journal of Animal Ecology,* 85 (4): 1025-1034.

HOMER, M.J. AGUILAR-DELFIN, I. TELFORD, S.R.,3RD KRAUSE, P.J. & PERSING, D.H. 2000. Babesiosis. *Clinical Microbiology Reviews,* 13 (3): 451-469.

HORAK, I.G. 1999. Parasites of domestic and wild animals in South Africa. XXXVII. Ixodid ticks on cattle on Kikuyu grass pastures and in Valley Bushveld in the Eastern Cape Province. *Onderstepoort Journal of Veterinary Research*, 66: 175-184.

HORAK, I.G. ANTHONISSEN, M. KRECEK, R.C. & BOOMKER, J. 1992. Arthropod parasites of springbok, gemsbok, kudus, giraffes and Burchell's and Hartmann's zebras in the Etosha and Hardap Nature Reserves, Namibia. *Onderstepoort Journal of Veterinary Research*, 59: 253-253.

HORAK, I.G. BOOMKER, J. & FLAMAND, J.R.B. 1995. Parasites of domestic and wild animals in South Africa. XXXIV. Arthropod parasites of nyalas in north-eastern KwaZulu-Natal. *Onderstepoort Journal of Veterinary Research*, 62: 171-179.

HORAK, I.G. JORDAAN, A.J. NEL, P.J. VAN HEERDEN, J. HEYNE, H. & VAN DALEN, E.M. 2015. Distribution of endemic and introduced tick species in Free State Province, South Africa. *Journal of the South African Veterinary Association,* 86 (1): 01-09.

HORAK, I.G. POTGIETER, F.T. WALKER, J.B. DE VOS, V. & BOOMKER, J. 1983. The ixodid tick burdens of various large ruminant species in South African nature reserves. *Onderstepoort Journal of Veterinary Research*, 50 (3): 221-228.

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

HUANG, H. UNVER, A. PEREZ, M.J. ORELLANA, N.G & RIKIHISA, Y. 2005. Prevalence and molecular analysis of *Anaplasma platys* in dogs in Lara, Venezuela. *Brazilian Journal of Microbiology*, 36 (3): 211-216.

HUNFELD, K. HILDEBRANDT, A. & GRAY, J. 2008. Babesiosis: recent insights into an ancient disease. *International Journal for Parasitology,* 38 (11): 1219-1237.

INOKUMA, H. OYAMADA, M. KELLY, P.J. JACOBSON, L.A. FOURNIER, P.E. ITAMOTO, K. OKUDA, M. & BROUQUI, P. 2005. Molecular detection of a new *Anaplasma* species closely related to *Anaplasma phagocytophilum* in canine blood from South Africa. *Journal of Clinical Microbiology,* 43 (6): 2934-2937.

INOKUMA, H. RAOULT, D. & BROUQUI, P. 2000. Detection of *Ehrlichia platys* DNA in brown dog ticks (*Rhipicephalus sanguineus*) in Okinawa Island, Japan. *Journal of Clinical Microbiology,* 38 (11): 4219-4221.

JENKINS, M.C. 2001. Advances and prospects for subunit vaccines against protozoa of veterinary importance. *Veterinary Parasitology,* 101 (3-4): 291-310.

JOAZEIRO, A.C. MARTINS, J. MASUDA, A. SEIXAS, A. & DA SILVA VAZ JUNIOR, ITABAJARA. 2015. A PCR for Differentiate between *Anaplasma marginale* and *A. centrale*. *Acta Scientiae Veterinariae,* 43:1270.

JONGEJAN, F. & UILENBERG, G. 2004. The global importance of ticks. *Parasitology,* 129 (S1): S3-S14.

JONGEJAN, F. MUSISI, F. MOORHOUSE, P. SNACKEN, M. & UILENBERG, G. 1986. *Theileria taurotragi* in Zambia. *Veterinary Quarterly,* 8 (3): 261-263.

JONGEJAN, F. NENE, V. DE LA FUENTE, J. PAIN, A. & WILLADSEN, P. 2007. Advances in the genomics of ticks and tick-borne pathogens. *Trends in Parasitology,* 23 (9): 391-396.

JONSSON, N.N. BOCK, R.E. JORGENSEN, W.K. MORTON, J.M. & STEAR, M.J. 2012. Is endemic stability of tick-borne disease in cattle a useful concept? *Trends in Parasitology,* 28 (3): 85-89.

JORI, F. BRAHMBHATT, D. FOSGATE, G.T. THOMPSON, P.N. BUDKE, C. WARD, M.P. FERGUSON, K. & GUMMOW, B. 2011. A questionnaire-based evaluation of the veterinary cordon fence separating wildlife and livestock along the boundary of the Kruger National Park, South Africa. *Preventive Veterinary Medicine,* 100 (3–4): 210-220.

KATENDE, J. MORZARIA, S. TOYE, P. SKILTON, R. NENE, V. NKONGE, C. & MUSOKE, A. 1998. An enzyme-linked immunosorbent assay for detection of *Theileria parva* antibodies in cattle using a recombinant polymorphic immunodominant molecule. *Parasitology Research*, 84 (5): 408-416.

KAWAHARA, M. RIKIHISA, Y. LIN, Q. ISOGAI, E. TAHARA, K. ITAGAKI, A. HIRAMITSU, Y. & TAJIMA, T. 2006. Novel genetic variants of *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma centrale*, and a novel *Ehrlichia* sp. in wild deer and ticks on two major islands in Japan. *Applied and Environmental Microbiology,* 72 (2): 1102-1109.

KHUMALO, Z.T. BRAYTON, K.A. COLLINS, N.E. CHAISI, M.E. QUAN, M. & OOSTHUIZEN, M.C. 2018. Evidence confirming the phylogenetic position of *Anaplasma centrale* (ex Theiler 1911) Ristic and Kreier 1984. *International Journal of Systematic and Evolutionary Microbiology*. 68 (2018): 2682-2691.

KHUMALO, Z.T. CATANESE, H.N. LIESCHING, N. HOVE, P. COLLINS, N.E. CHAISI, M.E. GEBREMEDHIN, A.H. OOSTHUIZEN, M.C. & BRAYTON, K.A. 2016. Characterization of *Anaplasma marginale* subsp. *centrale* Strains by Use of msp1aS Genotyping Reveals a Wildlife Reservoir. *Journal of Clinical Microbiology,* 54 (10): 2503-2512.

KIM, C. ISEKI, H. HERBAS, M.S. YOKOYAMA, N. SUZUKI, H. XUAN, X. FUJISAKI, K. & IGARASHI, I. 2007. Development of TaqMan-based real-time PCR assays for diagnostic detection of *Babesia bovis* and *Babesia bigemina*. *The American Journal of Tropical Medicine and Hygiene*, 77 (5): 837-841.

KIVARIA, F. HEUER, C. JONGEJAN, F. OKELLO-ONEN, J. RUTAGWENDA, T. UNGER, F. & BOEHLE, W. 2004. Endemic stability for *Theileria parva* infections in Ankole calves of the Ankole ranching scheme, Uganda. *Onderstepoort Journal of Veterinary Research,* 71 (3): 189-195.

KOCAN, K. DE LA FUENTE, J. BLOUIN, E. & GARCIA-GARCIA, J. 2004. *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host–pathogen adaptations of a tick-borne rickettsia. *Parasitology,* 129 (S1): S285-S300.

KOCAN, K.M. BLOUIN, E.F. & BARBET, A.F. 2000. Anaplasmosis control: past, present, and future. *Annals of the New York Academy of Sciences,* 916 (1): 501-509.

KOCAN, K.M. DE LA FUENTE, J. GUGLIELMONE, A.A. & MELENDEZ, R.D. 2003. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clinical Microbiology Reviews,* 16 (4): 698-712.

KOLO, A.O. SIBEKO-MATJILA, K.P. MAINA, A.N. RICHARDS, A.L. KNOBEL, D.L. & MATJILA, P.T. 2016. Molecular detection of zoonotic rickettsiae and *Anaplasma* spp. in domestic dogs and their ectoparasites in Bushbuckridge, South Africa. *Vector-Borne and Zoonotic Diseases*, 16 (4): 245-252.

KRYSTYNAK, R. 1986. A benefit-cost analysis of the programme to eradicate an outbreak of anaplasmosis in Canada. *Proceedings of the Fourth International Symposium on Veterinary Epidemiology and Economics*, 18-27 November 1985. Singapore.

KUMAR, S., KUMAR, R. AND SUGIMOTO, C., 2009. A perspective on Theileria equi infections in donkeys. *Japanese Journal of Veterinary Research*, 56 (4): 171-180.

