Molecular detection of tick-borne haemoparasites in cattle and buffalo samples from Mashonaland West and Masvingo Provinces, Zimbabwe

by

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DECLARATION

I hereby declare that this dissertation is my own original work and has not been previously submitted by me for a degree at this or any other tertiary institution. All sources of information used in this study has been acknowledged and referenced in accordance with the University of Pretoria's requirements.

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Annicky A. R. Modirwa Date

DEDICATION

This dissertation is dedicated to my lovely daughter Kagoentle ‘Leblomo-nyana la mama’ Modirwa.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Degrees Celsius
%	Percent
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CAT	Card Agglutination Test
CFT	Complement Fixation Test
C_p	Crossing point
C_t	Threshold cycle
C_q	Quantification cycle
DALRRD	Department of Agriculture, Land Reform and Rural Development
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DFVS	Department of Field Veterinary Services
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
ECF	East Coast fever
ECL	Enhanced chemiluminescence
EDAC	Ethyl-3-(-3-dimethylamininopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FTA cards	Flinders Technology Associates
GNP	Gonarezhou National Park
IFAT	Indirect Fluorescent Antibody Test
KCl	Potassium chloride
MAFFT	Multiple Alignment using Fast Fourier Transmorm

MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium chloride
µl	Microlitre
µM	Micromolar
ml	Millilitre
mm	Millimetre
mM	Millimolar
mg	Milligram
MSP	Major surface protein
NaHCO3	Sodium bicarbonate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
pH	Potential Hydrogen
POD	Peroxidase
PIM	Polymorphic immunodominant molecule
qPCR	Quantitative real-time polymerase chain reaction
Rap-1a	Rhoptry-associated protein-1a
RLB	Reverse line blot
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
sp.	Species
SSPE	Saline–sodium phosphate–EDTA
TBDs	Tick-borne diseases
TqM	TaqMan
Tris-HCL	Tris hydrochloride
UDG	Uracil DNA glycosylase
UNG	Uracil-N-glycosylase

STUDY SUMMARY

Molecular detection of tick-borne haemoparasites in cattle and buffalo samples from Mashonaland West and Masvingo Provinces, Zimbabwe

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Degree: MSc (Veterinary Science)

Tick-borne haemoparasite diseases caused by *Babesia*, *Theileria*, *Anaplasma* and *Ehrlichia* species are a major constraint to the beef and dairy cattle industry, causing the most economic losses of cattle in sub-Saharan Africa. The cattle industry in Zimbabwe is continuously threatened by the spread of tick-borne diseases, which significantly affect the economy not only through morbidity and mortality but also through the costs involved in the control of diseases and treatment of sick animals. However, there is a lack of current data on the distribution of tick-borne diseases in Hurungwe district, Mashonaland West Province. The current study used molecular tools to investigate the occurrence of haemoparasites in cattle from Hurungwe district in Mashonaland West Province and buffalo from Gonarezhou National Park in Zimbabwe. DNA was extracted from 87 whole blood samples including 80 cattle and seven buffalo. The DNA samples were subjected to the Reverse-line blot hybridization (RLB) and quantitative real-time polymerase chain reaction (qPCR) analyses. Haemoparasite infections were detected in 58 samples (67 %) by RLB, and 55 % of these only hybridized to the genus-specific probes. Tick-borne haemoparasites detected by RLB included three *Theileria* species (*T. mutans*, *T. velifera*, and *Theileria* sp. sable), detected in single and mixed-parasite infections. *Anaplasma centrale* (3 %) and *Babesia bigemina* (1 %) were also detected by the RLB assay. The most commonly occurring tick-borne pathogens in cattle

detected by qPCR assays were *A. marginale* (28 %) and *B. bigemina* (9 %); followed by *A. centrale* (8 %) and *B. bovis* (3 %). While in buffalo *A. marginale* (86 %), followed by *A. centrale* (14 %) were mostly detected. The results of the current study indicated that the species-specific qPCR assays used were more sensitive in detecting haemoparasites than the RLB assay. *Anaplasma marginale* and *Babesia bovis* were only detected by the species-specific qPCR assays and not by the RLB assay, which suggests that these haemoparasite infections were present at low levels thus could not be detected by RLB assay. The RLB assay suffers lower sensitivity when a sample is infected with more than one haemoparasite, especially when the levels of infection vary; the high infection will be preferentially detected over low infections of the same genus due to primer competition. Notably, *T. parva* or *E. ruminantium* was not detected from the investigated samples. The amplification and sequencing of the 16S and 18S rRNA genes from samples that hybridized exclusively to the RLB genus-specific probes yielded nine and one good quality sequences, for the 16S and 18S rRNA genes respectively. However, BLASTn analysis did not reveal hits to any haemoparasites expected to occur in cattle and buffalo. Our results did not follow the common trend for the prevalence of tick-borne diseases of cattle in Zimbabwe. Bovine theileriosis has recently been reported to be responsible for most cattle mortalities in Zimbabwe, followed by babesiosis, heartwater, and then anaplasmosis. Our results therefore suggest that the trend of occurrence of tick-borne diseases depends on the vector-parasite-host-environment dynamics for each province, thus may vary between provinces. Finally, this study confirms that buffalo in the sampled area are carriers of tick-borne diseases that pose risk to the cattle population.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Haemoparasite diseases such as theileriosis, babesiosis, anaplasmosis and heartwater are a major constraint to cattle production in sub-Saharan Africa, with ticks being the most important vectors (Perry and Young, 1995). Tick-borne diseases inflict major problems to the health and management of livestock in Zimbabwe, often resulting in at least 60% mortalities (Sungirai et al., 2015). Tick-borne diseases limit livestock farming by causing fertility problems, decreased meat and milk production and a reduction in growth rate (Simuunza et al., 2011; Sungirai et al., 2016). Moreover, tick-borne diseases restrict the introduction of more productive livestock and the improvement of existing livestock in developing countries in Africa (Simuunza et al., 2011).

Bovine theileriosis, babesiosis, anaplasmosis and heartwater, respectively caused by *Theileria parva, Babesia bigemina* and *B. bovis, Anaplasma marginale,* and *Ehrlichia ruminantium,* are considered the most important tick-borne diseases of cattle in Zimbabwe (Lawrence and Norval, 1979; 1987; Norval et al., 1992a). The epidemiology of these diseases has been studied in the past (Norval, 1979; Norval et al., 1983; 1984; 1985; Peter et al., 1998; Katsande et al., 1999; Latif et al., 2001), however, there is no recent data on the distribution of these tick-borne diseases in cattle at Hurungwe district.

Bovine theileriosis poses the greatest threat to cattle production across farming systems that are in the smallholder areas in Zimbabwe (Norval et al., 1985; Latif et al., 2001; Moyo et al., 2017). Three forms of theileriosis are known in Zimbabwe, namely East Coast fever, January disease and Corridor disease; caused by the cattle-derived or buffalo-derived *Theileria parva* (Lawrence et al., 2004). East Coast fever was successfully eradicated in Zimbabwe by 1954, through a combination of practices, which included compulsory short-interval dipping to

control ticks, control of animal movement, quarantine and slaughter (Lawrence and Norval, 1979). Following the control of theileriosis in Zimbabwe, babesiosis, anaplasmosis and heartwater were concurrently brought under control and eradicated (Lawrence and Norval, 1979; Norval, 1979). However, tick control was disrupted during the pre-independence war (1972 to 1980), resulting in a rapid increase of ticks and tick-borne diseases (Norval et al., 1983; 1984; 1985). To date, January disease caused by the cattle-derived *T. parva* continues to cause the most cattle mortalities in Zimbabwe (Lawrence et al., 2004; Moyo et al., 2017; Manyenyeka et al., 2021). The disease is acute and frequently fatal, and is spreading throughout Zimbabwe. Known to be a seasonal disease, recent reports show that January disease is apparently losing seasonality (Manyenyeka et al., 2021). However, there is no current data on the occurrence of the disease in Hurungwe district.

The reported factors that contribute to the increase in cases of bovine theileriosis in Zimbabwe include, poor disease surveillance as a result of budget constraints, the lack of disease awareness among rural farmers and the inaccessibility in resettlement areas (Moyo et al., 2017). In their study, conducted from the year 2000 to 2014, the majority of bovine theileriosis cases were recorded during the month of January when the adult *Rhipicephalus appendiculatus* ticks are most active (Koch, 1990); 33% of the cases were recorded post rainy season, while low cases were recorded during the cold dry season (Moyo et al., 2017). The decreased budget allocations by the Zimbabwean government resulted in the collapse of the dipping services, which mostly affected the communal farmers. Consequently, bovine theileriosis spread from the Highveld and Middleveld towards the Lowveld regions of Zimbabwe (Moyo et al., 2017). The increase of bovine theileriosis in the Lowveld regions of Zimbabwe might also be caused by the increased contact between cattle and buffalo from the national parks and game reserve parks that are found in the Lowveld region (Moyo et al., 2017).

Approximately 50 000 cattle died in Zimbabwe during 2017 and 2018 due to bovine theileriosis (Shekede et al., 2021). While the distribution of tick vectors that transmit important cattle diseases have been largely explored in Zimbabwe (Norval et al., 1994; Peter et al., 1998; Sungirai et al., 2015; 2016; 2017; 2018; Mandara, 2018; Moyo et al., 2018;

Manyenyeka et al., 2021; Shedeke et al., 2021), the distribution and occurrence of babesiosis, anaplasmosis and heartwater is lacking, especially at Hurungwe district.

In Zimbabwe, cattle are the most important livestock, not only as an investment and a status symbol but also as a source of income. They also play an important role in the socio-cultural activities (Tavirimirwa et al., 2013). Approximately 75% of households rely on livestock as a major contributor for meat and milk (Simuunza et al., 2011; Sungirai et al., 2016). In addition, the cattle industry contributes 35 – 38% to the economy of Zimbabwe (Manyenyeka et al., 2021). However, this industry is threatened by the continuous spread of tick-borne diseases. Recent studies in Zimbabwe have reported a shift in the distribution of ixodid ticks, which are vectors of tick-borne pathogens of economic importance. The spread of these ticks is caused by the fast-tracked land reform programme which led to movement of livestock, and thus the introduction of ticks to areas where they previously did not occur (Sungirai et al., 2015; 2017; Shekede et al., 2021).

The distribution of ticks that transmit a particular parasite and the availability of the respective host usually defines the occurrence and distribution of tick-borne diseases. However, changes in environmental conditions and the introduction of carrier animals into a herd can gradually extend the distribution of tick-borne diseases to new areas (Perry et al., 1991; Estrada-Peña et al., 2008). Tick-borne diseases in Africa are complex as a single tick species may transmit different pathogens or different tick species may transmit the same pathogen. Furthermore, different tick species interact with different hosts, as a result, they pick a wide range of pathogenic and non-pathogenic organisms, which in turn they transmit to susceptible hosts (Njiiri et al., 2015). For example, the African buffalo plays a significant role in the maintenance and transmission of many cattle diseases of economic importance which include foot-and-mouth disease, bovine tuberculosis and bovine brucellosis (Sibeko et al., 2008; Michel and Bengis, 2012; Eygelaar, 2015; Moumouni et al., 2015). As carriers of Corridor disease, one of the disease syndromes caused by the buffalo-derived *T. parva*, buffalo can introduce the pathogen across species especially in areas where cattle and buffalo share grazing land (Walker et al., 2014b).

Control measures for tick-borne diseases remains a problem for a developing country such as Zimbabwe due to the lack of infrastructure and resources for disease surveillance (Gadaga et al., 2016). As a result, there is no current data on the occurrence of tick-borne haemoparasite diseases in large parts of the country. It is therefore important to know which haemoparasites are currently present in Hurungwe district, since they cause cattle diseases of economic importance. Moreover, data on tick-borne haemoparasites that occur in Zimbabwe will assist in the design of effective disease management measures.

1.2. Aim of the Study

To investigate the occurrence of tick-borne haemoparasites in cattle at Hurungwe district in Mashonaland West Province and buffalo from Gonarezhou National Park, Zimbabwe.

1.3. Study Objectives

a) Screening of blood samples obtained from cattle and buffalo for the presence of haemoparasites DNA using Reverse line blot (RLB) hybridization assay.
b) Characterization of undescribed pathogen species detected from RLB hybridization assay by DNA sequencing of the 18S and 16S rRNA genes.
c) Specific pathogen detection using the following real-time PCR assays:
    - *Theileria parva* specific qPCR assay
    - *Babesia bovis* specific qPCR assay
    - *Babesia bigemina* specific qPCR assay
    - Duplex qPCR assay for specific detection of *Anaplasma marginale* and *Anaplasma centrale*
    - pCS20 Sol1^{TqM} qPCR assay for specific detection of *Ehrlichia ruminatium*

1.4. Literature Review

In 1914, short-interval dipping was introduced in Zimbabwe to control tick burden on cattle. Following which, theileriosis, babesiosis, anaplasmosis and heartwater were brought under control and eradicated in the 1950's (Lawrence and Norval, 1979; Norval et al., 1983). In 1970, dipping of cattle was disrupted by the escalation of the pre-independence war, which resulted in approximately one million cattle dying from tick-borne diseases (Norval, 1979). The Zimbabwean government financially supported the control of ticks which included dipping and spraying animals with acaricides, and this was done through the Department of Field Veterinary Services (DFVS) (Sungirai et al., 2016). However, due to budget constraints the services were disrupted, leading to outbreaks of ticks and tick-borne diseases (Ndhlovu et al., 2009). Moreover, the agricultural land redistribution by the Zimbabwean government resulted in movement of animals and migration of ticks, leading to the establishment of ticks in areas where they previously did not occur (Tavirimirwa et al., 2013; Sungirai et al., 2015; 2017; Mandara, 2018; Manyenyeka et al., 2021; Shekede et al., 2021).

Lack of epidemiological information, in developing countries such as Zimbabwe can result in inadequate control of major tick-borne diseases, with devastating effects to livestock production (Asiimwe et al., 2013; Moyo et al., 2017).

1.4.1. Bovine theileriosis

Theileriosis is an economically important tick-borne disease that infects domestic and wild animals in the tropical and subtropical regions (Uilenberg, 1995). Bovine theileriosis is endemic and a notifiable disease in Zimbabwe. The disease causes a significant problem in the cattle industry in Zimbabwe with mortality rates that can reach 90%, and the high cost of treatment of sick cattle and control measures (Lawrence and Norval, 1979; Perry and Young, 1995; Moyo et al., 2017; Manyenyeka et al., 2021).

Bovine theileriosis is caused by the apicomplexan parasites of the genus *Theileria*, which are transmitted by ticks of the genera *Rhipicephalus*, *Amblyomma*, *Haemaphysalis* and *Hyalomma* (Norval et al., 1992b; Bishop et al., 2004; Lawrence and Williamson, 2004; Walker

et al., 2014a). Among the *Theileria* species, *T. parva* and *T. annulata* are the most pathogenic causing severe disease in cattle. *Theileria parva* causes East Coast fever (ECF), January disease (also known as Zimbabwean theileriosis) and Corridor disease in the eastern, central and southern Africa (Perry et al., 1991; Sibeko et al., 2008; Moumouni et al., 2015). *Theileria annulata* causes tropical theileriosis which occurs in North Africa, southern Europe and Asia. Other *Theileria* species which can infect cattle and buffalo, but considered mild or non-pathogenic include *T. mutans, T. taurotragi*, *T. velifera*, and the *T. sergenti/ orientalis/ buffeli* group (Norval et al., 1992b; Gubbels et al., 2000; Uilenberg, 2011). These *Theileria* species cause only mild or subclinical diseases, however, they can interfere with the diagnosis of the pathogenic *Theileria* species and thus confuse epidemiology (Norval et al., 1992b).

Theileria parva

Theileria parva is a haemoprotozoan pathogen found within the genus *Theileria*. *Theileria parva* infections were previously divided into three subspecies. The causative agents of classical East Coast fever (ECF), Corridor disease and January disease, were previously known as *T. parva parva, T. parva lawrencei* and *T. parva bovis,* respectively (Uilenberg, 1999; Lawrence et al., 2004; Yusufmia et al., 2010). However, the three sub-species are morphologically and serologically indistinguishable. Due to this, and the lack of molecular evidence to justify different *T. parva* subspecies; this nomenclature had to be abandoned abandoned. Thus, the causative agents of ECF and January disease are now referred to as cattle-derived *T. parva*, and those of Corridor disease as buffalo-derived (Norval et al., 1992b; Perry and Young, 1993).

Theileria parva parasites are single celled eukaryotes that belong to the phylum Apicomplexa, in the order Piroplasmida (Norval et al., 1992b). *Theileria parva* transforms leukocytes of the host, causing disease syndromes that are different in pathogenicity, epidemiology and clinical symptoms (Norval et al., 1992b; Lawrence and Williamson, 2004). The African buffalo (*Syncerus caffer*) is considered the original host of *T. parva*, they become persistently infected with the parasite and can transmit it to cattle in the presence of transmitting vector ticks (Gadaga et al., 2016).

Transmission and life cycle of *Theileria parva*

Theileria parva is mainly transmitted by *Rhipicephalus appendiculatus* ticks, and *R. zambeziensis* in the field; however, *R. duttoni* has also been reported as a possible vector (Norval et al., 1992b; Uilenberg, 1999; Norval and Horak, 2004). Proliferation of these transmitting vector ticks relies on suitable environmental conditions and the availability of suitable hosts (Madder et al., 2005). In sub-Saharan Africa, the three-host tick *R. appendiculatus* has a strict seasonal life cycle and a wide host range including wild and domestic animals, although cattle are the most preferred hosts (Norval and Horak, 2004). *Rhipicephalus zambeziensis* and *R. appendiculatus* are morphologically similar, and have the same host range. *Rhipicephalus appendiculatus* is a common tick in Zimbabwe, confined to the high rainfall regions in the Highveld and Middleveld (Norval et al., 1982; Moyo et al., 2017; Sungirai et al., 2017). The adult *R. appendiculatus* ticks are most abundant during the rainy period from December to April, larvae in the late summer and cool periods from April to August, and nymphs in the winter and early spring from June to August (Latif et al., 2001). *Rhipicephalus zambeziensis* is more prominent in hotter and drier regions of the northern, Northwestern and southern parts of Zimbabwe (Norval et al., 1982; 1992a).