KUTTLER, K. 1980. Pharmacotherapeutics of drugs used in treatment of anaplasmosis and babesiosis. *Journal of the American Veterinary Medical Association,* 176 (10 Spec No): 1103-1108.

LAWRENCE, J. DE VOS, A. IRVIN, A. & COETZER, J. 1994. Zimbabwe theileriosis, p329-330. In Infectious Diseases of Livestock with Special Reference to Southern Africa*,* Second Edition, Oxford University Press, Southern Africa.

LAWRENCE, J.A. NORVAL, R.A.I. & UILENBERG, G. 1983. Rhipicephalus zambeziensis as a vector of bovine *Theileriae*. *Tropical Animal Health and Production*, 15 (1): 39-42.

LEGER, E. VOURE'H, G. VIAL, L. CHEVILLON, C. & MCCOY, K.D. 2013. Changing distributions of ticks: causes and consequences. *Experimental and Applied Acarology,* 59 (1): 219-244.

LEMPEREUR, L. GEYSEN, D. & MADDER, M. 2010. Development and validation of a PCR–RFLP test to identify African *Rhipicephalus* (*Boophilus*) ticks. *Acta Tropica,* 114 (1): 55-58.

LEW, A.E. BOCK, R.E. MINCHIN, C.M. & MASAKA, S. 2002. A *msp1α* polymerase chain reaction assay for specific detection and differentiation of *Anaplasma marginale* isolates. *Veterinary Microbiology*, 86 (4): 325-335.

LIU, Z. MA, M. WANG, Z. WANG, J. PENG, Y. LI, Y. GUAN, G. LUO, J. & YIN, H. 2012. Molecular survey and genetic identification of *Anaplasma* species in goats from central and southern China. *Applied and Environmental Microbiology,* 78 (2): 464-470.

LOFTIS, A.D. REEVES, W.K. SPURLOCK, J.P. MAHAN, S.M. TROUGHTON, D.R. DASCH, G.A. & LEVIN, M.L. 2006. Infection of a goat with a tick-transmitted *Ehrlichia* from Georgia, USA that is closely related to *Ehrlichia ruminantium*. *Journal of Vector Ecology*, 31 (2): 213-223.

M'GHIRBI, Y. YAÏCH, H. GHORBEL, A. & BOUATTOUR, A. 2012. *Anaplasma phagocytophilum* in horses and ticks in Tunisia. *Parasites and Vectors*, 5 (1): 180.

MADDER, M. ADEHAN, S. DE DEKEN, R. ADEHAN, R. & LOKOSSOU, R. 2012. New foci of *Rhipicephalus microplus* in West Africa. *Experimental and Applied Acarology*, 56 (4): 385-390.

MAKALA, L.H., MANGANI, P., FUJISAKI, K. & NAGASAWA, H. 2003. The current status of major tick borne diseases in Zambia. *Veterinary Research*, 34: 27-45.

MANS, B.J. PIENAAR, R. & LATIF, A.A. 2015. A review of *Theileria* diagnostics and epidemiology. *International Journal for Parasitology: Parasites and Wildlife,* 4 (1): 104-118.

MARCELINO, I. DE ALMEIDA, A.M. VENTOSA, M. PRUNEAU, L. MEYER, D.F. MARTINEZ, D. LEFRANÇOIS, T. VACHIÉRY, N. & COELHO, A.V. 2012. Tick-borne diseases in cattle: Applications of proteomics to develop new generation vaccines. *Journal of Proteomics,* 75 (14): 4232-4250.

MARCELINO, I. VERÍSSIMO, C. SOUSA, M.F. CARRONDO, M.J. & ALVES, P.M. 2005. Characterization of *Ehrlichia ruminantium* replication and release kinetics in endothelial cell cultures. *Veterinary Microbiology,* 110 (1): 87-96.

MARTINS, T.M. NEVES, L. PEDRO, O.C. FAFETINE, J.M. DO ROSARIO, V.E. & DOMINGOS, A. 2010. Molecular detection of *Babesia* spp. and other haemoparasitic infections of cattle in Maputo Province, Mozambique. *Parasitology*, 137 (6): 939-946.

MASIKA, P. SONANDI, A. & VAN AVERBEKE, W. 1997. Tick control by small-scale cattle farmers in the central Eastern Cape Province, South Africa. *Journal of the South African Veterinary Association,* 68 (2): 45-48.

MASON, C.A. & NORVAL, R.A.I. 1981. The transfer of *Boophilus microplus* (Acarina: *Ixodidae*) from infested to uninfested cattle under field conditions. *Veterinary Parasitology*, 8 (2): 185-188.

MBASSA, G.K. BALEMBA, O. MASELLE, R.M. & MWAGA, N.V. 1994. Severe anaemia due to hematopoietic precursor cell destruction in field cases of East Coast Fever in Tanzania. *Veterinary Parasitology*, 52 (3-4):243-256.

MBATI, P.A. HLATSHWAYO, M. MTSHALI, M.S. MOGASWANE, K.R. DE WAAL, T.D. & DIPEOLU, O.O. 2003. Ticks and tick-borne diseases of livestock belonging to resource-poor farmers in the eastern Free State of South Africa, p217-224. In Ticks and Tick-Borne Pathogens: Proceedings of the 4th International Conference on Ticks and Tick-Borne Pathogens The Banff Centre Banff, Alberta, Canada 21â€“26 July 2002. Edited by F. Jongejan & W.R. Kaufman, Dordrecht: Springer Netherlands.

MBIZENI, S. POTGIETER, F.T. TROSKIE, C. MANS, B.J. PENZHORN, B.L. & LATIF, A.A. 2013. Field and laboratory studies on Corridor disease (*Theileria parva* infection) in cattle population at the livestock/game interface of uPhongolo-Mkuze area, South Africa. *Ticks and Tick-borne Diseases,* 4 (3): 227-234.

MCHARDY, N. 1979. Experimental therapy of theileriosis. *Journal of the South African Veterinary Association,* 50 (4): 321-322.

MCHARDY, N. 1984. Recent advances in the chemotherapy of theileriosis. *Preventive Veterinary Medicine,* 2 (1-4): 179-192.

MEHLHORN, H. & SCHEIN, E. 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. *Parasitology Research*, 84 (6): 467-475.

MEHLHORN, H. SCHUMACHER, B. JATZLAU, A. ABDEL-GHAFFAR, F. AL-RASHEID, K.A. KLIMPEL, S. & POHLE, H. 2011. Efficacy of deltamethrin (Butox® 7.5 pour on) against nymphs and adults of ticks (*Ixodes ricinus*, *Rhipicephalus sanguineus*) in treated hair of cattle and sheep. *Parasitology Research*, 108 (4): 963-971.

MIGUEL, E. BOULINIER, T. DE GARINE-WICHATITSKY, M. CARON, A. FRITZ, H. & GROSBOIS, V. 2014. Characterising African tick communities at a wild–domestic interface using repeated sampling protocols and models. *Acta Tropica,* 138 5-14.

MIHALCA, A.D. COZMA, V. ŞUTEU, E. MARINCULIC, A. & BOIREAU, P. 2010. The quest for piroplasms: from Babeş and Smith to molecules. *Scientia Parasitologica*, 11: 14-19.

MINJAUW, B. & MCLEOD, A. 2003. Tick-borne diseases and poverty: the impact of ticks and tick-borne diseases on the livelihoods of small-scale and marginal livestock owners in India and eastern and southern Africa. *Research Report*, DFID Animal Health Programme, Centre for Tropical Veterinary Medicine, University of Edinburgh, UK.

MOSQUEDA, J. OLVERA-RAMIREZ, A. AGUILAR-TIPACAMU, G. & J CANTO, G. 2012. Current advances in detection and treatment of babesiosis. *Current Medicinal Chemistry,* 19 (10): 1504-1518.

MTSHALI, K. NAKAO, R. SUGIMOTO, C. & THEKISOE, O. 2017. Occurrence of *Coxiella burnetii*, *Ehrlichia canis*, Rickettsia species and *Anaplasma phagocytophilum*-like bacterium in ticks collected from dogs and cats in South Africa. *Journal of the South African Veterinary Association*, 88 (1): 1-6.

MTSHALI, M. DE WAAL, D. & MBATI, P. 2004. A sero-epidemiological survey of blood parasites in cattle in the north-eastern Free State, South Africa. *Onderstepoort Journal of Veterinary Research,* 71 (1): 67-75.