The mode of transmission of *T. parva* by *R. appendiculatus* is transstadial. *Rhipicephalus appendiculatus* larvae or nymphs acquire the pathogen while feeding on the infected host. The pathogen is transmitted in the next stage, by the nymphs if it was acquired by larvae or adults if it was acquired by nymphs. In the tick gut, the parasite piroplasms differentiate into male and female gametocytes (Figure. 1.1). The zygotes form from the fusion of the gametocytes and differentiate into kinetes, which then move to the salivary gland, where they develop to sporozoites. The sporozoites are inoculated into a vertebrate host during tick feeding (Marcelino et al., 2012). In this host, sporozoites enter lymphocytes and develop into schizonts, the pathogenic stage of the parasite. Some schizonts multiply and transform the infected lymphocytes, while others develop into merozoites that eventually invade erythrocytes. In these cells, the merozoites develop into the tick infective stage, the piroplasms (Marcelino et al., 2012).

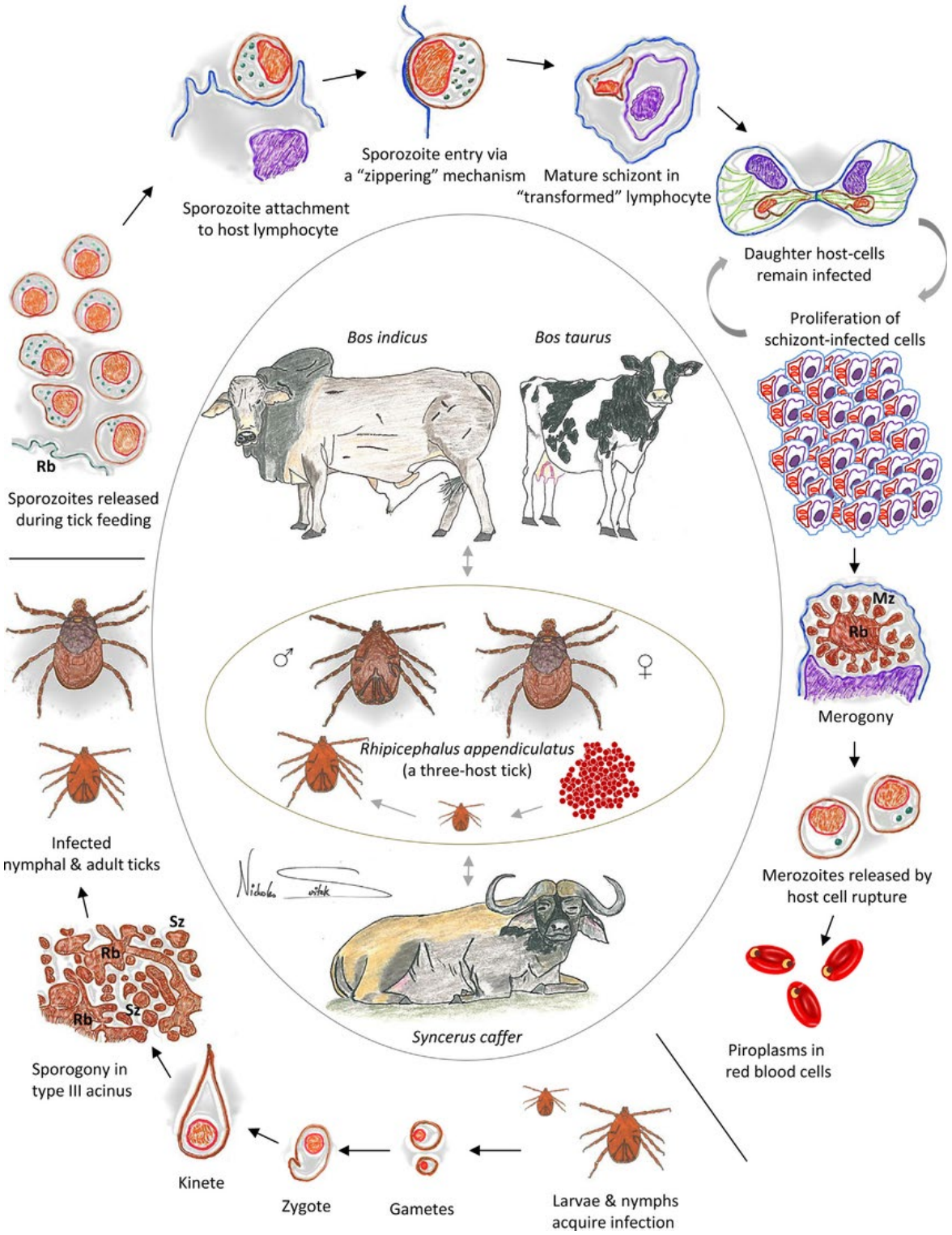


Figure1.1: The life cycle of *Theileria parva* in the mammalian host and the tick vector (Nene et al., 2016).

Different forms of cattle theileriosis caused by *Theileria parva* infections

East Coast fever is caused by the cattle-derived *T. parva*; transmitted from cattle to cattle by the brown ear tick, *R. appendiculatus*. Following its introduction in Zimbabwe, ECF was encountered in southern Mozambique and then the northern parts of South Africa. The disease spread southwards along the east coast of southern Africa, through Swaziland, KwaZulu-Natal and the Eastern Cape Province (Lawrence and Williamson, 2004).

The typical characteristic of ECF is the proliferation of lymphoblasts infected with schizonts in the lymph nodes, lymphoid aggregates, lungs, liver, spleen and kidneys. Clinical signs of ECF include fever, anaemia, enlarged lymph nodes, anorexia, difficulty breathing, nasal discharge, corneal opacity and diarrhoea. Animals that recover from the disease remain life-long carriers that become a source of infection for ticks (Norval et al., 1992b).

Corridor disease is caused by the buffalo-derived *T. parva.* The disease is acute and usually fatal. Corridor disease, although distinct, resembles ECF and it was first encountered in 1934 in the southern lowveld of Zimbabwe, affecting cattle grazing in the same area as the *T. parva*-infected African buffalo, in the presence of *R. appendiculatus* ticks and *R. zambeziensis* (Norval et al., 1992b). Corridor disease was subsequently recorded in South Africa between Hluhluwe and Umfolozi game reserves in KwaZulu-Natal (Neitz et al., 1955).

The African buffalo shows no disease symptoms, however, as natural reservoir hosts of *T. parva*, they play a major role as the source of infection for ticks and to the epidemiology of this disease (Norval et al., 1992b; Eygelaar, 2015). Although clinical signs and pathological changes of Corridor disease and ECF are similar, they are not identical (Neitz et al., 1955). Low schizont parasitosis and piroplasm parasitaemia are seen with Corridor disease and the schizonts are very scanty. The course of disease is usually shorter with death occurring three to four days after the onset (Sibeko et al., 2008; Tembo et al., 2018). Corridor disease remains a threat in areas where cattle and buffalo share grazing land, and cattle that recover from the disease become carriers of the parasite (Potgieter et al., 1988). Although the parasitaemia levels might be too low in carrier animals, a disease outbreak might occur in areas where the tick-vector occurs (Bishop et al., 1992).

Until 1936, ECF and Corridor disease were the two recognized theileriosis disease syndromes caused by *T. parva* infections. However, during 1936 Lawrence encountered a form of theileriosis that was different from both ECF and Corridor disease in that fewer schizonts and piroplasm were produced, and this was discovered in eastern Zimbabwe in Chipinge district (Koch, 1990). The disease was initially known as Specific disease because it occurred mainly during the rainy season each year and was later named January disease or Rhodesian-Zimbabwean theileriosis (Koch, 1990).

January disease is an acute and fatal disease caused by the cattle-derived *T. parva* infections. The disease occurs sporadically on the highveld of Zimbabwe, coinciding with the adult tick-vector occurrence and activities (Lawrence et al., 2004; Moyo et al., 2017). More than 95% of January disease outbreaks are recorded between December and April, during high rainfall (Latif et al., 2001). Although the disease is mild, the pathogenesis, pathology and clinical signs of January disease are similar to those of ECF (Lawrence et al., 2004).

1.4.2. Bovine babesiosis

Bovine babesiosis is caused by the intraerythrocytic protozoan parasites of the genus *Babesia*, phylum Apicomplexa, class Sporozoasida, order Eucoccidiorida, suborder Piroplasmorina and family Babesiidae (Bock et al., 2004). Babesiosis is distributed worldwide, infecting a wide range of domestic and wild animals, occasionally humans (Bock et al., 2004). Bovine babesiosis, also known as redwater fever, causes morbidity and mortality resulting in major economic losses in the cattle industry, and it is considered the second most important tick-borne disease following ECF in sub-Saharan Africa (Bock et al 2004). The two most important *Babesia* species infecting cattle in Africa are *Babesia bovis* and *B. bigemina* (De Vos et al., 2004). Other *Babesia* species that can infect cattle include *B. major, B. ovate, B. occultans, B. divergens* and *B. jakimovi* (Bock et al., 2004; Uilenberg, 2006).

Babesia bovis

Babesia bovis causes Asiatic redwater. This parasite is more pathogenic, causing severe disease in cattle (Uilenberg, 2006). *Babesia bovis* spread into the north-eastern and central areas of Zimbabwe from Mozambique (Norval et al., 1992a). Asiatic redwater is characterized

by haemolysis leading to anaemia, circulatory disorder, aggression, convulsion and paralysis, diarrhoea and death; animals may also abort (Bock et al., 2004).

Babesia bigemina

Babesia bigemina causes African redwater and has a wider distribution throughout Zimbabwe (Norval, 1979; Norval et al., 1983; Katsande et al., 1999; Smeenk et al., 2000). *Babesia bigemina* has been present in Zimbabwe since 1890, causing morbidity and mortality in imported cattle (Norval, 1979). African redwater is characterized by fever, weakness, icterus, haemoglobinaemia and haemoglobinuria (Bock et al., 2004).

Transmission and distribution of *Babesia bovis* and *B. bigemina*

The distribution of redwater is dependent on the distribution of tick vectors, *Rhipicephalus microplus* and *R. decoloratus* in a particular region. *Rhipicephalus microplus* ticks transmit both *B. bovis* and *B. bigemina* while *R. decoloratus* ticks are responsible for the transmission of *B. bigemina* (Bock et al., 2004). *Rhipicephalus microplus* ticks spread into Zimbabwe through livestock movements from Mozambique in the 1970s (Norval et al., 1983) while *R. decoloratus* is believed to be indigenous in Zimbabwe (Sungirai et al., 2015). *Rhipicephalus microplus* ticks are reported to be confined to the cooler and high rainfall areas of the eastern Highveld in Zimbabwe (Katsande et al., 1999), however, recent studies found that these ticks have also spread into the south-eastern and northern Lowveld parts of the country (Sungirai et al., 2017). *Rhipicephalus decoloratus* ticks tolerate broad climate conditions, hence the wider distribution in Zimbabwe (Sungirai et al., 2017). Although studies reported that *R. microplus* competes with and displaces *R. decoloratus* in the warm and humid conditions (Norval et al., 1983; Katsande et al., 1999; Sungirai et al., 2015), this was not apparent in a study conducted by Sungirai et al. (2017). These observations could be due to the presence of alternative hosts for *R. decoloratus* in the cold and dry areas or the increased resistance of *R. decoloratus* to acaracides, thus reducing the competitive advantage of *R. microplus* (Sungirai et al., 2017).

Life cycle of *Babesia* species

The transmission of *B. bovis* by *R. microplus* occurs transovarially. During the life cycle of babesiosis, as shown in Figure 1.2, the pathogen is acquired by adult ticks when feeding on

an infected host or carrier of the pathogen and the larvae of the following generation will transmit the pathogen while feeding. The larvae are no longer infected after feeding and can be re-infected in the adult stages when feeding. Similarly, *B. bigemina* is transmitted by *R. decoloratus* and *R. microplus* both transovarially and vertically. In the latter, infection is carried from one generation to another without re-infection taking place (Bock et al., 2004; Uilenberg, 2006).

The *Babesia* parasites are transmitted into the bovine host as sporozoites, which parasitize erythrocytes. Each sporozoite penetrates the cell membrane of an erythrocyte with the aid of the apical complex. Inside the erythrocyte, it transforms into a tropozoite from which two merozoites develop by binary fission (Bock et al., 2004). The merozoites that replicate within the host erythrocytes are then acquired by the adult female tick that transovarially transmits the kinete stage to the larvae (Bock et al., 2004; Uilenberg, 2006).

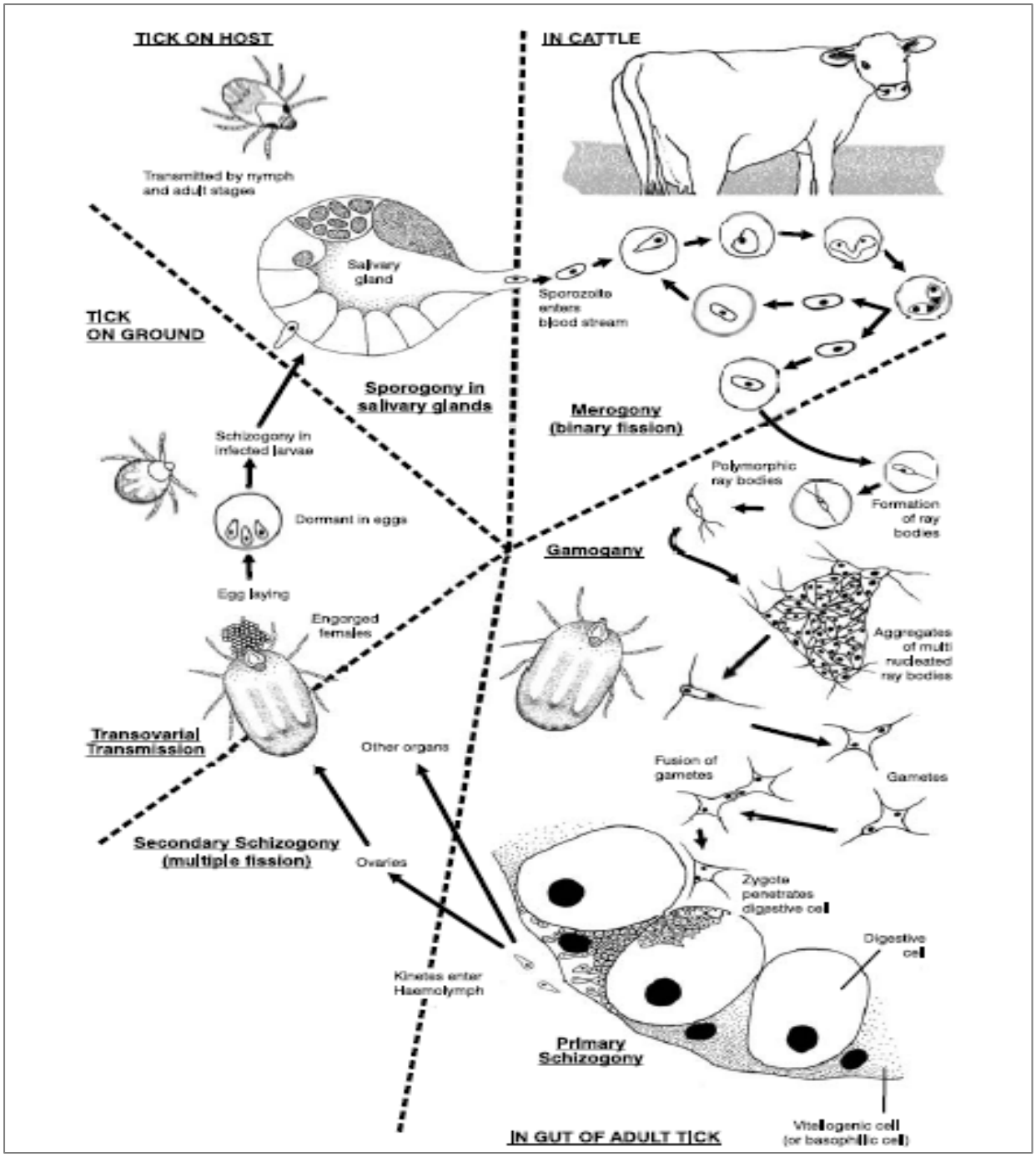


Figure1.2: The development lifecycle of *Babesia* parasites in cattle and the tick vector (Bock et al., 2004).

1.4.3. Bovine anaplasmosis

Bovine anaplasmosis, formerly known as gall sickness is caused by the intra-erythrocytic rickettsias of the genus *Anaplasma* (Norval et al., 1984). Bovine anaplasmosis is widely distributed around the world, including South Africa and Zimbabwe, where it is endemic in

most of the cattle-farming areas (Norval et al., 1984; Potgieter and Stoltsz, 2004; Marufu et al., 2010; Chaisi et al., 2017). Bovine anaplasmosis is the tick-borne disease that caused the majority of cattle deaths during 1965 in Zimbabwe (Matson, 1965). It is believed that the disease have been present in Zimbabwe prior to the 19th century, affecting imported cattle even before the first white settlers arrived (Lawrence and Norval, 1979). Bovine anaplasmosis was recorded throughout the year, especially during heavy tick infestations in the summer months in regions where cattle dipping had been suspended for over a year (Norval, 1979).

The causative agents of bovine anaplasmosis

The two important species of *Anaplasma* infecting cattle in Zimbabwe are *Anaplasma marginale* and *A. centrale* (Norval et al., 1984). *Anaplasma marginale* causes acute anaplasmosis, which is characterized by progressive anaemia and jaundice associated with the intra-erythrocytic inclusion bodies (Lew and Jorgensen, 2005; Eygelaar, 2015; Hove et al., 2018). *Anaplasma centrale* is less pathogenic and causes mild signs in cattle; it is often used as a vaccine against *A. marginale* (Uilenberg, 1995; Kocan et al., 2010).

Transmission and life cycle of *Anaplasma* species

Bovine anaplasmosis is not contagious, it spreads through tick-bite. Transmission can also occur mechanically through fresh erythrocytes from biting flies, or contaminated surgical equipment (Aubry and Geale, 2011; Marcelino et al., 2012). *Anaplasma* species are transmitted by *Rhipicephalus* ticks*, R. decoloratus, R. microplus*, *R. evertsi evertsi* and *R. simus,* as well as *Hyalomma rufipes* (Norval et al., 1984). Transmission of *Anaplasma* spp*.* is transstadial or intrastadial, with the larvae, nymph and adult *R. microplus* ticks transmitting the pathogen (Aubry and Geale, 2011; Marcelino et al., 2012). The tick acquires the pathogen while feeding on infected animal (Figure 1.3). Replication in the ticks occurs in the midgut epithelial cells and progress to the salivary glands, and the pathogen will then be transmitted during the next blood meal (Atif, 2015). The life cycle of *Anaplasma* spp*.* in cattle has an incubation period of 7 - 60 days (Aubry and Geale, 2011).

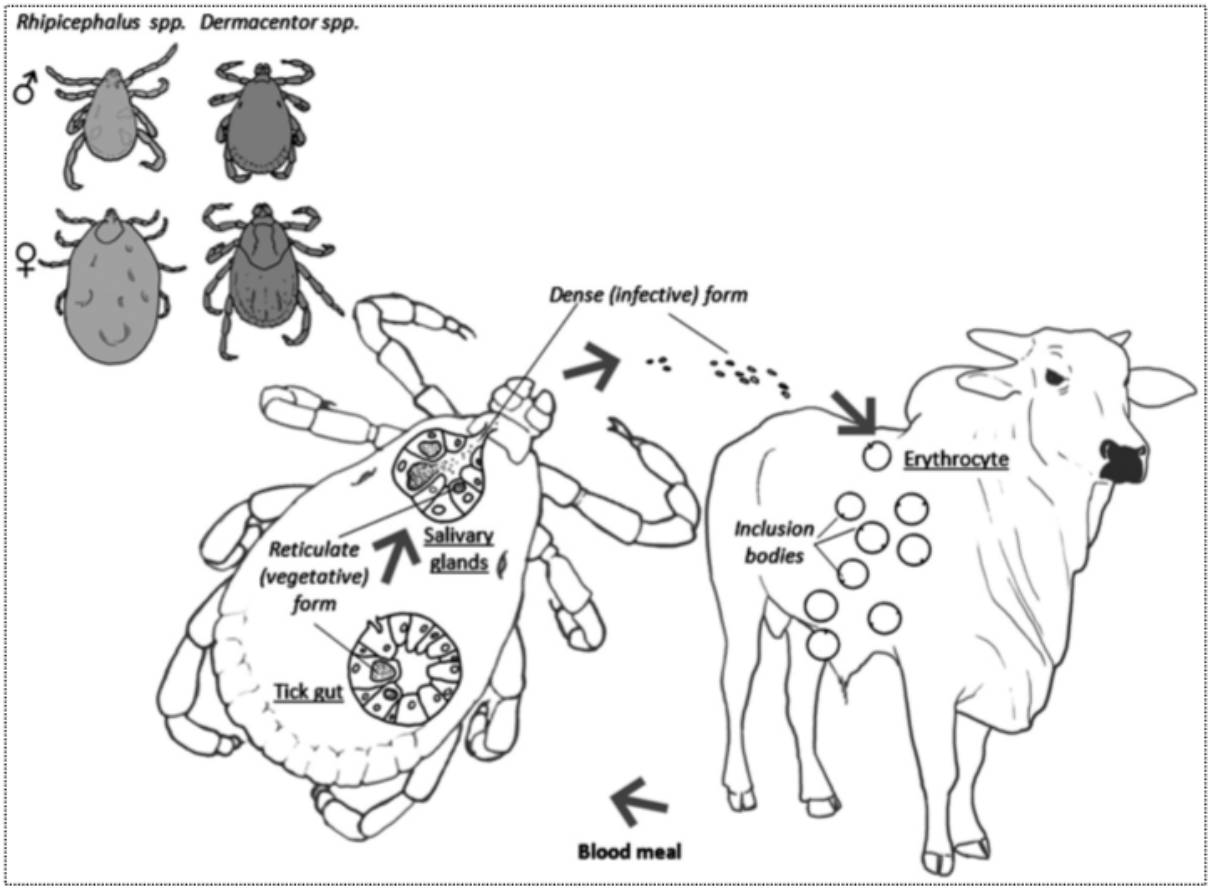


Figure1.3: The development lifecycle of *Anaplasma* parasites in cattle and the tick vector (Marcelino et al., 2012).

1.4.4. Heartwater

Heartwater (also known as Cowdriosis) is a fatal tick-borne disease affecting cattle, sheep, goats and some wild ruminants throughout sub-Saharan Africa (Allsopp, 2010). The disease is caused by the obligate intracellular bacterium *Ehrlichia ruminantium*, in the family Anaplasmataceae, order Rickettsiales (Dumler et al., 2001). *Ehrlichia ruminantium* was originally named *Rickettsia ruminantium* (Cowdry, 1925); the name was changed to *Cowdria ruminantium* and then reclassified as *Ehrlichia ruminantium* (Dumler et al., 2001). Heartwater causes constraints to improvement of livestock; and the severity of the disease varies from peracute to mild forms (Allsopp, 2010; 2015). The peracute form of the disease is characterized by fever, convulsion and sudden death while in acute forms, cattle develop a sudden high fever, followed by the loss of appetite, depression, breathing and nervous system problems, incoordination and circling. Death usually occurs within a week. In subacute forms cattle experience prolonged fever, coughing, mild incoordination and death occurs within 1 –

2 weeks. A brief fever in mild forms is seen and animals usually recover (Cowdry, 1925; Allsopp, 2015).

It is difficult to quantify the economic impact of heartwater in African countries, including Zimbabwe since the definitive diagnosis of the disease is often not performed (Allsopp, 2010). Nonetheless, the distribution of heartwater follows that of its tick-vector. Several *Amblyomma* tick species are capable of transmitting *E. ruminantium* in sub-Saharan Africa. The two most important ticks are *Amblyomma hebraeum*, which is the main vector in southern Africa while *A. variegatum* is distributed widely in Africa (Allsopp, 2010). Both tick species are present in Zimbabwe. *Amblyomma hebraeum* was thought to be confined to the low-lying regions of the South and *A. variegatum* in the low-lying regions of the northwest, however, reports indicate a slow but progressive spreading and expansion of *A. hebraeum* and *A. variegatum* ticks towards areas within their climate niche in Zimbabwe (Estrada-Peña et al., 2008; Sungirai et al., 2015; 2017; Mandara, 2018).

Disease transmission and life cycle of *E. ruminantium*

Transmission of *E. ruminantium* by *Amblyomma* ticks occurs transstadially (Prozesky and Duplessis, 1987). The ticks become infected when feeding on acute to subacute infected hosts. The larvae or nymph acquires the pathogen and transmit it as nymph, if acquired by larvae, or adults, if acquired by nymph. Heartwater can also be transmitted vertically or through colostrum of carrier dams and also by intravenous inoculation of blood (Prozesky and Duplessis, 1987; Allsopp, 2010; Marcelino et al., 2012).

Amblyomma tick spp. acquires *E. ruminantium* parasites while feeding on infected animals. The life cycle is shown in Figure 1.4, briefly, the parasite invades the gut epithelial cells of the tick vector with subsequent stages developing within the salivary glands of the ticks (Prozesky and Duplessis, 1987; Marcelino et al., 2012). The parasite is then transmitted to the host through the tick salivary glands while feeding. Proliferation of the parasite takes place in the vascular endothelial cells, neutrophils and macrophages; and a biphasic developmental cycle occurs, presenting two morphologically distinct forms, which are the elementary body and the reticulate body. The elementary bodies enter the cells by phagocytosis and divide within

the intracytoplasmic vacuoles by binary fission, forming large colonies of reticulate bodies (Prozesky and Duplessis, 1987; Marcelino et al., 2012).

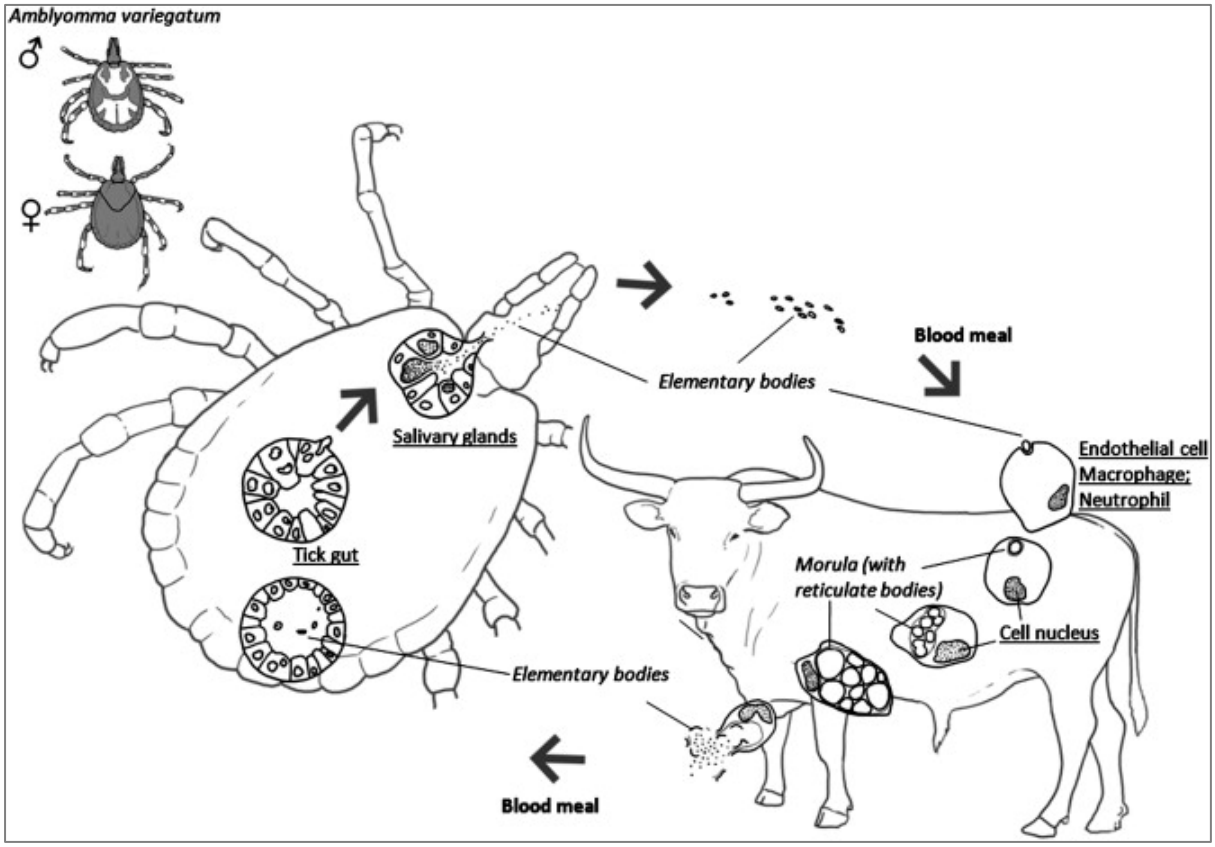


Figure1.4: The life cycle of *Ehrlichia ruminantium* in the tick vector and the vertebrate host. From Marcelino et al. (2012).

1.5. Diagnosis of tick-borne diseases

Diagnosis of tick-borne diseases in Zimbabwe is currently presumptive (Moyo et al., 2017; Manyenyeka et al., 2021). *Theileria parva* diagnosis in Zimbabwe is based on the identification of pathognomonic signs, followed by confirmatory diagnosis which includes microscopic demonstration of parasites in peripheral blood smears and in lymph node biopsy smear, and the epidemiological profile (Moyo et al., 2017; Manyenyeka et al., 2021). Since cattle can be infected with mixed haemoparasite infections, it is often impossible to diagnose diseases based only on the history, clinical signs and microscopic findings. In addition, misdiagnosis might also occur (Happi et al., 2020). Various methods that are used for the diagnosis of tick-borne diseases include:

Microscopic detection of haemoparasites

Microscopic detection of haemoparasites involves preparation, staining and examining blood smears and lymph node biopsy smears under a light microscope. This procedure is inexpensive and is the most commonly and routinely used method in most resource-limited countries (Happi et al., 2020). However, while *Anaplasma*, *Babesia*, *Ehrlichia* and *Theileria* can be identified in erythrocytes and leukocytes by morphological characteristics, it is difficult to accurately differentiate various species in each genus since piroplasms and shcizonts are very difficult to distinguish, especially with mixed haemoparasite infections (Norval et al., 1992b; Bock et al., 2004; Aubry and Geale, 2011). Moreover, the low sensitivity of microscopic diagnosis is also seen with the detection of carrier animals since the level of parasitaemia is often low (Happi et al., 2020).

Serological methods

Serological methods including the indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), card agglutination test (CAT) and complement fixation test (CFT) have been used for detection of haemoparasites (Bishop et al., 1992; Yunker, 1996; Katende et al., 1998; De Wall, 2000; Bock et al., 2004; Mans et al., 2015). However, limitations of these methods were linked to cross reactivity, false negative and false positive results, seen mostly in animals that remain seropositive while the parasite is cleared (Potgieter and Stoltsz, 2004). In addition, low sensitivity was reported with the detection of low parasitaemia and/or carrier animals (Bishop et al., 1992; Norval et al., 1992b; Yunker, 1996; Katende et al., 1998; De Wall, 2000; Mans et al., 2015).

Molecular methods

Molecular techniques such as the conventional polymerase chain reaction (PCR) assay, Reverse-line blot (RLB) hybridization assay, DNA sequencing and quantitative real-time PCR assay allow for the detection of parasite genomic material in blood, tissue or ticks. Molecular methods not only detect active infections but also very low parasitaemia especially in carrier animals. The sensitivity and specificity of molecular methods is higher than that of microscopy and serological methods (Salih et al., 2015).

- Conventional PCR assays

Conventional PCR assays involve the amplification of the target DNA. They consist of a series of repeated temperature changes whereby primers and DNA-polymerase enzyme are used for the replication of the target parasite genetic material. The assays are used for the detection of viral, bacterial and fungal pathogens, as well as gene analysis (Smeenk et al., 2000; Valones et al., 2009). The small ribosomal subunit (18 or 16S rRNA) is a frequently used molecular marker in PCR assay; however, other genes such as membrane proteins and cytochrome-b have also been used (Criado-Fornelio, 2007). Allsopp et al. (1993) detected six different *Theileria* species, which included *T. parva*, *T. annulata*, *T. mutans*, *T. taurotragi*, *T*. sp. Buffalo and *T*. sp. Marula in cattle using a PCR based on the V4 hypervariable region of the 18S r RNA gene. *Babesia bigemina* and *B. bovis* PCR assays that target the cytochrome b, rhoptry-associated protein-1a (Rap-1a) and spherical body protein 2 (SBP2) genes have been described (Fahrimal et al., 1992; Salem et al., 1999; Ringo et al., 2022). Polymerase chain reaction assays that target *msp1α*, *msp4*, and *groEL* genes have been used for detection and differentiation of rickettsial bacteria including *A. marginale* and *A. centrale* (de la Fuente et al., 2001; Lew et al., 2002; Shkap et al., 2002; Mtshali et al., 2007). A PCR assay targeting the pCS20 gene is often used for detection of *E. ruminantium* (Waghela et al., 1991; Van Heerden et al., 2004). Nested PCR assays were developed to improve the sensitivity and specificity of PCR. These assays involve two sequential PCR reactions using two sets of primer pairs (Green and Sambrook, 2019); whereby the amplicon of the first PCR reaction is used as a template for the second PCR reaction. Detection of tick-borne haemoparasites using nested PCR assays have been previously described (Allsopp et al., 1999; Smeenk et al., 2000; Decaro et al., 2008; Simuunza et al., 2011; Chaisi et al., 2017). Multiplex PCR assays have been developed to simultaneously detect various pathogen infections in a single sample. The technique uses two or more primer pairs to amplify different organisms simultaneously in a single PCR reaction (Bilgic et al., 2013). Figueroa et al. (1993) described a multiplex PCR assay for detection of *B. bigemina*, *B. bovis* and *A. marginale* in cattle. A limitation of multiplex PCR is that the use of multiple primer pairs in a single reaction can compromise the assay sensitivity (Lew and Jorgensen, 2005). Another PCR assay that allow analysis of multiple parasites in a single sample is the PCR-restriction fragment length polymorphism (PCR-RFLP) (Cacciò et al., 2000); and it has been used for detection and differentiation of *Theileria* spp. and *T. parva* species variants (Geysen et al., 1999; Bazarusanga et al., 2007; Sibeko et al., 2010). Agarose gel

electrophoresis is generally used to analyse amplicons at the end of the PCR reaction. The DNA molecular weight marker is used as a reference to estimate the size of the unknown DNA (Happi et al., 2020). Conventional PCR assays can be time consuming, and if the DNA was contaminated results could be ambiguous and misleading.

- Reverse-line blot hybridization assay

Reverse-line blot hybridization assay is used for the simultaneous detection and differentiation (especially within the same genus) of a wide range of haemoparasites. This method combines PCR amplification of the gene region of interest, followed by hybridization of PCR products with various genus- and species-specific oligonucleotide probes that are linked to the membrane. Oligonucleotide primers are designed based on the conserved region of the 18S or 16S rRNA gene to target tick-borne haemoparasites or rickettsial bacteria, respectively. These primers amplify all species within a specific genus, usually *Theileria, Babesia, Anaplasma* and *Ehrlichia* (Gubbels et al 1999; Bekker et al 2002). For detection of specific species, oligonucleotide probes are designed to target the V4 hypervariable region of the 18S rRNA gene for *Theileria* and *Babesia* species detection (Gubbels et al 1999), and the 16S rRNA gene V1 hypervariable region for detection of *Ehrlichia* and *Anaplasma* species (Bekker et al. 2002). The RLB assay has since been used for the identification and differentiation of *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species in various hosts and ticks; and also the identification of novel species or variants of these species (Georges et al., 2001; Nijhof et al., 2003; 2005; Bosman et al., 2007; Matjila et al., 2008; Oosthuizen et al., 2008; Berggoetz et al., 2014; Eygelaar et al., 2015; Njiiri et al., 2015; Byaruhanga et al., 2016; Tembo et al., 2018). However, the sensitivity of the RLB assay is limited as a result of competition for primers, especially in varying infection levels in mixed infections.