MUTSHEMBELE, A.M. CABEZAS-CRUZ, A. MTSHALI, M.S. THEKISOE, O.M. GALINDO, R.C. & DE LA FUENTE, J. 2014. Epidemiology and evolution of the genetic variability of *Anaplasma marginale* in South Africa. *Ticks and Tick-Borne Diseases,* 5 (6): 624-631.

NEITZ, W. ALEXANDER, R. & ADELAAR, T. 1947. Studies on immunity in heartwater. *Onderstepoort Journal of Veterinary Research,* 21: 243-252.

NEITZ, W.O. 1935. The blesbuck (*Damaliscus albifrons*) and the black wildebeest (*Conochaetes gnu*) as carriers of heartwater. *Onderstepoort Journal of Veterinary Research,* 5: 35–40.

NEITZ, W.O. 1950. The specific action of pamaquin on the haemotropic parasites of *Theileria parva*. *South African Journal of Science*, 46: 218-219.

NEITZ, W.O. 1953. Aureomycin in *Theileria parva* infection. *Nature*, 171:34-35.

NEITZ, W.O., 1957. Theileriosis, gonderioses and cytauxzoonoses: a review. *Onderstepoort Journal of Veterinary Research,* 27: 275–430.

NIJHOF, A.M. PENZHORN, B.L. LYNEN, G. MOLLEL, J.O. MORKEL, P. BEKKER, C.P. & JONGEJAN, F. 2003. *Babesia bicornis* sp. nov. and *Theileria bicornis* sp. nov.: tick-borne parasites associated with mortality in the black rhinoceros (*Diceros bicornis*). *Journal of Clinical Microbiology,* 41 (5): 2249-2254.

NIJHOF, A.M. PILLAY, V. STEYL, J. PROZESKY, L. STOLTSZ, W.H. LAWRENCE, J.A. PENZHORN, B.L. & JONGEJAN, F. 2005. Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. *Journal of Clinical Microbiology*, 43 (12): 5907-5911.

NJIIRI, N.E. COLLINS, N.E. STEYN, H.C. TROSKIE, M. VORSTER, I. THUMBI, S. SIBEKO, K.P. JENNINGS, A. VAN WYK, I.C. & MBOLE-KARIUKI, M. 2015. The epidemiology of tick-borne haemoparasites as determined by the reverse line blot hybridization assay in an intensively studied cohort of calves in western Kenya. *Veterinary Parasitology,* 210 (1): 69-76.

NORVAL, R. ANDREW, H. & YUNKER, C. 1990. Infection rates with *Cowdria ruminantium* of nymphs and adults of the bont tick *Amblyomma hebraeum* collected in the field in Zimbabwe. *Veterinary Parasitology,* 36 (3-4): 277-283.

NORVAL, R. DONACHIE, P. MELTZER, M. DEEM, S. & MAHAN, S. 1995. The relationship between tick (*Amblyomma hebraeum*) infestation and immunity to heartwater (*Cowdria ruminantium* infection) in calves in Zimbabwe. *Veterinary Parasitology,* 58 (4): 335-352.

NORVAL, R.A.I. FIVAZ, B.H. LAWRENCE, J.A. & BROWN, A.F. 1984. Epidemiology of tick-borne diseases of cattle in Zimbabwe. II. Anaplasmosis. *Tropical Animal Health and Production*, 16 (2): 63-70.

NORVAL, R.A.I., PERRY, B.D. and YOUNG, A., 1992. The epidemiology of theileriosis in Africa*.* 1st edition*.* Academic Press, London.

NUTTALL, P. TRIMNELL, A. KAZIMIROVA, M. & LABUDA, M. 2006. Exposed and concealed antigens as vaccine targets for controlling ticks and tick-borne diseases. *Parasite Immunology,* 28 (4): 155-163.

NYANGIWE, N. HORAK, I.G. VAN DER MESCHT, L. & MATTHEE, S. 2017. Range expansion of the economically important Asiatic blue tick, *Rhipicephalus microplus*, in South Africa. *Journal of the South African Veterinary Association*, 88 (1): 1-7.

O'DOHOGHUE, P.J. FRIEDHOFF, K.T. VIZCAINO, O.G. & WEYRETER, H. 1985. The detection of IgM and IgG antibodies against *Babesia bigemina* in bovine sera using semi-defined antigens in enzyme immunoassays. *Veterinary Parasitology*, 18 (1):1-12.

ODONGO, D.O. SUNTER, J.D. KIARA, H.K. SKILTON, R.A. & BISHOP, R.P. 2010. A nested PCR assay exhibits enhanced sensitivity for detection of *Theileria parva* infections in bovine blood samples from carrier animals. *Parasitology Research, 106* (2): 357.

OGDEN, N.H. GWAKISA, P. SWAI, E. FRENCH, N.P. FITZPATRICK, J. KAMBARAGE, D. AND BRYANT, M. 2003. Evaluation of PCR to detect *Theileria parva* in field-collected tick and bovine samples in Tanzania. *Veterinary Parasitology*, 112 (3):177-183.

OLWOCH, J.M. REYERS, B. ENGELBRECHT, F.A. & ERASMUS, B.F.N. 2008. Climate change and the tick-borne disease, Theileriosis (East Coast fever) in sub-Saharan Africa. *Journal of Arid Environments*, 72 (2): 108-120.

OOSHIRO, M. ZAKIMI, S. MATSUKAWA, Y. KATAGIRI, Y. & INOKUMA, H. 2008. Detection of *Anaplasma bovis* and *Anaplasma phagocytophilum* from cattle on Yonaguni Island, Okinawa, Japan. *Veterinary Parasitology,* 154 (3): 360-364.

OURA, C. BISHOP, R. WAMPANDE, E. LUBEGA, G. & TAIT, A. 2004. Application of a reverse line blot assay to the study of haemoparasites in cattle in Uganda. *International Journal for Parasitology,* 34 (5): 603-613.

PADGETT, K.A. & LANE, R.S. 2001. Life cycle of *Ixodes pacificus* (Acari: *Ixodidae*): timing of developmental processes under field and laboratory conditions. *Journal of Medical Entomology,* 38 (5): 684-693.

PARKER, D.M. & BERNARD, R.T.F. 2005. The diet and ecological role of giraffe (*Giraffa camelopardalis*) introduced to the Eastern Cape, South Africa. *Journal of Zoology*, 267 (2): 203-210.

PAROLA, P. & RAOULT, D. 2001. Ticks and Tickborne Bacterial Diseases in Humans: An Emerging Infectious Threat. *Clinical Infectious Diseases,* 32 (6): 897-928.

PAROLA, P. CORNET, J.P. SANOGO, Y.O. MILLER, R.S. VAN THIEN, H. GONZALEZ, J.P. RAOULT, D. TELFORD III, S.R & WONGSRICHANALAI, C. 2003. Detection of *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and other eubacteria in ticks from the Thai-Myanmar border and Vietnam. *Journal of Clinical Microbiology*, 41 (4):1600-1608.

PERRY, B.D. & YOUNG, A.S. 1995. The past and future roles of epidemiology and economics in the control of tick-borne diseases of livestock in Africa: the case of theileriosis. *Preventive Veterinary Medicine,* 25 (2): 107-120.

PETER, T.F. DEEM, S.L. BARBET, A.F. NORVAL, R. SIMBI, B.H. KELLY, P.J. & MAHAN, S.M. 1995. Development and evaluation of PCR assay for detection of low levels of *Cowdria ruminantium* infection in *Amblyomma* ticks not detected by DNA probe. *Journal of Clinical Microbiology*, 33 (1): 166-172.

PFÄFFLE, M. LITTWIN, N. MUDERS, S.V. & PETNEY, T.N. 2013. The ecology of tick-borne diseases. *International Journal for Parasitology,* 43 (12–13): 1059-1077.

PFITZER, S. LAST, R. & DE WAAL, D. 2004. Possible death of a buffalo calf (*Syncerus caffer*) due to suspected heartwater (*Ehrlichia ruminantium*). *Journal of the South African Veterinary Association*, 75 (1): 54-57.

PFITZER, S. OOSTHUIZEN, M.C. BOSMAN, A. VORSTER, I. & PENZHORN, B.L. 2011. Tick-borne blood parasites in nyala (*Tragelaphus angasii*, Gray 1849) from KwaZulu-Natal, South Africa. *Veterinary Parasitology,* 176 (2): 126-131.

PIENAAR, R. POTGIETER, F.T. LATIF, A.A. THEKISOE, O.M. & MANS, B.J. 2011. Mixed *Theileria* infections in free-ranging buffalo herds: implications for diagnosing *Theileria parva* infections in Cape buffalo (*Syncerus caffer*). *Parasitology,* 138 (7): 884-895.