- Quantitative real-time PCR assay

The quantitative real-time PCR assay is a highly sensitive and specific diagnostic tool. The assay detects and differentiate different species in a sample, using fluorescent hybridization or hydrolysis probes (Criado-Fornelio, 2007). Various qPCR assays have been used previously for the detection of *Anaplasma marginale*, *A. centrale*, *Ehrlichia ruminantium*, *Babesia bovis*, *B. bigemina* and *Theileria parva* (Carelli et al., 2007; Kim et al., 2007; Sibeko et al., 2008; Cangi et al., 2017; Chaisi et al., 2017). Unlike the conventional PCR, qPCR assay is able to detect and

quantify the parasite DNA in a single tube, eliminating the need for post amplification manipulation; thus reducing the risk of contamination (Sibeko et al., 2008).

1.6. Molecular characterization of haemoparasites by DNA sequencing

Molecular characterization by DNA sequencing is increasingly applied to study phylogenetic relationships and taxonomy of tick-borne pathogens. DNA sequencing allows for the classification of novel species, as well as the Identification of species variants (Collins et al., 2002; Lew and Jorgensen, 2005; Allsopp and Allsopp, 2006; Kumar et al., 2021). In the procedure, polymerase chain reaction assay is used for the amplification of DNA using primers that are specific for the target gene, followed by cloning and sequencing. Briefly, the PCR product is cloned into a plasmid vector, to allow differentiation of multiple amplicons and to generate more copies of the specific amplicon. The specific amplicon is multiplied by transforming bacterial cells treated chemically or by electroporation to allow introduction of foreign DNA such as recombinant plasmid. The latter replicates in synchrony with transformed bacterial cells, thus increasing the copies of the amplicon. The recombinant plasmid DNA is then purified from bacterial cells and the ligated amplicon released using restriction enzymes for sequencing or directly subjected to sequencing using amplicon-specific primers. Various genes have been targeted for molecular characterization of tick-transmitted pathogens. For molecular characterization of *Anaplasma* and *Ehrlichia* species, genes that are considered include 16S rRNA, *msp4*, *msp1α*, *msp1-β*, *pCS20* and the heat shock protein *groEL* genes (Byaruhanga et al. 2018; Lew et al., 2002; Van Heerden et al., 2004; Shkap et al., 2002; Sisson et al., 2017). Characterization of *Babesia* and *Theileria* species has largely targeted the 18S rRNA and ITS genes (Gubbels et al., 2000; Nijhof et al., 2005; Matjila et al., 2008; Oosthuizen et al., 2008; Bosman et al., 2010; Chaisi et al., 2011). Molecular characterization has also been performed for differentiation of sub-species. In *Theileria parva,* genes encoding antigens such as *p67, p104, p150* and the polymorphic immunodominant molecule (*PIM*) have been used to differentiate cattle- and buffalo-associated *T. parva* strains (Bishop et al., 2001; Geysen et al., 2004; Sibeko et al., 2010; 2011). For *Babesia* species, molecular markers such as rap-1, gp45 and SBP-4 genes have been used have been used (Mtshali and Mtshali, 2013; Moumoni et al. 2015).

1.7. Control of tick-borne diseases in Zimbabwe

The control of tick-borne diseases in Zimbabwe was previously done by the Department of Veterinary services, financed by the government. However, due to budgetary constraints, this responsibility was transferred to the livestock owners; and this shift has changed the perception and attitude of farmers concerning control programs of tick-borne disease (Sungirai et al., 2016). The control of tick-borne diseases is usually achieved through a multifaceted approach, which includes control of tick infestations through the application of acaricides, control of animal movement, the use of therapeutic agents, vaccination and quarantine (Norval, 1979; Lawrence and Norval, 1987; Moyo et al., 2017). In Zimbabwe currently, there are no consistent strategies that effectively control and prevent tick-borne diseases (Shekede et al., 2021). However, the government is planning to prioritize the control of bovine theileriosis in resource-limited cattle farmers by dipping animals in acaricides (Manyenyeka et al., 2021).

CHAPTER 2

MATERIALS AND METHODS

2.1. Study area

Samples for this study were collected from Hurungwe district and Gonarezhou National Park (GNP), Zimbabwe (Figure 2.1). Hurungwe district is situated in Mashonaland West province in the North western part of Zimbabwe. The North western region of Zimbabwe has an altitude of 500 - 1000 m above sea level with 650 – 800 mm average annual rainfall; and temperatures ranging from 18 – 24 °C (Sungirai et al., 2016). Hurungwe district is divided into agricultural regions IIA (intensive farming), III (semi-intensive farming), IV (semi-extensive farming) and V (extensive farming); consisting of small, medium and large scale commercial farms, including safari and game reserves (Mbereko et al., 2015).

Gonarezhou National Park is situated in Masvingo province, Southeastern Zimbabwe. The National Park is the second largest National Park in Zimbabwe, following Hwange National Park. There is quite a number of large herbivores and carnivores which include the African buffalo, African elephant, giraffe, zebra, hippopotamus, blue wildebeest, sable antelope, roan antelope, waterbuck, nyala, kudu, impala, eland, African lion, spotted hyena and leopards, among others (Gandiwa, 2012, Gandiwa et al., 2013). Adjacent to the northern side of GNP, communities practice crop farming and livestock production (Gandiwa, 2011).

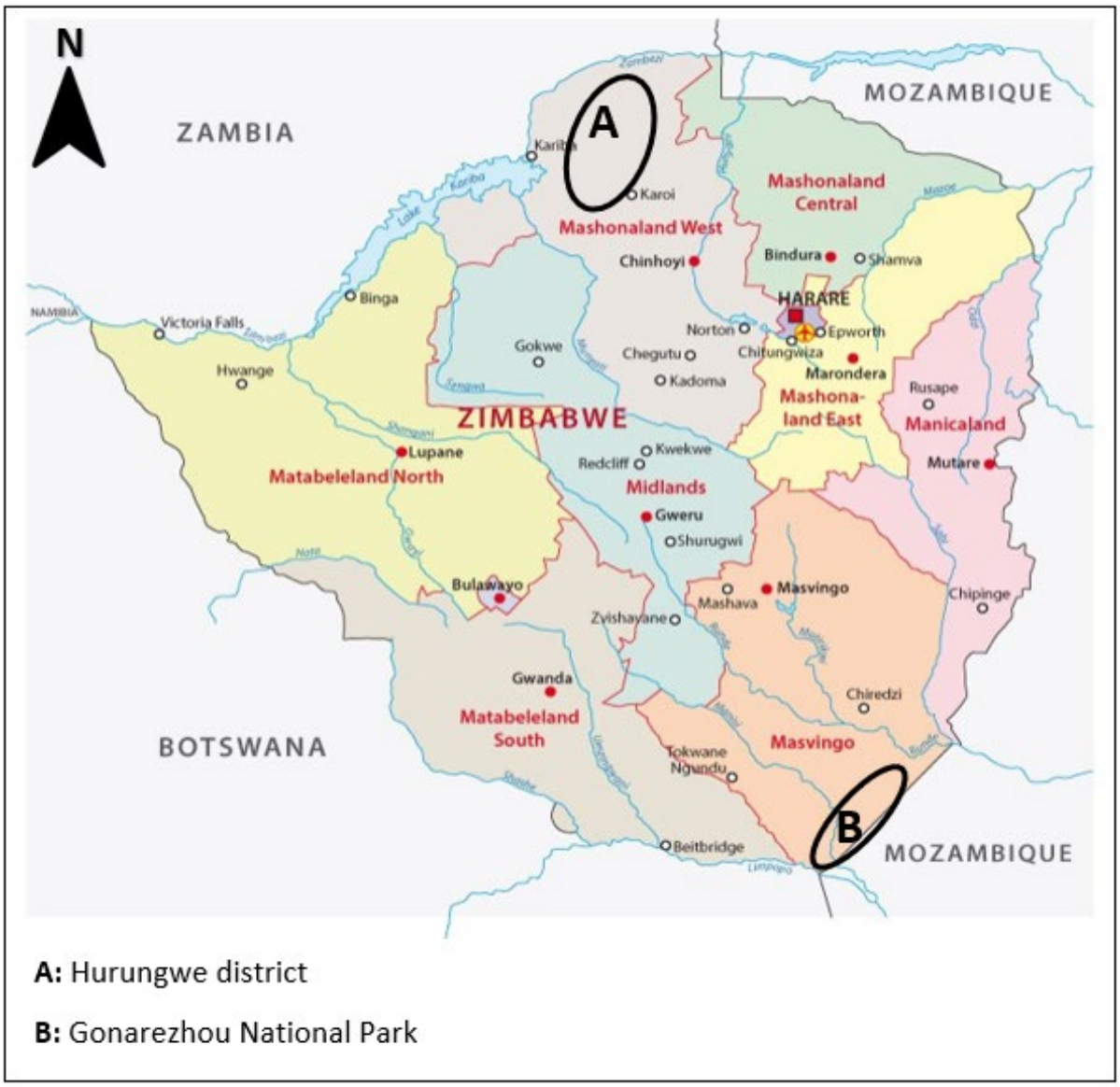


Figure 2. 1: Map of Zimbabwe showing the study area where samples originate.

Source: https://www.worldatlas.com/maps/zimbabwe

2.2. Sample collection

The blood samples used in the current study were provided by Chinhoyi University of Technology, Zimbabwe, with permission from the Department of Agriculture, Land Reform and Rural Development (DALRRD) (Section 20 Reference: 12/11/1/1/6; Import permit number: 201809002588). These included archived blood samples that were previously collected from cattle brought to the diptanks in Hurungwe district and buffalo from

Gonarezhou National Park, during the year 2016. Table 2.1 summarises the number of samples that were available for this study.

Table 2.1: The origin of blood samples used in the study

Province	District / Region	Host	Number of samples
Mashonaland West Province	Hurungwe district	Cattle	80
Masvingo Province	Gonarezhou National Park	Buffalo	7
Total			**87**

2.3. DNA extraction from blood spotted on FTA cards

DNA was extracted from 87 blood samples spotted on FTA (Flinders Technology Associates) cards, using the DNeasy® blood and tissue kit (Qiagen, Hilden, Germany), following the manufacturers protocol. Briefly, small pieces of dried blood spots were cut out of FTA cards using sterile surgical blades and placed into labelled 1.5 ml eppendorf tubes. One hundred and eighty (180) microlitres of the tissue lysing buffer, ATL, was added into tubes containing pieces of dried blood spots and incubated for 10 minutes at 85°C on a heating block. Twenty microlitres of Proteinase K (20 mg/ml) was added to all the tubes and the solution was mixed by vortexing. The tubes were then incubated for 1 hour at 56°C on a heating block. Subsequently, 200 µl of AL buffer was added to each tube and mixed again by vortexing. The mixture was incubated on a heating block at 70°C for 10 minutes, followed by centrifugation for 2-4 seconds. Two hundred (200) microlitres of 100 % ethanol was added to the tubes and mixed thoroughly by vortexing for 15 seconds. The mixture was then transferred into labelled QIAamp mini columns and centrifuged for 1 minute at 8 000 rpm. The filtrate was discarded, and 500 µl of the wash buffer, Buffer AW1 was added to the Qiagen Mini spin column and centrifuged at 8000 rpm for 1 minute. This was followed by a second wash using 500 µl of Buffer AW2 and centrifugation at 14000 rpm for 3 minutes. The filtrate was discarded and an additional 1 minute centrifugation was performed to remove residual wash buffer. The DNA was eluted in 80 µl of elution buffer, Buffer AE and stored at -20°C until further analysis.

2.4. Reverse-line blot hybridization assay

The 87 DNA samples were subjected to the reverse-line blot (RLB) hybridization assay for simultaneous detection of *Theileria*, *Babesia, Ehrlichia* and *Anaplasma* infections. The assay is based on the hybridization of biotinylated PCR products with a peroxidase-labelled streptavidin, which results in a chemiluminescence reaction indicating detection of a specific haemoparasite species or species variant. The (RLB) hybridization assay involves three steps that include 1. membrane preparation, 2. polymerase chain reaction, and 3. hybridization. The RLB assay used for this study followed the procedure reported by (Gubbels et al., 1999; Bekker et al., 2002; Nijhof et al., 2003; 2005).

2.4.1. Preparation of the RLB membrane

A Biodyne® C membrane (Separations, South Africa) was measured to the size of the support cushion of a MN45 miniblotter apparatus (Immunetics, Cambridge). The membrane was activated by incubation for 10 minutes in 10 ml freshly prepared 16% EDAC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) (Sigma, South Africa). The membrane was then placed on a support cushion in the miniblotter apparatus to load the oligonucleotide probes. *Theileria, Babesia, Anaplasma* and *Ehrlichia* genus- and species-specific oligonucleotide probes used are listed in Table 2.2. Each oligonucleotide probe was diluted in 0.5 M $NaHCO_3$ (pH 8.4) to a final concentration of 5 pmol/µl. One hundred and fifty microlitres of each diluted oligonucleotide probe was covalently attached onto the membrane in parallel lines followed by incubation for 2 minutes at room temperature. The membrane was inactivated with a freshly prepared 100 mM NaOH (Sodium hydroxide) for 8 minutes on a shaker at room temperature, and then washed in 100 ml 2X SSPE/0.1% SDS at 60°C for 5 minutes.

Table 2.2: Genus and species-specific oligonucleotide probes incorporated onto the RLB membrane. R=A/G, W=A/T and Y=C/T symbols were used to indicate degenerate positions

Pathogen	**Sequence (5'-3')**	**Reference**
Ehrlichia/*Anaplasma* group genus-specific probe	GGG GGA AAG ATT TAT CGC TA	Bekker et al. (2002)
Anaplasma centrale	TCG AAC GGA CCA TAC GC	Bekker et al. (2002)
Anaplasma marginale	GAC CGT ATA CGC AGC TTG	Bekker et al. (2002)
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG	Bekker et al. (2002)
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG	Bekker et al. (2002)
Anaplasma bovis	GTA GCT TGC TAT GRG AAC A	Bekker et al. (2002)
Anaplasma platys	CGG ATT TTT GTC GTA GCT TGC TAT GAT	Nijhof et al. (2003)
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT	Gubbels et al. (1999)
Anaplasma sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC	Bekker et al. (2002)
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA	Gubbels et al. (1999)
Theileria/*Babesia* group genus-specific probe	TAA TGG TTA ATA GGA RCR GTT G	Gubbels et al. (1999)
Theileria genus-specific probe	ATT AGA GTG TTT CAA GCA GAC	Nijhof (unpublished)
Babesia 1 genus-specific probe	ATT AGA GTG TTT CAA GCA GAC	Nijhof (unpublished)
Babesia 2 genus-specific probe	ACT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
Babesia felis	TTA TGC GTT TTC CGA CTG GC	Bosman et al. (2007)
Babesia divergens	ACT RAT GTC GAG ATT GCA C	Nijhof et al. (2003)
Babesia microti	GRC TTG GCA TCW TCT GGA	Nijhof et al. (2003)
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels et al. (1999)
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels et al. (1999)
Babesia rossi	CGG TTT GTT GCC TTT GTG	Matjila et al. (2004)
Babesia canis	TGC GTT GAC CGT TTG AC	Matjila et al. (2004)
Babesia vogeli	AGC GTG TTC GAG TTT GCC	Matjila et al. (2004)
Babesia lengau	CTC CTG ATA GCA TTC	Bosman et al. (2010)
Babesia bicornis	TTG GTA AAT CGC CTT GGT C	Nijhof et al. (2003)
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT	Butler et al. (2008)
Babesia gibsoni	TAC TTG CCT TGT CTG GTT T	Yisaschar-Mekuzas et al. (2013)
Babesia sp. (sable)	GCG TTG ACT TTG TGT CTT TAG C	Oosthuizen et al. (2008)
Theileria sp. kudu	CTG CAT TGT TTC TTT CCT TTG	Nijhof et al. (2005)
Theileria sp. sable	GCT GCA TTG CCT TTT CTC C	Nijhof et al. (2005)

Theileria bicornis	GCG TTG TGG CTT TTT TCT G	Nijhof et al. (2003)
Theileria annulata	CCT CTG GGG TCT GTG CA	Georges et al. (2001)
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT	Gubbels et al. (2000)
Theileria sp. buffalo	CAG ACG GAG TTT ACT TTG T	Oura et al. (2004)
Theileria mutans	CTT GCG TCT CCG AAT GTT	Gubbels et al. (1999)
Theileria parva	GGA CGG AGT TCG CTT TG	Nijhof et al. (2003)
Theileria taurotragi	TCT TGG CAC GTG GCT TTT	Gubbels et al. (1999)
Theileria veifela	CCT ATT CTC CTT TAC GAG T	Gubbels et al. (1999)
Theileria equi	TTC GTT GAC TGC GYT TGG	Butler et al. (2008)
Theileria lestoquardi	CTT GTG TCC CTC CGG G	Schnittger et al. (2004)
Theileria ovis	TTG CTT TTG CTC CTT TAC GAG	Bekker et al. (2002)
Theileria annae	CCG AAC GTA ATT TTA TTG ATT G	Yisaschar-Mekuzas et al. (2013)
Babesia leo	TTA TGC TTT TCC GAC TGG C	Bosman et al. (2007)
Babesia occultans	CCT CTT TTG GCC CAT CTC GTC	Anderson et al. (2013)

2.4.2. Polymerase chain reaction (PCR)

Two separate PCR master mixes were prepared for the specific amplification of either *Theileria*/*Babesia* or *Ehrlichia*/*Anaplasma* species. Primers RLB-F2 and a biotin-labelled RLB-R2 were used to amplify the V4 hypervariable region of the 18S rRNA gene of *Theileria* and *Babesia* (Gubbels et al., 1999; Nijhof et al., 2003) (Table 2.3). Another set of primers Ehr-F and a biotin-labelled Ehr-R were used to amplify the V1 hypervariable region of the 16S rRNA gene of *Anaplasma* and *Ehrlichia* (Bekker et al., 2002) (Table 2.3). The Platinum® Quantitative PCR SuperMix-UDG (Life Technologies™, South Africa) was used to prepare the PCR reaction mixture. For each 25 µl PCR reaction mixture, 12.5 µl of Platinum® Quantitative PCR SuperMix-UDG (containing 60 U/ml Platinum Taq DNA polymerase, 40 U/ml UDG, 100 mM KCl, 40 mM Tris-HCl (pH 8.4), 6 mM $MgCl_2$, 400 µM dATP, 400 µM dGTP, 400 µM dUTP and 400 µM dCTP), 0.2 µM of each primer, 7 µl PCR-grade water and 5 µl of DNA template was included. To monitor false positive and false negative results, *A. centrale* and *B. bigemina* were included as controls for 16S and 18S rRNA PCR reactions respectively, while nuclease free water was used as a no-template control. A touchdown thermal cycling programme shown in Table 2.4 was used for the amplification of *Ehrlichia*/*Anaplasma* and *Theileria*/*Babesia* species (Nijhof et al., 2005).

Table 2.3: Oligonucleotide primers used for the amplification of *Theileria*/*Babesia* and *Ehrlichia*/*Anaplasma* species.

Genus	Target gene	Primer	Sequence (5'-3')	Reference
Theileria/ *Babesia*	18S rRNA	RLB F2	GACACAGGGAGGTAGTGACAAG	Nijhof et al. (2003)
		RLB R2	Biotin-CTAAGAATTTCACCTCTAACAGT	Nijhof et al. (2003)
Anaplasma/ *Ehrlichia*	16S rRNA	Ehr-F	GGAATTCAGAGTTGGATCMTGGYTCAG	Bekker et al. (2002)
		Ehr-R	Biotin-CGGGATCCCGAGTTTGCCGGGACTTYTTCT	Bekker et al. (2002)

Table 2.4: Touchdown PCR program used for the amplification of *Theileria*/*Babesia* and *Ehrlichia*/*Anaplasma* species.

Cycle	Time	Temperature	Purpose
1 cycle	3 min	37°C	UDG activation
1 cycle	10 min	94°C	UDG inactivation and Taq polymerase activation
2 cycles	20 sec	94°C	Denaturing of double stranded DNA template
	30 sec	67°C	Primer annealing
	30 sec	72°C	PCR product extension by Taq polymerase
2 cycles	20 sec	94°C	Denaturing of double stranded DNA template
	30 sec	65°C	Primer annealing
	30 sec	72°C	PCR product extension by Taq polymerase
2 cycles	20 sec	94°C	Denaturing of double stranded DNA template
	30 sec	63°C	Primer annealing
	30 sec	72°C	PCR product extension by Taq polymerase
2 cycles	20 sec	94°C	Denaturing of double stranded DNA template
	30 sec	61°C	Primer annealing
	30 sec	72°C	PCR product extension by Taq polymerase

2 cycles	20 sec 30 sec 30 sec	94°C 59°C 72°C	Denaturing of double stranded DNA template Primer annealing PCR product extension by Taq polymerase
40 cycles	20 sec 30 sec 30 sec	94°C 57°C 72°C	Denaturing of double stranded DNA template Primer annealing PCR product extension by Taq polymerase
1 cycle	7 min	72°C	Final extension

2.4.3 Reverse-line blot hybridization

The hybridization step was performed as previously described by Nijhof et al. (2005). The previously prepared Biodyne®C membrane was activated with approximately 50 ml 2X SSPE/0.1% SDS at room temperature under gentle shaking for 5 minutes. The PCR products from the 18S and 16S rRNA gene amplification reactions (targeting *Theileria*/*Babesia* and *Ehrlichia*/*Anaplasma* species, respectively) were diluted with 130 µl of 2X SSPE/0.1% SDS. The diluted PCR products were denatured for 10 minutes using a thermal cycler, at 99.9°C then cooled on ice immediately. One hundred and fifty microlitres of denatured PCR products were loaded onto the membrane, such that they run across all the oligonucleotide probes previously attached to the membrane, and all empty slots were filled with 2X SSPE/0.1% SDS. The miniblotter was subsequently incubated for 60 minutes at 42°C to allow hybridization. The samples were then removed by aspiration and the membrane was removed from the miniblotter and placed in a plastic container. The membrane was covered with preheated 2X SSPE/0.5% SDS and then incubated with gentle shaking for 10 minutes at 50°C. This washing step was repeated twice before the membrane was incubated with 10 ml 2X SSPE/0.5% SDS and 12.5 µl (1.25 U) streptavidin-POD (peroxidase-labelled) conjugate (Roche Diagnostics, South Africa) for 30 minutes at 42°C. The membrane was further washed twice with preheated 2X SSPE/0.5% SDS for 10 minutes at 42°C and again twice with 2X SSPE for 5 minutes at room temperature. Six millilitres of enhanced chemiluminescence (3 ml ECL1 + 3 ml ECL2) (DNA Thunder™, Separation Scientific, South Africa) was added onto the membrane and mixed by gentle shaking for 1 min at room temperature. The membrane was then

exposed onto an X-ray film (X-OMAT™ Blue XB-1, Kodak, Separation Scientific, South Africa) for 5-20 minutes. Subsequently, the X-ray film was developed in the developer solution for approximately 30 seconds and rinsed with water; then fixed in the fixer solution for approximately 30 seconds, rinsed with water and allowed to dry. A positive reaction was visualized by dark spots on the X-ray film as a result of the chemiluminescence reaction (Gubbels et al., 1999). The membrane was stripped with 2 washes of pre-heated 1% SDS in a water bath set at 80°C for 30 minutes, with gentle shaking. Subsequently washed for 15 minutes at room temperature in 20 mM EDTA (pH 8.0) and then stored at 4°C in 20 mM EDTA.

2.5. Real-time quantitative polymerase chain reaction (qPCR) assays

Various quantitative real-time polymerase chain reaction assays were performed for the specific detection of various haemoparasites of cattle and buffalo that include *Anaplasma marginale*, *Anaplasma centrale*, *Babesia bigemina*, *Babesia bovis*, *Ehrlichia ruminantium* and *Theileria parva*.

2.5.1. Duplex qPCR assay for specific detection of *Anaplasma marginale* and *Anaplasma centrale*

The detection of *A. marginale* and *A. centrale* was accomplished using a published Taqman™ duplex qPCR assay (Decaro et al., 2008), with a modification of the *A. centrale* probe for analysis by the Lightcycler qPCR machine (Chaisi et al., 2017). The assay allows simultaneous, specific detection and quantification of both *A. marginale* and *A. centrale* targeting the *msp1β* and *groEL* genes, respectively.

Briefly, the 20 µl PCR reaction mixture consisted of 4 µl Fast Start Taqman™ mix (Roche Diagnostics), 0.5 µl UDG, 0.6 µM of each *A. marginale*-specific primer (Table 2.5), 0.9 µM of each *A. centrale*-specific primer (Table 2.5), 0.2 µM of each species-specific probe (Table 2.5), 5 µL of template DNA and nuclease-free water. Nuclease-free water was included as a no-template control. The DNA that was extracted from an *A. centrale* vaccine strain (Onderstepoort Biological Products) was used as a positive control. A field sample, C14, collected from a cow in the Mnisi Community area (Mpumalanga Province, South Africa), and

confirmed to be *A. marginale* positive by sequencing of the *msp1β* gene, was used as *A. marginale* positive control (Khumalo et al., 2016; Chaisi et al., 2017).

Table 2.5: Oligonucleotide primers and probes used for the *Anaplasma marginale* and *Anaplasma centrale* duplex qPCR assay.

***Anaplasma* species**	**Target gene**	**Primer**	**Sequence (5'-3')**	**Reference**
A. marginale	*msp1β*	AM-For	TTGGCAAGGCAGCAGCTT	Carelli et al. (2007)
		AM-Rev	TTCCGCGAGCATGTGCAT	Carelli et al. (2007)
		AM-Pb	6FAM-TCGGTCTAACATCTCCAGGCTTTCAT-BHQ1	Carelli et al. (2007)
A. centrale	*groEL*	AC-For	CTATACACGCTTGCATCTC	Decaro et al. (2008)
		AC-Rev	CGCTTTATGATGTTGATGC	Decaro et al. (2008)
		AC-Pb	LC610-ATCATCATTCTTCCCCTTTACCTCGC-BHQ2	Chaisi et al. (2017)

The amplification reaction was performed using the Roche LightCycler® real-time PCR machine with thermal cycling conditions that included UDG activation at 40°C for 10 minutes, pre-incubation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute and annealing-extension at 60°C for 1 minute (using the quantitative analytical mode). The cooling step was performed at 40°C for 30 seconds. Results were analyzed using the Lightcycler® Software version 4.0 (Roche Diagnostics, Mannheim, Germany). A positive result was indicated by a quantification cycle (Cq value) at which the fluorescence from amplification exceeded the background fluorescence. The cut-off threshold was set at 36 cycles. A lower Cq value corelated with a higher starting concentration of DNA in a sample (Decaro et al., 2008; Chaisi et al., 2017).

2.5.2. pCS20 Sol1^{TqM} qPCR assay for specific detection of *Ehrlichia ruminantium*

A pCS20 Sol1^{TqM} qPCR assay was used for the detection of *E. ruminantium* parasite DNA in cattle and buffalo DNA samples (Cangi et al., 2017). The assay targets a highly conserved pCS20 gene region of *E. ruminantium*. Reactions prepared in a final volume of 25 µl contained 12 µl TaqMan® Universal PCR Master Mix (composed of AmpliTaq Gold® DNA polymerase LD, dNTPs with dUTPs/dTTP blend and optimized buffer components) (LTC Tech South Africa (Pty)

Ltd), 0.625 µM of each pCS20 Sol1^{TqM} primer (Table 2.6), 0.5 µM of the probe (Sol1P) (Table 2.6) and 5 µl of template DNA. Nuclease-free water was used as a no-template control and DNA extracted from an *E. ruminantium* vaccine strain (Onderstepoort Biological Products) was included as a positive control.

Table 2.6: Oligonucleotide primers and probe used for *Ehrlichia ruminantium* pCS20 Sol1 qPCR assay.

Species	**Primer and probe**	**Sequence (5'-3')**	**Reference**
E. ruminantium	Sol1 F	ACAAATCTGGYCCAGATCAC	Cangi et al. (2017)
	Sol1 R	CAGCTTTCTGTTCAGCTAGT	Cangi et al. (2017)
	Slo1^{TqM} probe	6-FAM-ATCAATTCACATGAAACATTACATGAAG-BHQ1	Cangi et al. (2017)

The assay was performed using the StepOnePlus^{TM} real-time PCR machine (Applied Biosystems, Life Technologies, South Africa), with thermal cycling conditions including UNG incubation at 50°C for 2 minutes, followed by a cycle of AmpliTaq Gold pre-activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 55°C for 1 minute. Results were analyzed using Applied Biosystems StepOnePlus^{TM} Real-time PCR system software version 2.3 (Life Technologies, South Africa). The cut-off threshold for *E. ruminantium* positive samples was set at 37 cycles (Cangi et al., 2017).

2.5.3. *Babesia bigemina* and *Babesia bovis* qPCR assays

Two Taqman-based real-time PCR assays were performed, each for the specific detection of *B. bigemina* and *B. bovis* parasite DNA. The two assays were performed as previously described by Kim et al. (2007), however, the forward primer for *B. bovis* qPCR was modified (Table 2.7) and the annealing temperature was increased from 55°C to 57°C (Byaruhanga et al., 2022). For each of the qPCR assays, a reaction mixture was prepared as follows:

Babesia bigemina qPCR assay reaction mixture consisted of 8 µl of TaqMan® Universal PCR Master mix, 0.5 µM of each *B. bigemina*-specific primers (BiF and BiR) and 0.25 µM of *B. bigemina* probe (BiP) (Table 2.7), 5 µl of DNA template and nuclease-free water, making up a total volume of 20 µl per reaction. Nuclease-free water was used as a no-template control and DNA extracted from *B. bigemina* vaccine strain (Onderstepoort Biological Products, Pretoria, South Africa) was used for the positive control.

Babesia bovis qPCR assay reaction mixture consisted of 8 µl of TaqMan® Universal PCR Master mix, 0.5 µM each of *B. bovis*-specific primers (BoF2 and BoR) and 0.25 µM of *B. bovis* probe (BoP) (Table 2.7), 5 µl of DNA template and nuclease-free water to make up a total volume of 20 µl per reaction. Nuclease-free water was used as a no-template control and DNA extracted from *B. bovis* vaccine strain (Onderstepoort Biological Products, Pretoria, South Africa) was used for the positive control.

Table 2.7: Oligonucleotide primers and probes used for *Babesia bigemina* qPCR and *Babesia bovis* qPCR assays.

Species	Primer or probe	Sequence (5'-3')	Reference
B. bigemina	BiF	AATAACAATACAGGGCTTTCGTCT	Kim et al. (2007)
	BiR	AACGCGAGGCTGAAATACAACT	Kim et al. (2007)
	BiP probe	VIC-TTGGAATGATGGTGATGTACAACCTCA-TAMRA	Kim et al. (2007)
B. bovis	BoF2	GGTTTCGCCTGTATAATTG	Byaruhanga et al. (2022)
	BoR	AGTCGTGCGTCATCGACAAA	Kim et al. (2007)

	BoP probe	FAM-CCTTGTATGACCCTGTCGTACCGTTGG-TAMRA	Kim et al. (2007)

The StepOnePlus™ real-time PCR machine (Applied Biosystems, Life Technologies, South Africa) was used, and the thermal cycling conditions included a UNG incubation at 50°C for 2 minutes, followed by AmpliTaq Gold pre-activation at 95°C for 10 minutes and 45 cycles of denaturation at 95°C for 20 seconds followed by a 1-minute annealing-extension at 55°C for the *B. bigemina* qPCR assay. The annealing temperature for the *B. bovis* qPCR assay was adjusted to 57°C. Results for both assays were analyzed using the StepOnePlus™ Real-Time PCR software version 2.3 (Life Technologies, South Africa). The cut-off threshold for *B. bigemina* and *B. bovis* positive samples was set at 37.1 and 39 cycles, respectively (Kim et al., 2007; Byaruhanga et al., 2022). DNA samples that gave a Cq value above the cut-off value were considered false positive.

2.5.4. *Theileria parva*-specific qPCR assay

All the DNA samples were subjected to *T. parva*-specific qPCR assay as described by Sibeko et al. (2008). The oligonucleotide primers and probes used are listed in Table 2.8. The 20 µl PCR reaction mixture consisted of 4 µl FastStart DNA Masterplus Hybprobe (Roche Diagnostics, Mannheim, Germany), 0.5 µl Uracil-deoxy-glycosylase (UDG) (Roche Diagnostics, Mannheim, Germany), 0.5 mM of each primer, 0.1 mM of each hybridization probe, 4 µl of DNA template and nuclease-free water. Nuclease-free water was used as a no-template control and a *T. parva* positive sample KNP102 (Sibeko et al., 2008) was used for the positive control reaction.

Table 2.8: Primers and probes used in the hybridization probed-based T. parva-specific real-time PCR assay

Species/genus	Primer/probe	Sequence (5'-3')	Reference
T. parva	Forward primer	CTGCATCGCTGGTGTCCCTT	Sibeko et al. (2008)
Theileria-genus	Reverse primer	ACCAACAAAATAGAACCAAAGTC	Sibeko et al. (2008)
T. parva	Anchor probe	GGGTCTCTGCATGTGGCTTAT-FL	Sibeko et al. (2008)

	Sensor probe	LCRed640-TCGGACGGAGTTCGCT-PH	
Theileria-genus	Anchor probe	AGAAAATTAGAGTGCTCAAAGCAGGCTTT-FL	Sibeko et al. (2008)
	Sensor probe	LCRed705-GCCTTGAATAGTTTAGCATGGAAT-PH	

The assay was performed using the Roche LightCycler® real-time PCR machine (Roche Diagnostics, Mannheim, Germany) with thermal cycling conditions that included UDG activation at 40°C for 10 minutes, followed by the pre-incubation step at 95°C for 10 minutes. The amplification step consisted of 45 cycles of denaturing at 95°C for 10 seconds, annealing at 58°C for 10 seconds and extension at 72°C for 15 seconds. For the melting curve analysis, the temperature was gradually increased from 40°C to 95°C at 0.2°C/second heating rate. The fluorescence values were measured at 640 and 750 nm. The melting peak at Tm 63°C ± 0.62°C was indicative of the presence of *T. parva* nucleic acid in a sample (Sibeko et al., 2008).

2.6. 16S rRNA and 18S rRNA gene cloning and sequencing

The DNA samples that hybridized exclusively to the genus-specific probes on the RLB hybridization assay and tested negative on all the species-specific qPCR assays, were subjected to cloning and sequencing of the near full-length small subunit ribosomal RNA (16S rRNA and 18S rRNA) gene for species characterization.

2.6.1. Amplification of the 16S rRNA gene

Nine DNA samples (Table 2.9) were selected for the amplification of the near full-length 16S rRNA gene (1600 bp) for *Anaplasma* species characterization. The DNA samples were amplified by conventional PCR as previously described by Weisburg et al. (1991). Each reaction mixture consisted of 12.5 µl Phusion Flash High-Fidelity PCR Mastermix (Thermo Fisher Scientific, South Africa), 0.2 µM each of fD1 and rP2 primers (Table 2.10), 5 µl DNA and nuclease-free water to make up a total volume of 25 µl. A field sample (C14) and nuclease-free water were used as positive and no-template controls, respectively. Thermal cycling conditions included an initial denaturation at 98°C for 10 seconds, 30 cycles of denaturation

at 98°C for 1 second, annealing at 55°C for 5 seconds and extension at 72°C for 15 seconds, followed by a final extension at 72°C for 1 minute.