POTGIETER, F. & STOLTSZ, W. 1994. Bovine anaplasmosis, p408-430. In Infectious Diseases of Livestock-With Special Reference to Southern Africa. Edited by J.A.W. Coetzer, G.R. Thompson & R.C. Tustin., Oxford University Press, Cape Town, South Africa).

POTGIETER, F.T. & VAN RENSBURG, L. 1987. Tick transmission of *Anaplasma centrale*. *The Onderstepoort Journal of Veterinary Research*, *54* (1): 5-7.

POTGIETER, F.T. 1979. Epizootiology and control of anaplasmosis in South Africa. *Journal of the South African Veterinary Association,* 50 (4): 367-372.

POTGIETER, F.T. 1981. Recent findings on the transmission of anaplasmosis in cattle. South *African Journal of Science*, 77: 572.

POTGIETER, F.T. STOLTSZ, W.H. BLOUIN, E.F. & ROOS, J.A. 1988. Corridor disease in South Africa: a review of the current status. *Journal of the South African Veterinary Association,* 59 (3): 155-160.

PRESTON, P.M. 2001. Theilerioses: In: The Encyclopedia of Arthropod Transmitted infections. *The Encyclopedia of Arthropod transmitted infections,* N.W. Service. CABI Publishing, pp. 487-504.

PURNELL, R. GUNTER, T. & SCHRODER, J. 1989. Development of a prophylactic regime using long-acting tetracycline for the control of redwater and heartwater in susceptible cattle moved into an endemic area. *Tropical Animal Health and Production,* 21 (1): 11-19.

RAR, V. & GOLOVLJOVA, I. 2011. *Anaplasma*, *Ehrlichia*, and "*Candidatus Neoehrlichia*" bacteria: pathogenicity, biodiversity, and molecular genetic characteristics, a review. *Infection, Genetics and Evolution,* 11 (8): 1842-1861.

RECHAV, Y. 1982. Dynamics of tick populations (Acari: *Ixodidae*) in the Eastern Cape Province of South Africa. *Journal of Medical Entomology*, 19(6): 679-700.

REGASSA, A. PENZHORN, B. & BRYSON, N. 2003. Attainment of endemic stability to *Babesia bigemina* in cattle on a South African ranch where non-intensive tick control was applied. *Veterinary Parasitology,* 116 (4): 267-274.

RHYAN, J.C. & SPRAKER, T.R. 2010. Emergence of diseases from wildlife reservoirs. *Veterinary Pathology,* 47 (1): 34-39.

RIKHOTSO, B.O. STOLTSZ, W.H. BRYSON, N.R. & SOMMERVILLE, J.E.M. 2005. The impact of 2 dipping systems on endemic stability to bovine babesiosis and anaplasmosis in cattle in 4 communally grazed areas in Limpopo Province, South Africa. *Journal of the South African Veterinary Association*, 76 (4): 217-223.

RINGO, A.E. MOUMOUNI, P.F.A. TAIOE, M. JIRAPATTHARASATE, C. LIU, M. WANG, G. GAO, Y. GUO, H. LEE, S. & ZHENG, W. 2018. Molecular analysis of tick-borne protozoan and rickettsial pathogens in small ruminants from two South African provinces. *Parasitology International,* 67 (2): 144-149.

RISTIC, M. & KREIER, J.P. 1984. Family. III. Anaplasmataceae Philip 1957, 980AL, p 719-729. In Bergey's Manual of Systematic Bacteriology. 1. Edited by Krieg, N.R. & Holt, J. G. Baltimore, Williams & Wilkins.

RISTIC, M. 1981. Babesiosis, p443-468. In Diseases of Cattle in the Tropics. Springer, Dordrecht.

ROSS, J.P. AND LÖHR, K.F., 1972. A capillary-tube agglutination test for the detection and titration of *Theileria parva* and *Theileria mutans* antibodies in bovine serum. *Research in Veterinary Science*, 13 (5): 405-410.

RYMASZEWSKA, A. & GRENDA, S. 2008. Bacteria of the genus *Anaplasma*–characteristics of *Anaplasma* and their vectors: a review. *Veterinary Medicine,* 53 (11): 573-584.

SAHINDURAN, S., 2012. Protozoan diseases in farm ruminants, p473–497. In A Bird's-Eye View of Veterinary Medicine. Edited by Perez-Marin, C. InTech Publishers, Rijeka, Croatia

SALEM, G.H. LIU, X.J. JOHNSRUDE, J.D. DAME, J.B. & REDDY, G.R. 1999. Development and evaluation of an extra chromosomal DNA-based PCR test for diagnosing bovine babesiosis. *Molecular and Cellular Probes*, 13 (2): 107-113.

SALIH, D.A. EL HUSSEIN, A.M. AND SINGLA, L.D. 2015. Diagnostic approaches for tick-borne haemoparasitic diseases in livestock. *Journal of Veterinary Medicine and Animal Health*, *7* (2): 45-56.

SAMISH, M. PIPANO, E. & HADANI, A. 1993. Intrastadial and interstadial transmission of *Anaplasma marginale* by *Boophilus annulatus* ticks in cattle. *American Journal of Veterinary Research*, 54(3): 411-414.

SCHEIN, E. & VOIGT, W. 1979. Chemotherapy of bovine theileriosis with Halofuginone. *Acta Tropica,* 36 (4): 391-394.

SCHNITTGER, L. RODRIGUEZ, A.E. FLORIN-CHRISTENSEN, M. & MORRISON, D.A. 2012. *Babesia*: a world emerging. *Infection, Genetics and Evolution,* 12 (8): 1788-1809.

SCOLES, G.A. BROCE, A.B. LYSYK, T.J. & PALMER, G.H. 2005. Relative efficiency of biological transmission of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) by *Dermacentor andersoni* (Acari: Ixodidae) compared with mechanical transmission by *Stomoxys calcitrans* (Diptera: Muscidae). *Journal of Medical Entomology*, 42 (4): 668-675.

SCOLES, G.A. MILLER, J.A. & FOIL, L.D. 2008. Comparison of the efficiency of biological transmission of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) by *Dermacentor andersoni* Stiles (Acari: Ixodidae) with mechanical transmission by the horse fly, *Tabanus fuscicostatus* Hine (Diptera: Muscidae). *Journal of Medical Entomology*, 45 (1): 109-114.

SHKAP, V. DE VOS, A.J. ZWEYGARTH, E. & JONGEJAN, F. 2007. Attenuated vaccines for tropical theileriosis, babesiosis and heartwater: the continuing necessity. *Trends in Parasitology, 23* (9): 420-426.

SHKAP, V. KOCAN, K. MOLAD, T. MAZUZ, M. LEIBOVICH, B. KRIGEL, Y. MICHOYTCHENKO, A. BLOUIN, E. DE LA FUENTE, J. SAMISH, M. & MTSHALI, M. 2009. Experimental transmission of field *Anaplasma marginale* and the *A. centrale* vaccine strain by *Hyalomma excavatum*, *Rhipicephalus sanguineus* and *Rhipicephalus* (*Boophilus*) *annulatus* ticks. *Veterinary Microbiology*, *134* (3-4): 254-260.

SIBEKO, K.P. OOSTHUIZEN, M.C. COLLINS, N.E. GEYSEN, D. RAMBRITCH, N.E. LATIF, A.A. GROENEVELD, H.T. POTGIETER, F.T. & COETZER, J.A. 2008. Development and evaluation of a real-time polymerase chain reaction test for the detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle. *Veterinary Parasitology,* 155 (1): 37-48.

SISSON, D. HUFSCHMID, J. JOLLES, A. BEECHLER, B. & JABBAR, A. 2017. Molecular characterisation of *Anaplasma* species from African buffalo (*Syncerus caffer*) in Kruger National Park, South Africa. *Ticks and Tick-borne Diseases,* 8 (3): 400-406.

SIVAKUMAR, T. HAYASHIDA, K. SUGIMOTO, C. & YOKOYAMA, N. 2014. Evolution and genetic diversity of Theileria. *Infection, Genetics and Evolution*, 27: 250-263.

SKILTON, R.A. BISHOP, R.P. KATENDE, J.M. MWAURA, S. & MORZARIA, S.P. 2002. The persistence of *Theileria parva* infection in cattle immunized using two stocks which differ in their ability to induce a carrier state: analysis using a novel blood spot PCR assay. *Parasitology*, 124 (3): 265-276.

SMITH, E.R. & PARKER, D.M. 2010. Tick communities at the expanding wildlife/cattle interface in the Eastern Cape Province, South Africa: implications for Corridor disease. *Journal of the South African Veterinary Association-Tydskrif Van Die Suid-Afrikaanse Veterinere Vereniging,* 81 (4): 237-240.

SMITH, T. & KILBORNE, F.L. 1893. Investigations into the nature, causation, and prevention of Texas or Southern cattle tick fever, p177–304. In Bureau of Animal Industries, bulletin no. 1. U.S. Department of Agriculture, Washington, D.C.

SOUTH AFRICAN NATIONAL DEPARTMENT OF AGRICULTURE (2014). - Disease database. Available at: https://www.daff.gov.za/daffweb3/Branches/Agricultural-Production-Health-Food-Safety/Animal-Health/Epidemiology/diseasedatabase (accessed on 20 September 2018).

SPICKETT, A.M. HEYNE, I.H. & WILLIAMS, R. 2011. Survey of the livestock ticks of the North West province, South Africa. *Onderstepoort Journal of Veterinary Research*, 78 (1): 1-12.

STEYN, H.C. PRETORIUS, A. MCCRINDLE, C.M.E. STEINMANN, C.M.L. & VAN KLEEF, M. 2008. A quantitative real-time PCR assay for *Ehrlichia ruminantium* using pCS20. *Veterinary Microbiology*, 131 (3-4): 258-265.

STOLTSZ, W. 1989. Theileriosis in South Africa: a brief review. *Revue Scientifique et Technique, Office International des Epizooties,* 8 (1): 93-102.

STOLTSZ, W.H. 2012. Aspects of the epidemiology of *Theileria parva* infections in cattle and African buffalo (*Syncerus caffer*) in South Africa revealed by tick transmission and sub-inoculation of blood. MSc (Veterinary Science) Dissertation. University of Pretoria, Pretoria, South Africa

SUNGIRAI, M. MOYO, D.Z. DE CLERCQ, P. & MADDER, M. 2016. Communal farmers' perceptions of tick-borne diseases affecting cattle and investigation of tick control methods practiced in Zimbabwe. *Ticks and Tick-borne Diseases*, 7 (1): 1-9.

TELFORD III, S.R. GORENFLOT, A. BRASSEUR, P. & SPIELMAN, A. 1993. Babesial infections in humans and wildlife. *Parasitic Protozoa,* 5: 1-47.

TESHALE, S. GEYSEN, D. AMENI, G. ASFAW, Y. & BERKVENS, D. 2015. Improved molecular detection of *Ehrlichia* and *Anaplasma* species applied to *Amblyomma* ticks collected from cattle and sheep in Ethiopia. *Ticks and Tick-borne Diseases,* 6 (1): 1-7.

TESHALE, S. GEYSEN, D. AMENI, G. DORNY, P. & BERKVENS, D. 2018. Survey of *Anaplasma phagocytophilum* and *Anaplasma* sp.'Omatjenne'infection in cattle in Africa with special reference to Ethiopia. *Parasites & Vectors*, 11 (1): 162.

THEILER, A. 1911. Further investigations into anaplasmosis of South African cattle, p7-46. In First Report of the Director of Veterinary Research, Union of South Africa. Johannesburg, South Africa.

THOMPSON, B.E. LATIFA, A. OOSTHUIZEN, M.C. TROSKIE, M. & PENZHORN, B.L. 2008. Occurrence of *Theileria parva* infection in cattle on a farm in the Ladysmith district, KwaZulu-Natal, South Africa. *Journal of the South African Veterinary Association,* 79 (1): 31-35.

TONETTI, N. BERGGOETZ, M. RÜHLE, C. PRETORIUS, A. & GERN, L. 2009. Ticks and tick-borne pathogens from wildlife in the Free State Province, South Africa. *Journal of Wildlife Diseases,* 45 (2): 437-446.

TØNNESEN, M.H. PENZHORN, B.L. BRYSON, N.R. STOLTSZ, W.H. & MASIBIGIRI, T. 2004. Displacement of *Boophilus decoloratus* by *Boophilus microplus* in the Soutpansberg region, Limpopo province, South Africa. *Experimental & Applied Acarology*, 32 (3): 199-208.

UETI, M.W. KNOWLES, D.P. DAVITT, C.M. SCOLES, G.A. BASZLER, T.V. & PALMER, G.H. 2009. Quantitative differences in salivary pathogen load during tick transmission underlie strain-specific variation in transmission efficiency of *Anaplasma marginale*. *Infection and Immunity*, 77 (1): 70-75.

UETI, M.W. PALMER, G.H. KAPPMEYER, L.S. STATDFIELD, M. SCOLES, G.A. & KNOWLES, D.P. 2005. Ability of the vector tick *Boophilus microplus* to acquire and transmit *Babesia equi* following feeding on chronically infected horses with low-level parasitemia. *Journal of Clinical Microbiology*, 43 (8): 3755-3759.

UETI, M.W. REAGAN, J.O. KNOWLES, D.P. SCOLES, G.A. SHKAP, V. & PALMER, G.H. 2007. Identification of midgut and salivary glands as specific and distinct barriers to efficient tick-borne transmission of *Anaplasma marginale*. *Infection and Immunity*, 75 (6): 2959-2964.

UILENBERG, G. 1983. Heartwater (*Cowdria ruminantium* infection): current status. *Advances in Veterinary Science and Comparative Medicine,* 27: 427-480.

UILENBERG, G. 1995. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Veterinary Parasitology,* 57 (1–3): 19-41.

UILENBERG, G. 1999. Immunization against diseases caused by *Theileria parva*: a review. *Tropical Medicine and International Health,* 4 (9): A12-A20.

UILENBERG, G. 2006. *Babesia*—a historical overview. *Veterinary Parasitology*, 138 (1-2): 3-10.

UILENBERG, G. CORTEN, J. & DWINGER, R. 1982. Heartwater (*Cowdria ruminantium* infection) on Sao Tome. *Veterinary Quarterly,* 4 (3): 106-107.

UILENBERG, G. DOBBELAERE, D.A.E. DE GEE, A.L.W. & KOCH, H.T., 1993. Progress in research on tick-borne diseases: Theileriosis and heartwater. *Veterinary Quarterly*, 15 (2): 48-54.

UILENBERG, G. PERIE, N.M. LAWRENCE, J.A. DE VOS, A.J. PALING, R.W. & SPANJER, A.A.M. 1982. Causal agents of bovine theileriosis in southern Africa. *Tropical Animal Health and Production*, 14 (3): 127-140.

VAN WINKELHOFF, A. & UILENBERG, G. 1981. Heartwater: Cross-immunity studies with strains of *Cowdria ruminantium* isolated in west and South Africa. *Tropical Animal Health and Production,* 13 (1): 160-164.

VLAHAKIS, P.A. CHITANGA, S. SIMUUNZA, M.C. SIMULUNDU, E. QIU, Y. CHANGULA, K. CHAMBARO, H.M. KAJIHARA, M. NAKAO, R. TAKADA, A. & MWEENE, A.S. 2018. Molecular detection and characterization of zoonotic *Anaplasma* species in domestic dogs in Lusaka, Zambia. *Ticks and Tick-borne Diseases*, *9* (1):39-43.

WALKER, A.R. BOUATTOUR, A. CAMICAS, J.L. ESTRADA-PEÑA, A. HORAK, I.G. LATIF, A.A. PEGRAM, R.G. PRESTO, P.M. 2003. *Ticks of domestic animals in Africa: a guide to identification of species.* Bioscience Reports Edinburgh.

WATT, D. KIARA, H. & SPARAGANO, O.A.E. 1998. A PCR-based Field Evaluation of *Theileria* Infections in Cattle and Ticks in Kenya. *Annals of the New York Academy of Sciences*, 849 (1): 69-77.

WESONGA, F.D. MUKOLWE, S.W. & GROOTENHUIS, J. 2001. Transmission of *Cowdria ruminantium* by *Amblyomma gemma* from infected African buffalo (*Syncerus caffer*) and eland (*Taurotragus oryx*) to sheep. *Tropical Animal Health and Production*, 33 (5): 379-390.

WOLDEHIWET, Z. 2010. The natural history of *Anaplasma phagocytophilum*. *Veterinary Parasitology,* 167 (2-4): 108-122.

WOOLHOUSE, M.E. THUMBI, S.M. JENNINGS, A. CHASE-TOPPING, M. CALLABY, R. KIARA, H. OOSTHUIZEN, M.C. MBOLE-KARIUKI, M.N. CONRADIE, I. HANDEL, I.G. & POOLE, E.J. 2015. Co-infections determine patterns of mortality in a population exposed to parasite infection. *Science Advances*, *1* (2): e1400026.