Table 2.9: DNA samples selected for the amplification, cloning and sequencing of the 16S and 18S rRNA gene

District / Region	Sample ID	Host	Gene for cloning and sequencing
Hurungwe district	NDT28	Cattle	16S rRNA
	NDT24		
	N45		
	MDT9		
	MDT23		
	M24		
	M21		
	PDT9		
	X31		
	NDT12	Cattle	18S rRNA
	NDT26		
	N25		
	MDT28		
	MDT37		
	M16		
	M34		
Gonarezhou National Park	BSC	Buffalo	18S rRNA

Table 2.10: Oligonucleotide primers used for the amplification of the 16S and 18S rRNA gene

Gene	Primer	Sequence (5'-3')	Reference
16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	Weisburg et al. (1991)
	rP2	ACGGCTACCTTGTTACGACTT	Weisburg et al. (1991)
18S rRNA	Nbab1F	AAGCCATGCATGTCTAAGTATAAGCTTTT	Oosthuizen et al. (2008)
	TB 18S-R	GAATAATTCACCGGATCACTCG	Matjila et al. (2008)

A 1.5% agarose gel stained with ethidium bromide was prepared and used to analyze the PCR products. Five microliters of PCR products mixed with 1 µl loading dye were loaded on the gel and electrophoresis was performed at 120 volts for 40 minutes. A ready-to-use 100 bp plus GeneRuler DNA ladder (Thermo Fisher Scientific, South Africa) was used as a marker to allow

estimation of the size of the PCR product. The gel was analyzed using the GelDoc imaging system (Bio-Rad, CA United States).

2.6.2. Amplification of the 18S rRNA gene

Eight DNA samples (Table 2.9) selected for cloning and sequencing of the near full-length 18S rRNA gene (1 700bp) were amplified by conventional PCR using primers previously described (Matjila et al., 2008; Oosthuizen et al., 2008) for *Babesia* and *Theileria* species characterization. Each reaction mixture consisted of 12.5 µl Phusion Flash High-Fidelity PCR Mastermix (Thermo Fisher Scientific, South Africa), 0.2 µM of each primer (Table 2.10), 5 µl of DNA template and nuclease-free water to make up a total volume of 25 µl. Nuclease-free water was used as a no-template control. Positive controls included cDNA isolated from *T. parva* Muguga strain and DNA extracted from the *B. bigemina* vaccine strain (Onderstepoort Biological Products, Pretoria, South Africa). Thermal cycling conditions included initial denaturation set at 98°C for 5 minutes, 40 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 1 minute, and a final extension at 72°C for 1 minute. Due to low levels of *T. parva* infection in field samples, following the gel electrophoresis analysis, the sensitivity of the PCR was increased by using 1 µl of the primary PCR products as a template in a re-amplification PCR reaction. Thermal cycling conditions were kept the same as for the primary PCR except that the amplification cycles were reduced to 25.

2.6.3. Purification and cloning of the 16S and 18S rRNA gene

Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacture's protocol. The PCR products were eluted in 30 µl instead of the recommended 50 µl, to increase the DNA concentration. To determine the purity and concentration of the purified products, a 1.5 % agarose gel containing ethidium bromide was prepared and analyzed as described in 2.6.1. Five microlitres of the purified PCR products were ligated onto the pJET1.2 cloning vector using the CloneJET PCR cloning kit (Thermo Fisher Scientific, South Africa) following the manufacture's protocol. The 20 µl ligation

reaction consisted of 10 µl 2x reaction buffer, 1 µl pJet 1.2/blunt cloning vector, 1 µl T4 DNA ligase, 5 µl purified PCR product and nuclease free water. The ligation reactions were incubated for 15 minutes at room temperature. Five microlitres of the ligated products were transformed into NEB 5-alpha F' Iq Competent *E. coli* cells (C2992I) (New England Biolabs, MA, USA) following the manufacture's protocol. Transformants were screened on Luria Bertani (LB) plates containing 100 µg/mL ampicillin (Thermo Fisher Scientific, South Africa). The plates were incubated overnight at 37°C.

Up to 20 single colonies were picked from each plate and screened for the presence of the insert by performing colony PCR in a 20 µl reaction consisting of 10 µl DreamTaq Green PCR Mastermix (Thermo Fisher Scientific, South Africa), 0.4 µl each of fD1 and rP2 primers and nuclease-free water. For the 18S rRNA gene, colony PCR was performed using 18S rRNA primers (Nbab1F and TB 18S-R). Thermal cycling conditions included an initial denaturation at 95°C for 3 minutes, 25 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes. Colony PCR products were visualized on gel electrophoresis and the clones with the correct insert were re-grown in LB liquid medium (Thermo Fisher Scientific, South Africa) overnight at 37°C. Plasmid isolation was performed using the Thermo-Fisher Scientific GeneJET Plasmid miniprep kit following the manufacturer's protocol. The purified plasmids were subjected to the conventional PCR described in section 2.6.1 (16S rRNA) and 2.6.2 (18S rRNA) to confirm the presence of the insert. Following gel electrophoresis analysis, plasmids with the correct insert were sent to Inqaba Biotechnologies (South Africa) for sequencing.

2.6.4. Sequencing and sequence data analyses

Sequencing was performed bidirectionally using the 16S rRNA gene primers (fD1 and rP2) and the 18S rRNA gene primers (Nbab1F and TB 18S-Rev). The quality of the sequences was evaluated using Chromas version 2.6.6 (2018) (Technelysium Pty Ltd, South Brisbane, Queensland Australia). The forward and reverse sequences were assembled using Pregap4 of the Staden package (version 1.6-r for Windows) (Bonfield et al., 1995). The assembled sequences were edited in Gap4 (Staden package) and a consensus sequence was created. The consensus sequence was subjected to the Basic Local Alignment Search Tool (BLAST) to search

for homologous sequences from GenBank. Multiple sequence alignments of the consensus sequences and reference sequences (Table 2.11) were performed using MAFFT (Katoh et al., 2019). The alignment was exported in Fasta format and truncated to the size of the shortest sequence using Bioedit version 7.2.5 (Hall, 1999). To determine the genetic differences between sequences, a pairwise distance analysis based on the number of nucleotide differences was performed using Mega7 (Kumar et al., 2016).

Table 2.11: GenBank reference sequences used for creating a multiple sequence alignment for the 16S and 18S rRNA gene

GenBank accession number	**Classification**	**Origin**	**Source / Host**
KM114613	*Anaplasma bovis*	Malaysia	*Macaca fascicularis*
LC269823	*Anaplasma* sp. ZAM dog-181	Lusaka, Zambia	Dog
KX505295	*Anaplasma* sp. isolate HN670	China	Cattle
KU586172	*A. platys*	China, Wuhan City	*Anopheles sinensis*
MG869532	*A. capra*	South Korea	Korean Water Deer
JQ917885	*A. ovis*	China	*Dermacentor niveus*
KU686792	*A. marginale*	Uganda	Cattle
AF414873	*A. marginale*	South Africa	Cattle
AF414878	*A. marginale*	Zimbabwe	Cattle
MF289481	*A. centrale*	China	Cattle
KP006404	Uncultured *Anaplasma* clone	Philippines	Dog
MT197260	*Cutibacterium acnes*	China	Human, Infants
MH699352	Propionibacteriaceae bacterium	Canada	Food
NR_041517	*Microlunatus panaciterrae*	South Korea	Soil
KU922133	*Neisseria flavescens*	China	Infant formula
MT482687	*Streptococcus infantis*	South Korea	Human
MN134515	*B. gibsoni*	India	Canine
MN134507	*B. gibsoni*	India	Canine

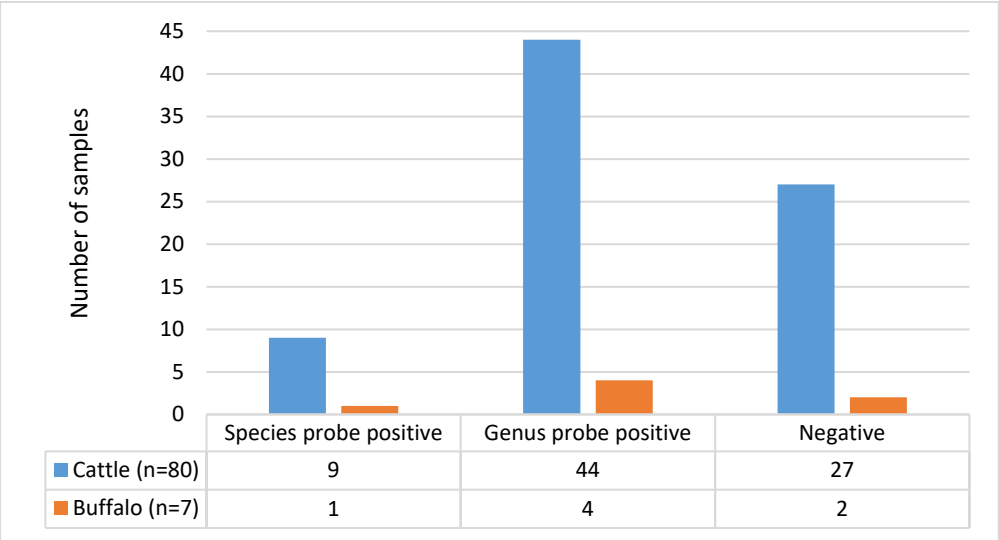
JX962779	*B. microti*	China	Fox
KY805840	*B. odocoilei*	China	Goat
MH651211	*T. equi*	China	Horse
MT903302	*T. bicornis*	South Africa	Black rhinoceros
KX115426	*T. buffeli*	China	Cattle
MH208639	*T. orientalis*	China	*Haemaphysalis longicornis*

CHAPTER 3

RESULTS

3.1. Reverse line blot (RLB) hybridization assay results

A total of 87 DNA samples were screened for the presence of *Theileria*, *Babesia*, *Anaplasma,* and *Ehrlichia* infections using the RLB hybridization assay. Of these, 58 (67%) tested positive for haemoparasite infections, while 29 (33%) samples were negative or had an infection below the detection limit of the test (Figure 3.1).



	Species probe positive	Genus probe positive	Negative
Cattle (n=80)	9	44	27
Buffalo (n=7)	1	4	2

Figure 3.1: A bar graph representing the RLB hybridization analysis, indicating the species-specific and genus-specific probe hybridization with various host DNA.

3.1.1. Tick-borne pathogens detected in cattle

Of the 80 DNA samples from cattle, 53 (66 %) were positive for tick-borne pathogens when screened using the RLB hybridization assay. These consisted of samples that hybridized exclusively to the genus-specific probes (n=44) and samples that also hybridized to the species-specific probes (n=9). Single parasite infections were indicated for 6 samples, while 3

samples hybridized to more than one species-specific probe, indicating a mixed infection (Figure 3.2). Neither *Ehrlichia ruminantium* nor *Theileria parva* could be detected in any of the cattle samples tested using the RLB hybridization assay.

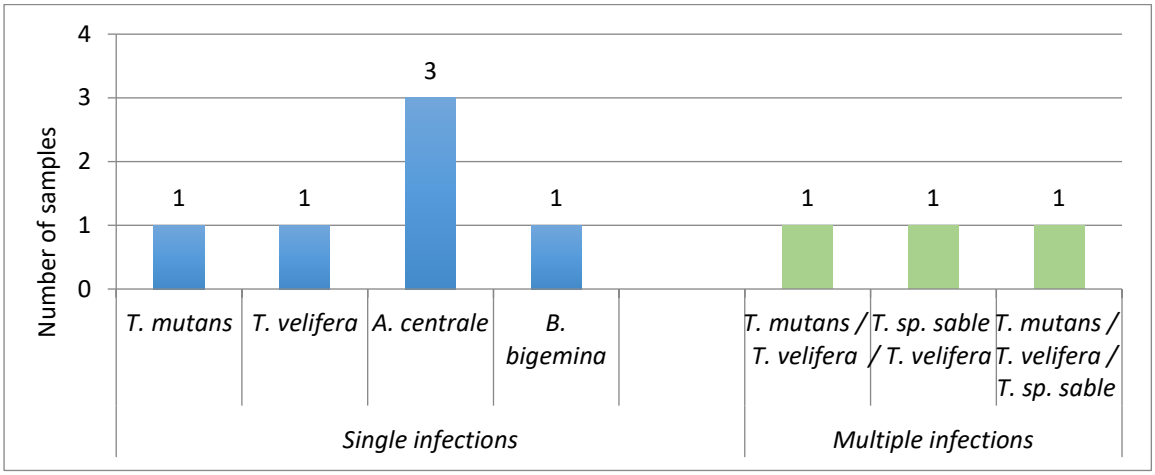


Figure 3.2: A bar graph representing the tick-borne pathogens detected in cattle DNA samples using the RLB hybridization assay. Three *Theileria* species, one *Babesia* species and one *Anaplasma* species were detected.

Forty-four DNA samples hybridized exclusively to the genus-specific probes. The various combinations detected are shown in Figure 3.3 below.

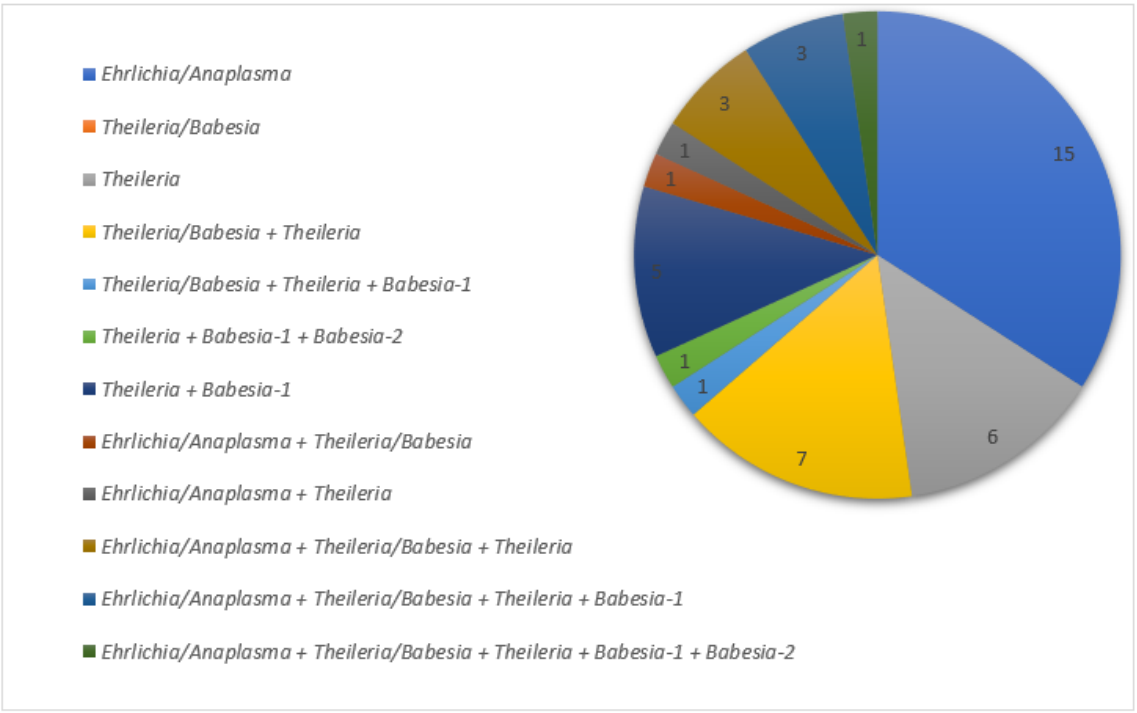


Figure 3. 3: An illustration of the genus-specific probes that were detected in cattle using the RLB hybridization assay.

3.1.2. Tick-borne pathogens detected in buffalo

Five (71 %) buffalo samples tested positive for tick-borne pathogens using the RLB hybridization assay. Of these positive samples, 1 (14 %) hybridized to the *Theileria mutans* species-specific probe and 4 (57 %) hybridized to genus-specific probes only as shown in Figure 3.4. Mixed parasite infections were not detected in buffalo using the RLB hybridization assay.

Ehrlichia ruminantium and *Theileria parva* were not detected in any of the buffalo DNA samples tested using the RLB hybridization assay.

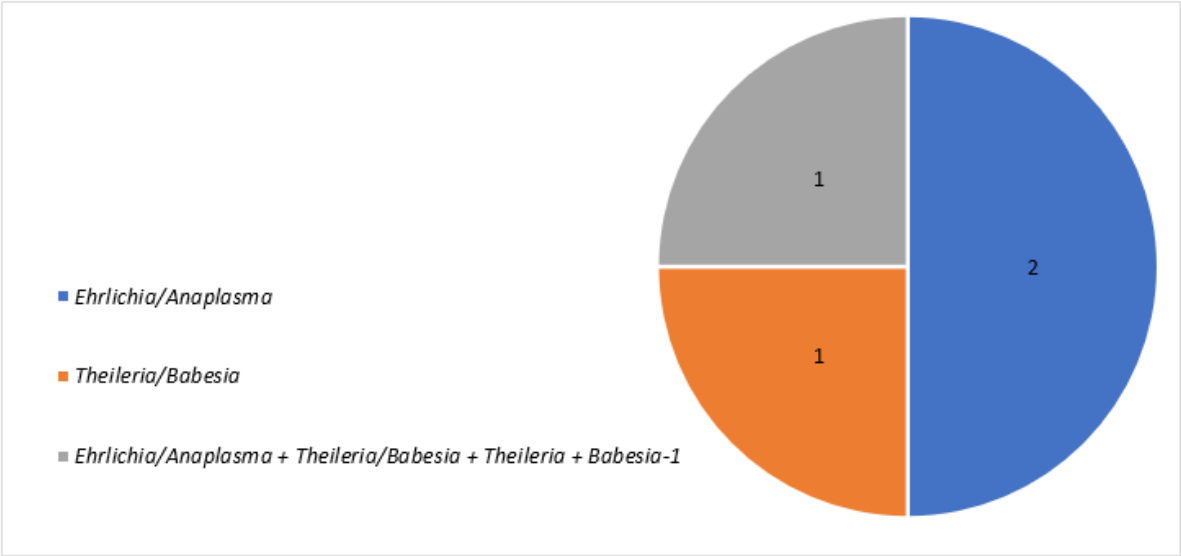


Figure 3.4: The genus-specific probes detected in buffalo using the RLB hybridization assay.