World Organisation for Animal Health (OIE) (2014). – Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris. Available at: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/A_index.htm (accessed on 04 September 2018).

YANG, J. LI, Y. LIU, Z. LIU, J. NIU, Q. REN, Q. CHEN, Z. GUAN, G. LUO, J. & YIN, H. 2015. Molecular detection and characterization of *Anaplasma* spp. in sheep and cattle from Xinjiang, northwest China. *Parasites & Vectors,* 8 (1): 108.

YOUNG, A. LEITCH, B. DOLAN, T. MBOGO, S. NDUNGU, S. GROOTENHUIS, J. & DE CASTRO, J. 1990. Evaluation of infection and treatment methods in immunization of improved cattle against theileriosis in an endemic area of Kenya. *Veterinary Parasitology,* 35 (3): 239-257.

YUNKER, C.E. 1996. Heartwater in sheep and goats: a review. *Onderstepoort Journal of Veterinary Research*, 63 (2): 159-170.

YUNKER, C.E. MAHAN, S.M. WAGHELA, S.D. MCGUIRE, T.C. RURANGIRWA, F.R. BARBET, A.F. & WASSINK, L.A., 1993. Detection of *Cowdria ruminantium* by means of a DNA probe, pCS20 in infected bont ticks, *Amblyomma hebraeum*, the major vector of heartwater in southern Africa. *Epidemiology & Infection*, 110 (1): 95-104.

ZINTL, A. GRAY, J.S. SKERRETT, H.E. & MULCAHY, G. 2005. Possible mechanisms underlying age-related resistance to bovine babesiosis. *Parasite Immunology,* 27 (4): 115-120.

ZOBBA, R. ANFOSSI, A.G. VISCO, S. SOTGIU, F. DEDOLA, C. PARPAGLIA, M.P. BATTILANI, M. PITTAU, M. & ALBERTI, A. 2015. Cell tropism and molecular epidemiology of *Anaplasma platys*-like strains in cats. *Ticks and Tick-borne Diseases,* 6 (3): 272-280.

ZWART, D. 1985. Hemoparasitic diseases of bovines. *Revue Scientifique et Technique de l'Office International des Epizooties,* 4 (3): 447-478.

APPENDICES

Appendix 1.1: V041-16 University of Pretoria Animal Ethics Committee approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Tick-borne disease dynamics in calves at the wildlife-livestock interface in the Mnisi Community Programme (MCP) area, Mpumalanga Province, South Africa (PILOT)**
PROJECT NUMBER	**V041-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Mr. SM Makgabo**

STUDENT NUMBER (where applicable)	**UP 11150752**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

The animals will go back to the owners after the pilot study is completed

ANIMAL SPECIES	**Bovine**	
NUMBER OF ANIMALS	**14**	
Approval period to use animals for research/testing purposes		**May 2016 –May 2017**
SUPERVISOR	**Prof. T Matjila**	

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	30 May 2016
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15

Appendix 1.2: V041-16 University of Pretoria Animal Ethics Committee approval Extension 1

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Tick-borne disease dynamics in calves at the wildlife-livestock interface in the Mnisi Community Programme (MCP) area, Mpumalanga Province, South Africa (PILOT)**
PROJECT NUMBER	**V041-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Mr. SM Makgabo**

STUDENT NUMBER (where applicable)	**UP_11150752**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

The animals will go back to the owners after the pilot study is completed

ANIMAL SPECIES	**Bovine**	
NUMBER OF ANIMALS	**14**	
Approval period to use animals for research/testing purposes		**January 2017-January 2018**
SUPERVISOR	**Dr. N Collins**	

KINDLY NOTE:

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

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

S4285-15

Appendix 1.3: V041-16 University of Pretoria Animal Ethics Committee approval Extension 2

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 2

PROJECT TITLE	Tick-borne disease dynamics in calves at the wildlife-livestock interface in the Mnisi Community Programme (MCP) area, Mpumalanga Province, South Africa
PROJECT NUMBER	V041-16
RESEARCHER/PRINCIPAL INVESTIGATOR	SM Makgobo

STUDENT NUMBER (where applicable)	U 11150752
DISSERTATION/THESIS SUBMITTED FOR	MSc

This is sentinel animals that will be bought in. All other animals that do get ill have to be treated

ANIMAL SPECIES	Cattle
NUMBER OF ANIMALS	14
Approval period to use animals for research/testing purposes	March 2018 – March 2019
SUPERVISOR	Dr. NE Collins

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 6 April 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

Appendix 2: Permission to conduct research in terms of section 20 of the ANIMAL DISEASE ACT, 1984 (ACT NO.35 of 1984) by the Department of Agriculture Fishery and Forestry (DAFF)

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

Enquiries: Mr Henry Golcio • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za
Reference: 12/11/1/1/6

Sekgota Marcus Makgabo
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

Dear Mr/Ms Makgabo,

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 24 August 2016, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

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

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 1982);
4. Bovine blood and tick samples may only be transported to Hans Hoheisen Wildlife Research Station (HHWRS) under a veterinary movement permit;
5. Genomic DNA extracted from the collected bovine blood and tick samples may be transported to the Department of Veterinary Tropical Diseases laboratory under a veterinary movement permit;
6. All samples must be packaged and transported in accordance with the National Road Traffic Act, 1996 (*Act* No. 93 of 1996).

Title of research/study: Tick-borne disease dynamics in calves at the wildlife-livestock interface in the Mnisi Community Programme (MCP) area,

– 1 –

Mpumalanga Province, South Africa.

Researcher (s): Sekgota Marcus Makgabo
Institution: Department of Veterinary Tropical Diseases
Your Ref./ Project Number: VO41-16
Our ref Number: 12/11/1/1/6

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2016 -09- 23

SUBJECT: Tick-borne disease dynamics in calves at the wildlife-livestock interface in the Mnisi Community Programme (MCP) area, Mpumalanga Province, South Africa.

Appendix 3: Consent form translated into local language informing the farmers and owners of the calves about the study. Owners signed the form to give permission to the study group to collect samples from the calves for a period of a year.

Appendix I: Consent form (adult)

Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
P/Bag X04
Onderstepoort 0110
South Africa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

PAPILA RAMPFUMELELANO WOLAVA KU DYONDZA HIMAVABYI YO HAMBANA HAMBANA

UNIVHESITI YA PITORI

Faculty of Veterinary Science

VALAVASISI VA MAVABYI:

Mr Marcus Makgabo: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria P/Bag X04 Onderstepoort 0110. **Tel:** +27 12 529 8382, **E-mail:** mmakgabo@gmail.com

Dr Nicola E Collins: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, P/Bag X04 Onderstepoort 0110. **Tel:** +27 12 529 8000. **E-mail:** nicola.collins@up.ac.za

Dr Kelly Brayton: Department of Microbiology and Pathology, Washington State University (WSU), PO Box 647040 , Washington State University, Pullman WA 99164-7040. Tel: 509 335 6340. **E-mail:** kbrayton@vetmed.wsu.edu

Prof Marinda C Oosthuizen: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, P/Bag X04 Onderstepoort 0110. **Tel:** + 27 12 529 8390. **E-mail:** marinda.oosthuizen@up.ac.za

Dr Louise Biggs: Hluvukani Animal Health Clinic Stand 554, Clare, Section B, Hluvukani. Tel: +27 12 529 8208. **E-mail:** wazzw@hotmail.com

NHLOKO MHAKA:

I vuvabyi bya swingalana hiku angarhelaeka marhole yaleka swifuwo swanhova na swa le kaya hikuhlangana kaswona eka ndawu ya hosi Mnis kwala Mpumalanga, South Africa.

PAPILA RO TIVISA (eka vanhu vamalembe yakusukela khume nhungu nakuya e henhla)

XIYENGE XA A: MONGO WA KONA

Hlaya hi vurhon'wana lebyikulu ungasehleketa hiku nghenelela tidyondzoletitavulavisisi.

Warhambiwa kunghenelela eka tidyondzo tavulavisisi byamavabyi eka marhole ya vufuwi byala makaya na byanhova hikuhlangana ka swona eka ndhawu ya ka Mnisi.