3.2. Quantitative real-time Polymerase Chain Reaction (qPCR) assays

For the specific detection of tick-borne pathogens, various quantitative real-time polymerase chain reaction assays were performed.

3.2.1. Specific detection of *Anaplasma marginale* and *Anaplasma centrale*

The modified duplex qPCR assay successfully detected and differentiated between *A. marginale* and *A. centrale*, which were detected in the FAM and LC610 channels, respectively (Figure 3.5 and 3.6). *Anaplasma marginale* was detected in 28 samples and *A. centrale* was detected in 7 samples (Figure 3.7).

Mixed infections of *Anaplasma marginale* and *Anaplasma centrale* were detected in cattle and buffalo (Figure 3.7).

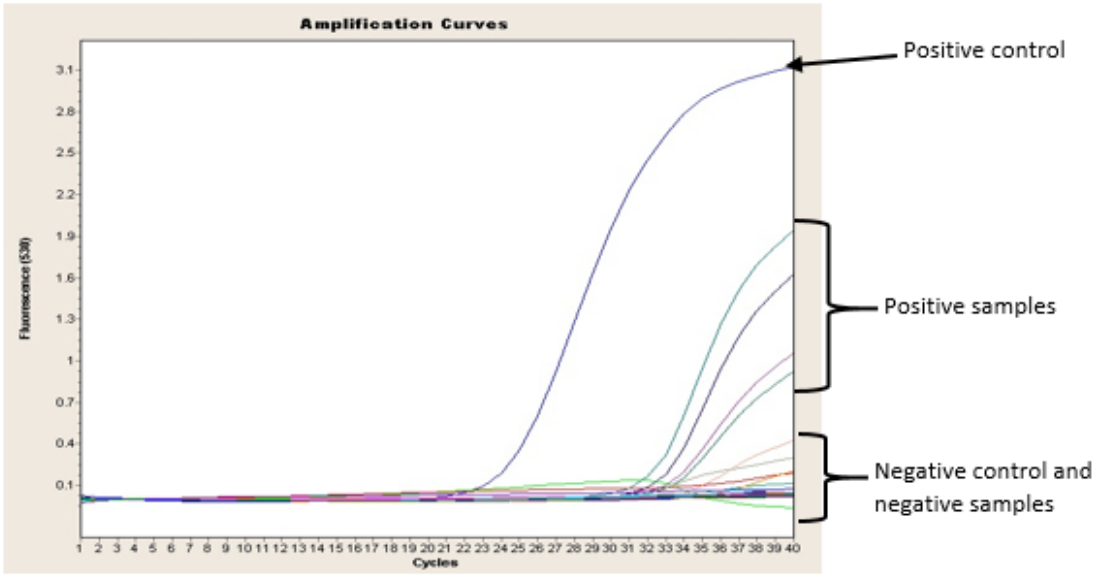


Figure 3.5: Detection of *Anaplasma marginale* in positive control and positive samples using the duplex qPCR assay, indicated by an increase in the fluorescence signal detected in the FAM channel (530 nm).

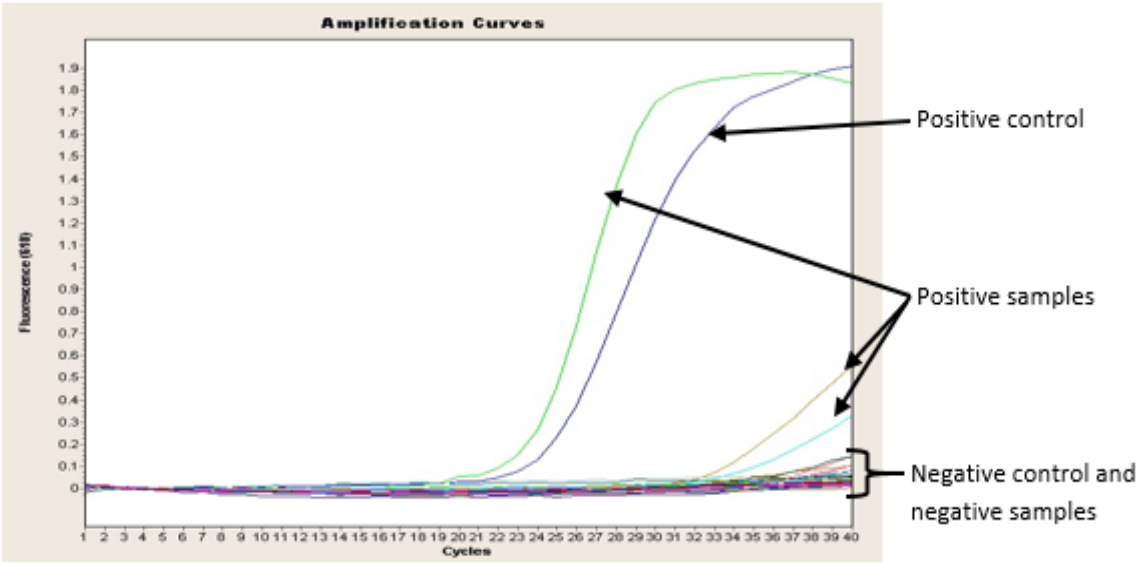


Figure 3.6: Detection of *Anaplasma centrale* in positive control and positive samples using the duplex qPCR assay, indicated by an increase in the fluorescence signal detected in the LC610 channel (610 nm).

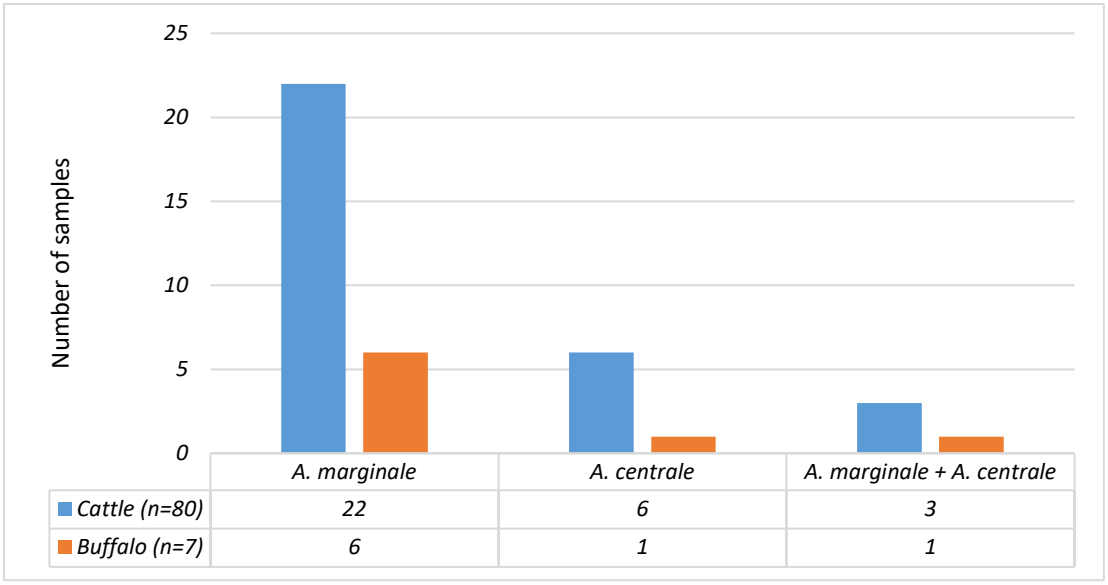


Figure 3.7: Distribution of single and mixed *Anaplasma marginale* and *Anaplasma centrale* infections in cattle and buffalo samples, detected using the modified duplex qPCR assay (Chaisi et al., 2017).

3.2.2. Detection of *Babesia bigemina* and *Babesia bovis*

Babesia bigemina (n=7) and *B. bovis* (n=2) were detected in cattle samples (Figure 3.8). All the buffalo samples tested negative for *B. bigemina* and *B. bovis*. The amplification plots generated for *B. bigemina* and *B. bovis* positive samples are shown in Figure 3.9 and 3.10 respectively.

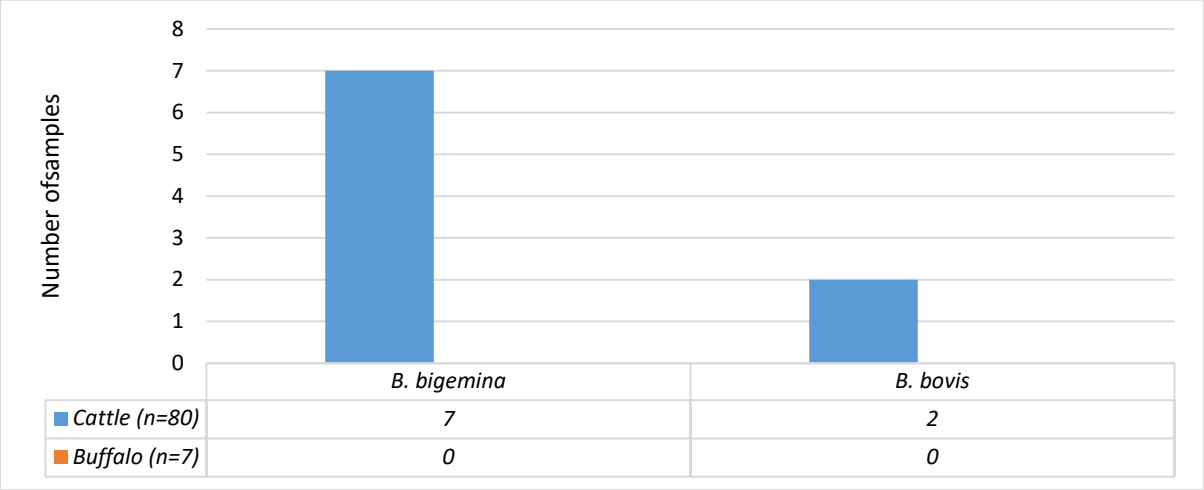


Figure 3.8: Distribution of *Babesia bigemina* and *Babesia bovis* infections in cattle and buffalo detected using the qPCR assays (Kim et al., 2007; Byaruhanga et al., 2022).

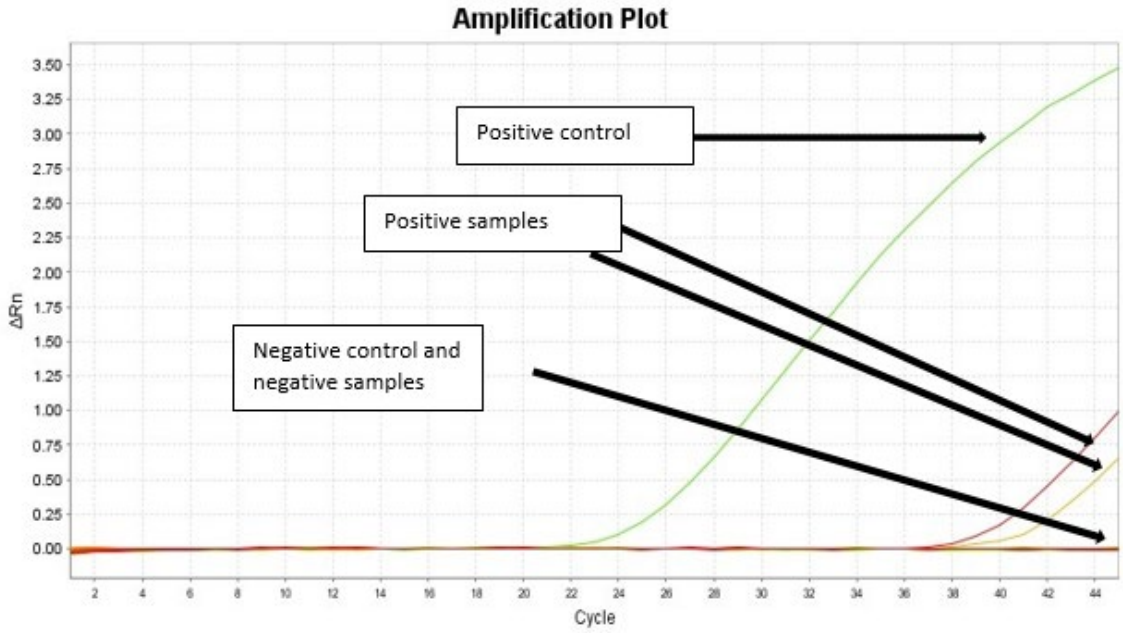


Figure 3.9: Detection of *Babesia bigemina* in positive control and positive samples using the qPCR assay, indicated by an increase in the fluorescence signal.

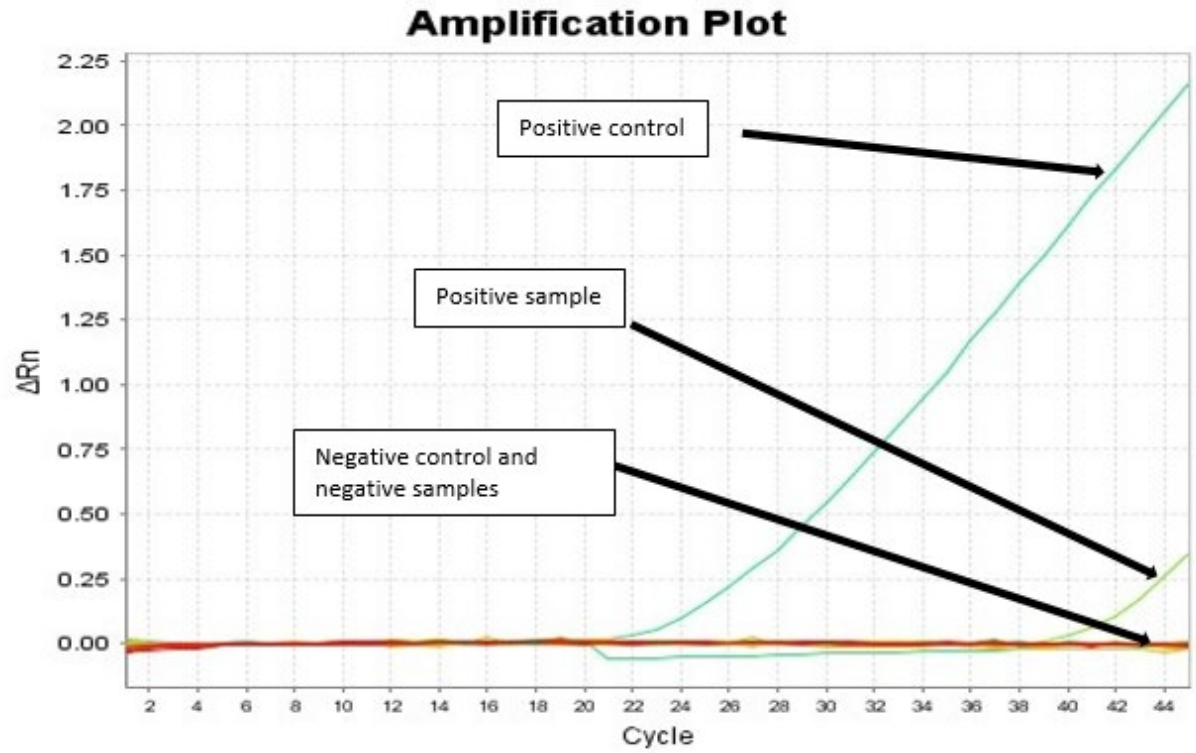


Figure 3.10: Detection of *Babesia bovis* in positive control and positive samples using the qPCR assay, indicated by an increase in the fluorescence signal.

Babesia bigemina and *B. bovis* mixed infections could not be detected in any of the samples tested. However, mixed infection consisting of *B. bigemina* and *A. marginale* were detected from two cattle samples. *Babesia bovis* and *A. marginale* mixed infection were detected in one cattle sample.

3.2.3. Detection of *Ehrlichia ruminantium*

All 87 DNA samples subjected to the pCS20 Sol1 qPCR assay were negative for *E. ruminantium* (Figure 3.11).

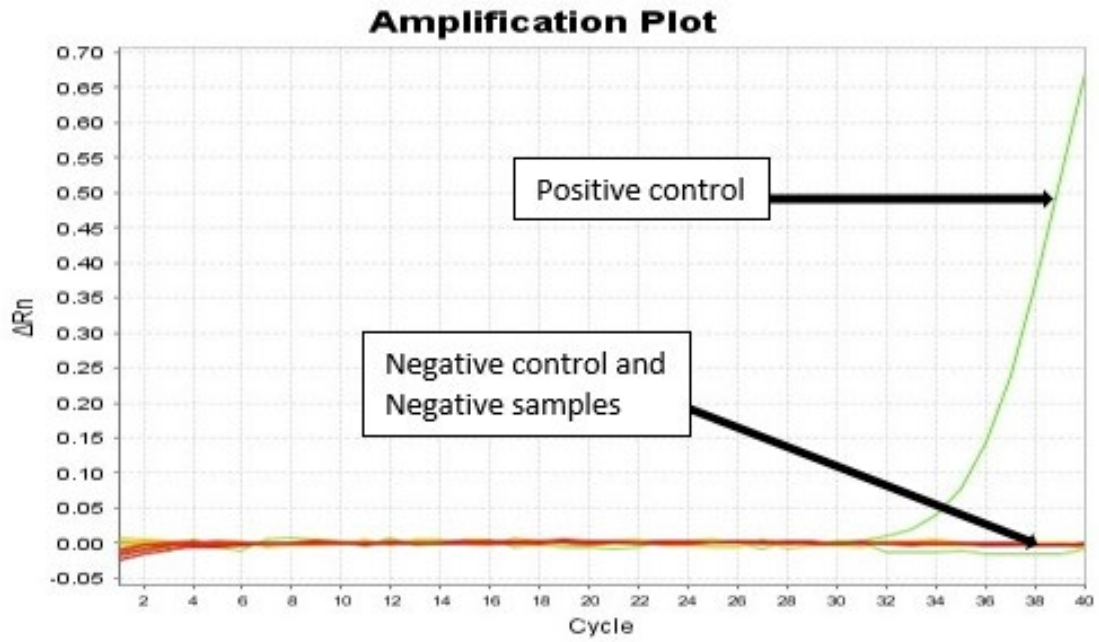


Figure 3.11: Detection of *Ehrlichia ruminantium*. The amplification curve shows the fluorescence that was generated in the *E. ruminantium* positive control sample. There was no increase of the fluorescence signal for the negative control and tested samples.