Mhakankulu leyi yiyisaka mavabyi e hansi e misaveni hinkwayo mara ngopfungopfu la Afrika I swi galena na vuvabyi bya swigalana ku hlangana ka tsongo ka swiharhi swanhova na swalemakaya eka misava hinkwayo switikomba nhlayo yi tlakuka, tani hi mbuyelo, ku hangala ka vuvabyi eka tindhawu hinkwato swi te keriwa enhlokweni eka ku hlangana ka swuharhi swalemakaya eka vanhu. Hita tsakela ku xava rhole ra wena nkarhi wa ku ringana lembe hixikongomelo xa ku dyondza eka nkarhi lowu. Rhole leri ritavariri e mavokweni ya wena. Hitateka vuxokoxoko hivhiki rin'wana na rin'wana ra vumbirhi e makamu ka lembe, n'wini wa xirhodyana itavuyiseliwa xona kutani a xitirhisa hila ha a tsakela hakona.

Appendix I: Consent form (adult)

Vuxokoxoko bya ngati na swingalana swita tekiwa ka mbhirhi hi nyangha kuringana lembe kulava kuvona nkarhi lowu xirhodyana xiwutekaka kukuma mavabyi eka ndhawu ya ka Mnisi. Switeka nkarhi ku lava ku vona muxaka ya mavabyi la vangi waka hi switsongwa tsongwana endhawini ya leyo. Swingava swa nkoka ngopfu eka vaaka tiko ku tiva na kutwisisa hi ndlela ya kutlulela ka switsongwa tsongwa swa mixaka xaka eka tihomu leti tinga la ndhawini. Ku nghenelela eka dyondzo swi endlekile hiku pfumelelana k ava tirhi n ava dyondzo ya yunivhesiti ya Pitori na Mpumalanga Veterinary Services (va madhibani) ku teka ngati na vuxokoxoko eka swifuwo na ku teka swingalana. Nkari lowu lavekaka ku teka ngati I makume mbirhi wa timinete eka xifuwo xin'wana na xin'wana, na le henhla ka awara ku teka swingalana

Ku nghenelela iku switsakela kambe loko wuhi pfumelela ku nghenelela projeke leyi swi vula leswakau u hipfumelela ku tirhisa rhole ra wena lembe hinkwaro.

A kuna tinghozi ku va u nghenelela eka projeke leyi. Hambi leswi ku teka vuxokoxoko bya nghati eka swifuwo swi nga vanga ku chav aka tsongo la xifuwo, hita ringeta himatimba hinkwawo ku va xifuwo xa wena xi nga tshikeleleki.

A kuna mbuyelo wo kongoma eka vangheneleri, handle ka ku vuyeriwa hi vutivi bya vuvabyi bya swi fuwo. U ta byeriwa hi mbuyelo bya vuxokoxoko lebyi nga tekiwa.

Tidyonzo leti ti fambisiwa hi va tirhisi va swi fuwo na lava va swi langutisaka, Univhesiti ya Pitori (Afrika-Dzonga).

XIYENGE XA B: MPFUMELELO WA VU NGHENELERI

Ndza pfumela leswaku ndzi tivisiwile hi ti dyondzo leti na swaku ndzi kumile phephe ra vuxokoxoko bya vu ngheneleri na papila ra vu ngheneleri. Ndzi hlayile ndzi tihela ndzi twisisa vuxokoxoko lebyi.

- Nza pfumela ku nghenelela eka ti dyondzo leti.
- Nza pfumela ku xavisa rhole ra mina eka tindzondzo leti kuringana lembe na ku teka ngati na voxokoxoko bya swi ghalana eka swifuwo xa mina.
- Ndza pfumela ku tisa swifuwo swa mina edibhini leswaku ku tatekiwa vuxokoxoko.
- Ndza pfumela leswaku vuriwini bya rhole byi ta vuyiseriwa eka mina emaheteleleni ya dyondzo.

Ndzi switwile swi vutiso swa mina swi hlamuriwa hi ku hetiseka.

(a) Mr Mlambo
Name of respondent

(b) .....
Signature or thumb print of respondent

(c) .....
Signature of Researcher Or Clinician

(d) .....
Signature of witness

(e) Eglington Village
Place

(f) 02/11/2017
Date

Appendix 4: Movement permit for moving samples from Mnisi Community Programme study area to Hans Hoheisen Wildlife Research Station.

MPUMALANGA PROVINCE

Permit in	Permit out

Permit number: 20171023GRK-RC1

DEPARTMENT OF AGRICULTURE | DEPARTEMENT LANDBOU | LITIKO LETEKULIMA

VETERINARY PERMIT TO MOVE ANIMALS/ ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIKHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Dieresiektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngeligunya leMtsetfo we Tifo te Tilwane No. 35 wa 1984:

Name: Naam: Libito:	MARCUS HUNGSBO	ID No:	9 1 1 2 2 6 5 5 1 2 C 5 1
Address: Adres: Likheli:	HANS HOHEISEN WILDLIFE RESEARCH STATION		

to move with: om te beweeg met: uvunyelwa yekuhambisa:	AMOUNT IN WORDS: ELEVEN	SPECIES: BOVINE TICKS	LIVE / PRODUCTS: BIOLOGICAL SAMPLES
identified as follows: geïdentifiseer as volg: lelikhonjiswe nga:	6 BOVINE BLOOD SAMPLES & 5 BOTTLES OF TICKS		
from the ~~farm/diptank area~~/place: vanaf die plaas/dipbak gebied/plek: kusuka epulanzini/edibhini/endzaweni:	EGLINGTON DIPTANK B7 BOC CERT STOCK CARD NUMBER	in the district of: in die distrik: esigodzini sase:	EMLANGENI N
to the ~~farm/diptank area/abattoir~~/place: na die plaas/dipbak gebied/abattoir/plek: kuya epulanzini/edibhini/emadzeleni/endzaweni:	HANS HOHEISEN WILDLIFE RESEARCH STATION	in the district of: in die distrik: esigodzini sase:	EMLANGENI N

~~Conditions~~
1. ~~This permit~~
- ~~is valid for 10 days from date of issue and for one consignment only~~
- ~~must accompany the animals / products listed above~~
- ~~must be produced for inspection on demand by any land or animal owner or police or veterinary official~~
- ~~must be kept at a destination until collected by a veterinary official~~

~~3. For game / game products a nature conservation permit is also needed~~
~~4. Animals and products must be visibly free from external parasites~~
~~4. Animals are to be kept ... in accordance with animal welfare principles~~
~~5. Other conditions:~~

Op die volgende voorwaardes:
1. Hierdie permit:
- is geldig vir 10 dae vanaf datum van uitreiking en slegs vir een besending;
- moet die diere / produkte hierbo vermeld vergesel;
- moet op aanvraag van enige grondeienaar of polisie- of veeartsenykundige beampte;
- moet by bestemming ... word totdat dit deur 'n veeartsenykundige beampte gekollekteer word.
2. Vir wild / wildsprodukte word ... OK benodig.
3. Diere en produkte moet vry wees van uitwendige parasiete wees.
4. Diere moet volgens dierewelsyn riglyne vervoer word.
5. Ander voorwaardes:

Ngaphansi kwemibandezelo lechazelwe ngaphezulu:
1. Lemvumo:
- Iseberita esalanga-kanga 10 ... futsi yekuhambisa ...
- Kwehambisa ... / kwetfo lethabulisako ... ngamuphi umnikati wendzawo kumbe umnikati wetilwane kumbe ngabophiya kumbe loshhulu letinesilwane kwetilwane, loyondzelako;
- Kwesibhelwe endzaweni yekutfola khona tilwane / tintfo letihabulisa ... futsi ... ngaseluhlu sekutewulelwa tilwane ...
2. Kutfumbiswe kwetilwane lesigange / kwetintfo letihabulisako ... futsi ... yeTelongeti kwaTeevelo.
3. Tilwane ... tihabulisako tetilwane tifanele kutsi ... tibangandvodze letingabonakali.
4. Tilwane ... ngendlela ...
5. Letibandzela lesinye:

6. Retention for / Quarantine for / Direct slaughter within: ..... days after arrival at destination

Seal no/s:	N/A		Place / Plek / Indzawo:	MLUMATHARI A C	
Vehicle reg. no/s:	OM 77 UH GP		Date / Datum / Lusuku:	2017/10/23	

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

PRINT NAME	SIGNATURE
DR HEIN KRUGER	
Issuing Veterinary Official:	State Veterinarian / Animal Health Technician/AHT Technician

IMPORTANT NOTE / BELANGRIKE NOTA / SATISO LESIBALULEKILE
Permit holder who does not comply with the permit or any condition thereof is guilty of an offence.
Permit houer wat in stryd met hierdie permit of enige voorwaarde daarvan optree, is skuldig aan 'n misdryf.
Lonalemvumo longalandzala lemvumo kumbe inuphi umbandzela wayo uphula umtsetfo.

DISTRIBUTION: VETERINARY OFFICIAL AT DESTINATION

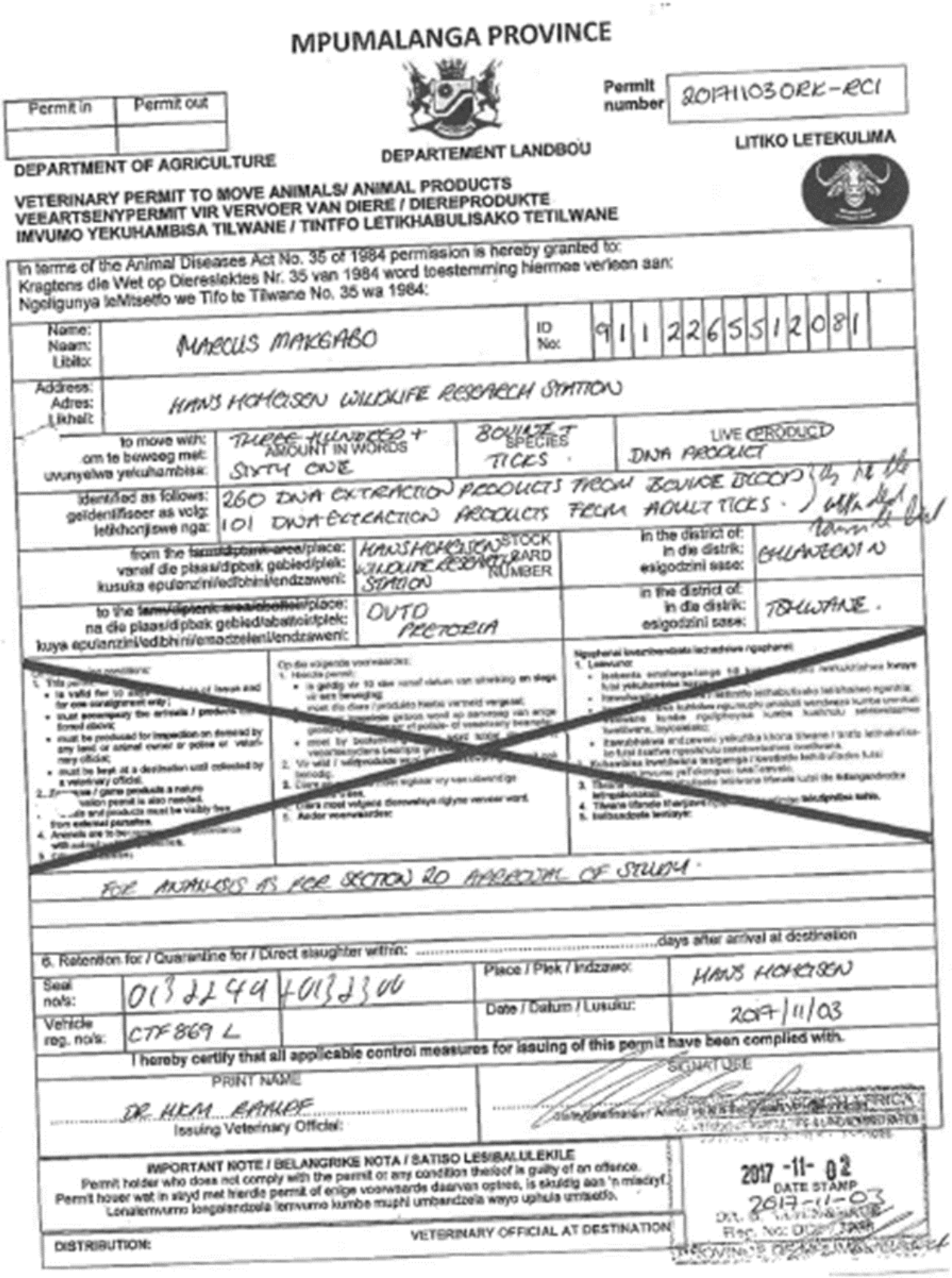
DATE STAMP
2017-10-23

Appendix 5: QIAmp DNA Extraction Log Sheet for samples in an FMD control zone.

QIAmp DNA Extraction Log Sheet (TBD's- Makgabo SM)

Date of Extraction	Number of Samples	Permit Number & Date	Step 1	Step 2	Step 3	Step 4 & 5	Step 6	Step 7	Step 8	Step 9	Step 10	Step 11	Signature of Researcher
			20 µl QIAGEN Protease	200 µl sample	200 µl Buffer AL	Incubate at 56°C for 30 min & Briefly centri	Add 200 µl ethanol (96–100%)	Apply (6) to QIAamp Mini spin column	500 µl Buffer AW1, Centri for 1min	500 µl Buffer AW2, Centri for 3min	Centrifuge for 1 min- full speed	Add 100 µl Buffer AE,	
27/09/17	7 blood Samples	B2/04/2018 B2/13/2018	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
28/10/17	8 blood Samples	2017100204RCI	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
23/10/17	7 blood Samples	2017100308RCI	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
23/10/17	6 blood Samples	2017102308RCI	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
27/10/17	2 tick Samples	B2/01/2019	✓	✓	✓	overnight ✓	✓	✓	✓	✓	✓	✓	
29/10/17	8 tick Samples	2017051108RCI 2017052908RCI	✓	✓	✓	overnight ✓	✓	✓	✓	✓	✓	✓	
27/10/17	15 tick Samples	2017061208RCI 2017062308RCI 2017062908RCI	✓	✓	✓	overnight ✓	✓	✓	✓	✓	✓	✓	
27/10/17	11 tick Samples	2017030308RCI 2017041708RCI 2017041808RCI	✓	✓	✓	overnight ✓	✓	✓	✓	✓	✓	✓	

Appendix 6: Movement permit for moving samples from the Hans Hoheisen Wildlife Research Station to the Department of Veterinary tropical Diseases, University of Pretoria.



MPUMALANGA PROVINCE

Permit in	Permit out

Permit number: 20171103ORK-RC1

DEPARTMENT OF AGRICULTURE

DEPARTEMENT LANDBOU

LITIKO LETEKULIMA

VETERINARY PERMIT TO MOVE ANIMALS/ ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIKHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Diereslektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngoligunya leMtsetfo we Tifo te Tilwane No. 35 wa 1984:

Name: Naam: Libito:	MARCUS MAKGABO	ID No:	9111226551208 1
Address: Adres: Likhaya:	HANS HOHEISEN WILDLIFE RESEARCH STATION		
to move with: om te beweeg met: uvuyelwa yekuhambisa:	THREE HUNDRED + SIXTY ONE (AMOUNT IN WORDS)	BOVINE T SPECIES TICKS	LIVE PRODUCT DNA PRODUCT
identified as follows: geidentifiseer as volg: letikhonjiswe nga:	260 DNA EXTRACTION PRODUCTS FROM BOVINE BLOOD 101 DNA EXTRACTION PRODUCTS FROM ADULT TICKS.		
from the farm/dipping area/place: vanaf die plaas/dipbak gebied/plek: kusuka epulazini/edibini/endzaweni:	HANS HOHEISEN WILDLIFE RESEARCH STATION (STOCK CARD NUMBER)	in the district of: in die distrik: esigodzini sase:	MHLANGENI N
to the farm/dipping area/abattoir/place: na die plaas/dipbak gebied/abattoir/plek: kuya epulazini/edibini/emacitelen/endzaweni:	OUTO PRETORIA	in the district of: in die distrik: esigodzini sase:	TSHWANE.

FOR ANALYSIS AS PER SECTION 20 APPROVAL OF STUDY.

6. Retention for / Quarantine for / Direct slaughter within: ..... days after arrival at destination

Seal no's:	0132144 / 0132300	Place / Plek / Indzawo:	HANS HOHEISEN
Vehicle reg. no's:	CTF 869 L	Date / Datum / Lusuku:	2017/11/03

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

PRINT NAME: DR HEM RAMKE
Issuing Veterinary Official:

SIGNATURE

IMPORTANT NOTE / BELANGRIKE NOTA / SATISO LESIBALULEKILE
Permit holder who does not comply with the permit or any condition thereof is guilty of an offence.
Permit houer wat in stryd met hierdie permit of enige voorwaarde daarvan optree, is skuldig aan 'n misdryf.
Lonalemvumo longalandzela lemvumo kumbe muphi umbandzela wayo uphula umtsetfo.

DISTRIBUTION:

VETERINARY OFFICIAL AT DESTINATION

2017 -11- 02
DATE STAMP
2017-11-03