3.2.4. *Theileria parva* real-time PCR results

All 87 DNA samples subjected to the *T. parva* real-time polymerase chain reaction assay were negative for *T. parva*.

3.3. Detection of single and mixed haemoparasite infections

The overall haemoparasite infections detected in the current study are summarized in Table 3.1. A high number of single infections were detected using the species-specific qPCR assays (n=30) compared to the RLB (n=7). Of the single infections detected, *A. marginale* was identified in 16 (20 %) cattle and 5 (71 %) buffalo samples. *Babesia bigemina* was detected in 5 (6 %) cattle samples using qPCR assay, while only 1 (1 %) sample was positive for *B. bigemina* with RLB assay. Nine (11.3 %) cattle samples were positive with two or three haemoparasite infection, indicating mixed parasite infections (Table 3.1). Only 1(14 %) buffalo sample was positive for *A. marginale* and *A. centrale* mixed infection.

Table 3. 1: The single and mixed haemoparasite infections detected in the current study

Haemoparasites detected	**Cattle (n=80)**		**Buffalo (n=7)**	
	RLB assay	**qPCR assay**	**RLB assay**	**qPCR assay**
Single infections	**6 (7.5 %)**	**25 (31 %)**	**1 (14 %)**	**5 (71 %)**
A. marginale	0	16	0	5
A. centrale	3	3	0	0
B. bigemina	1	5	0	0
B. bovis	0	1	0	0
T. mutans	1	0	1	0
T. velifera	1	0	0	0
Mixed infections	**3 (3.8 %)**	**6 (7.5 %)**	**0**	**1 (14 %)**
A. marginale + *A. centrale*	0	3	0	1
A. marginale + *B. bigemina*	0	2	0	0
A. marginale + *B. bovis*	0	1	0	0
T. velifera + *T. mutans*	1	0	0	0
T. velifera + *T*. sp. sable	1	0	0	0
T. velifera + *T. mutans* + *T*. sp. sable	1	0	0	0

3.4. DNA sequence analysis

Seventeen DNA samples that hybridized exclusively to the genus-specific probes on the RLB hybridization assay were selected for further analysis. These included nine DNA samples for the 16S rRNA and eight for the 18S rRNA gene cloning and sequencing. Nine 16S rRNA amplicons were obtained and only one 18S rRNA amplicon (Figures 3.12).

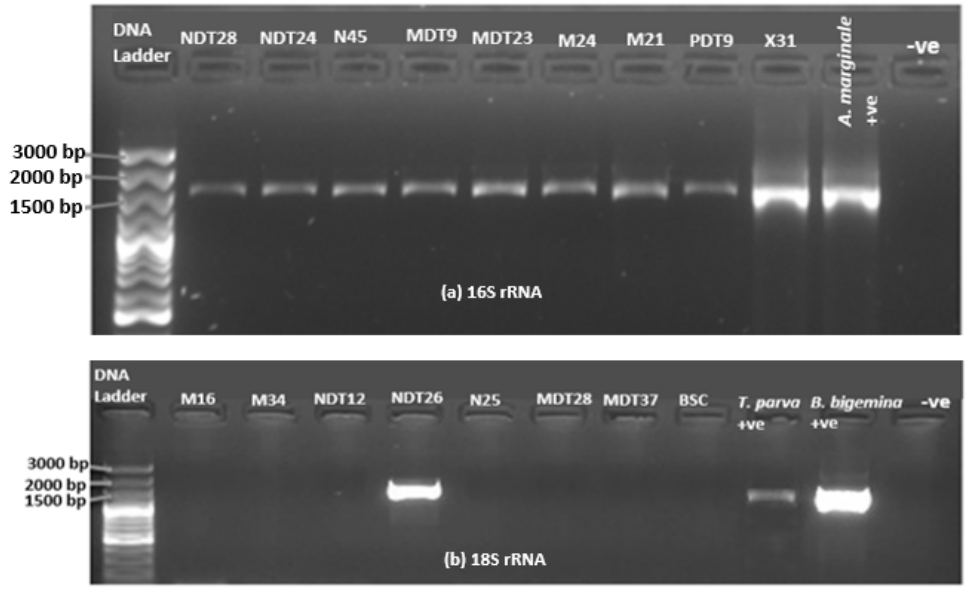


Figure 3.12: PCR products of the 16S rRNA (a) (1600 bp) and 18S rRNA (b) (1700 bp) genes. The products were separated on a 1,5 % agarose gel stained with ethidium bromide. A 100bp plus DNA ladder (Thermo Fisher Scientific, South Africa) was used to determine the size of the PCR products.

3.4.1. 16S rRNA sequence data analysis

Eight good-quality sequences were obtained from the nine samples that were sequenced. The BLASTn homology search targeting the genus *Anaplasma* revealed various species with percentage identities ranging from 78.94 - 81.51. These included *A. marginale* strain from Uganda (accession KU686792) 78.94 % identity, *A. marginale* strain from Zimbabwe (accession AF414878) 79.88 % identity, *A. marginale* strain from South Africa (accession AF414873) 79.83 % identity, *A. bovis* (accession KM114613) 79.24 % identity, *A. bovis* (accession MH255928) 79.17 % identity, *Anaplasma* sp. Isolate (accession KX505295) 79.17 % identity, *A. platys* (accession KU586172) 79.12 % identity, *A. platys* (accession KU586183) 79.04 % identity, *A. capra* (accession MG869532) 79.75 % identity, *A. capra* (accession MH762075) 81.51 % identity and *A.* sp. ZAM dog-181 (accession LC269823) *80.36* % identity. The general sequence similarity search revealed a high percentage identity with *Cutibacterium acnes* (accession MT197260) for samples M21, NDT28, PDT 9, and X31. Samples N45, M24, MDT9 and MDT23 were highly identical to *Streptococcus infantis* (99.79 % identity), *Neisseria flavescens* (100 % identity), *Microlunatus ginsengisoli* (96.98 % identity) and *Janthinobacterium svalbardensis* (100 % identity) respectively.

Pairwise distance analysis conducted using sequences obtained in this study and NCBI reference sequences are shown in Tables: 3.2, 3.3, 3.4 and 3.5. The analysis revealed a high number of nucleotide differences between the sequences obtained from the current study against the *Anaplasma* species. Conversely, high sequence similarities were seen with other bacterial species such as the *Cutibacterium acnes*, *Streptococcus infantis*, *Neisseria flavescent*, *Microlunatus ginsengisoli,* and *Janthinobacterium svalbardensis*.

The multiple sequence alignment of samples sequenced in this study, indicated high levels of nucleotide differences in the RLB *Ehrlichia/Anaplasma* genus-specific probe region (Figure 3.12). The probe region was 100 % identical to published *Anaplasma* spp. sequences that were detected in the Blastn homology searches.

Table 3.2: Pairwise distance analysis showing the number of nucleotide differences (from 1356 bp) between the sequences obtained in the current study and the reference sequences from GenBank

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Anaplasma marginale* Uganda strain_KU686792														
2	*Anaplasma marginale* South Africa_AF414873	0													
3	*Anaplasma marginale* Zimbabwe_AF414878	1	1												
4	*Anaplasma bovis*_KM114613	64	64	65											
5	*Anaplasma bovis*_MH255928	58	58	59	12										
6	*Neisseria flavescens*_KU922133	303	303	302	315	317									
7	*Neisseria flavescens*_KC178495	303	303	302	315	317	0								
8	M24	303	303	302	315	317	0	0							
9	*Cutibacteriumacnes*_MT197260	338	338	339	340	340	336	336	336						
10	M21	338	338	339	340	340	336	336	336	0					
11	*Propionibacteriaceae bacterium*_MH699352	338	338	339	340	340	336	336	336	0	0				
12	PDT9	337	337	338	339	339	335	335	335	1	1	1			
13	NDT28	337	337	338	339	339	335	335	335	1	1	1	0		
14	X31	338	338	339	340	340	336	336	336	0	0	0	1	1	

The number of base differences per sequence between sequences are shown. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1356 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Table 3.3: Pairwise distance analysis showing the number of nucleotide differences between sample MDT9 and the reference sequences from GenBank

		1	2	3	4	5	6	7	8	9	10	11	12
1	MDT9												
2	Uncultured *Microlunatus* sp. clone SGR11_JQ793387	7											
3	Uncultured_soil_bacterium_clone_451_AY493981	8	11										
4	*Microlunatus panaciterrae*_NR-041517	33	32	33									
5	*Microlunatus_ginsengisoli*_NR-041383	31	30	35	33								
6	*Anaplasma* sp. ZAM dog-181_LC269823	293	292	294	289	290							
7	Uncultured_*Anaplasma*_sp._clone_Dog8_KP006404	294	293	295	290	294	23						
8	*Anaplasma platys*_KU586183	296	295	297	292	296	22	5					
9	*Anaplasma platys*_KU586172	294	293	295	290	294	22	5	4				
10	*Anaplasma ovis*_JQ917885	296	293	297	285	297	45	40	39	39			
11	*Anaplasma ovis*_KJ410245	297	294	298	286	298	43	40	39	39	2		
12	*Anaplasma* sp. isolate GZ74_KX505300	297	294	298	286	298	43	40	39	39	2	0	

The number of base differences per sequence between sequences is shown. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1311 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Table 3.4: Pairwise distance analysis showing the number of nucleotide differences between sample MDT23 and the reference sequences from GenBank

		1	2	3	4	5
1	*Janthinobacterium svalbardensis*_MZ311851					
2	MDT23	0				
3	*Janthinobacterium* sp. MS-Y-S4_KU671185	0	0			
4	*Anaplasma capra*_MG869532	279	279	279		
5	*Anaplasma capra*_MH762075	282	282	282	5	

The number of base differences per sequence between sequences are shown. The analysis involved 5 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1355 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Table 3.5: Pairwise distance analysis showing the number of nucleotide differences between sample N45 and the reference sequences from GenBank

		1	2	3	4	5
1	*Streptococcus infantis*_MT482687					
2	N45	2				
3	*Streptococcus infantis*_MT512092	4	4			
4	*Anaplasma platys*_KU586183	301	301	300		
5	*Anaplasma platys*_KU586172	300	300	299	4	

The number of base differences per sequence between sequences is shown. The analysis involved 5 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1333 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

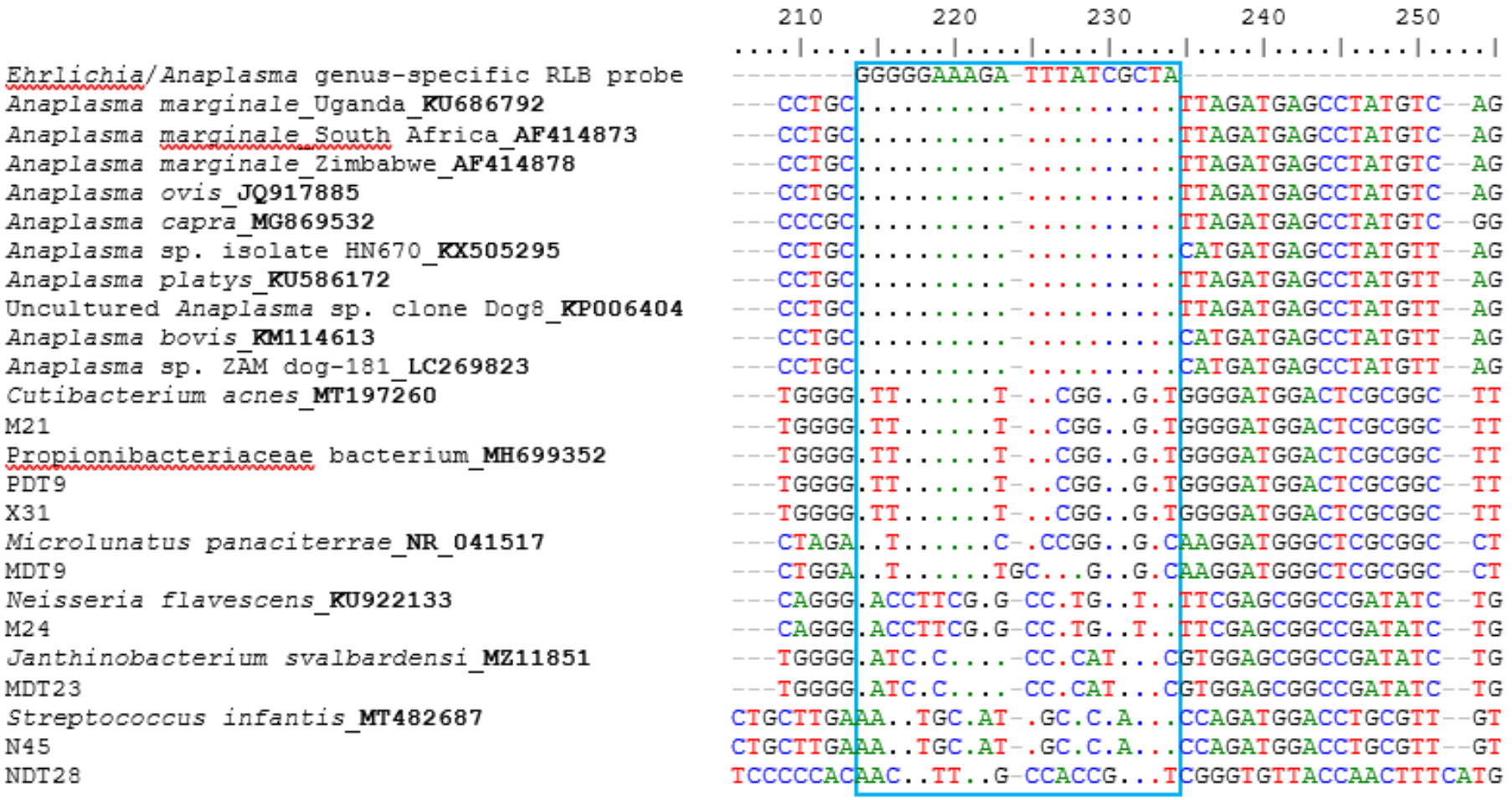


Figure 3.13: Multiple sequence alignment of the 16S rRNA gene. The region enclosed in the blue box represents the RLB Ehrlichia/Anaplasma genus-specific probe region. The alignment is made up of the 16S rRNA gene sequences obtained from the current study (M21, PDT9, X31, MDT9, M24, MDT23, N45, NDT28), various *Anaplasma* spp. and other bacterial species sequences downloaded from GenBank. Sequence similarities in the RLB probe region are represented by dots. Nucleotide differences in the probe region are shown with nucleotide letters, i.e. A, C, G, or T.

3.4.2. 18S rRNA sequence data analysis

One good quality sequence was obtained for the analysis of the 18S rRNA gene. BLASTn homology searches targeting the genus *Babesia* revealed various *Babesia* species, including *B. gibsoni* (accession MN134515) 86.72 % identity, *B. gibsoni* (accession MN134507) 86.72 % identity, *B. microti* (accession JX962779) 86.72 % identity, *B. odocoilei* (accession KY805840) 86.50 % identity. Homology searches targeting the genus *Theileria* revealed *T. equi* (accession MH651211) 83.67 % identity, *T. tachyglossi* (accession KY953258) 83.67 % identity, *T. bicornis* (accession MT903302) 83.07 % identity, *T. orientalis* (accession MH208639) 85.30 % identity and *T. buffeli* (accession KX115426) 83.67 % identity. The general sequence similarity search revealed high % identities with *Hausmanniella discoidea* (accession EU039900) 97.80 % identity, *Colpoda* sp. strain (accession HFCC1224) 97.00 % identity, *Kalometopia duplicata* (accession KJ873050) 97.01 % identity, *Exocolpoda augustini* (accession KJ607919) 97.21 % identity and *Kreyellidae* sp. MD-2012 (accession JQ723971) 96.34 % identity.

A pairwise distance analysis revealed a high number of nucleotide differences (204 – 219 nucleotide differences) between the sequence obtained from the current study compared with the *Babesia* and *Theileria* species in GenBank (Table 3.6). Species known to infect cattle, such as *Theileria parva, Babesia bigemina* and *B. bovis* were not detected in the BLASTn homology searches. The multiple sequence alignment revealed two nucleotide differences in the experimental sequence compared to the RLB *Theileria/Babesia* genus-specific probe region used in the study (Figure 3.13).

Table 3.6: Pairwise distance analysis showing the number of nucleotide differences between the sequences obtained in the current study against Babesia spp. Theileria spp. and other protozoan parasite sequences from GenBank

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	NDT26															
2	*Hausmanniella discoidea*_EU039900	17														
3	*Colpoda* sp._strain HFCC1224_MT001198	17	27													
4	*Kalometopia duplicata*_KJ873050	18	23	20												
5	*Kreyellidae* sp. MD-2012_JQ723971	25	26	27	24											
6	*Exocolpoda augustini*_KJ607919	18	25	17	12	25										
7	*Babesia gibsoni*_MN134515	216	216	220	216	219	209									
8	*Babesia gibsoni*_MN134507	217	217	221	217	220	210	1								
9	*Babesia microti*_JX962779	214	215	218	215	216	208	12	13							
10	*Babesia odocoilei*_KY805840	214	219	221	217	220	212	48	49	46						
11	*Theileria equi* MH651211	204	206	210	201	210	203	129	130	128	120					
12	*Theileria bicornis*_MT903302	219	219	220	214	217	213	115	116	111	100	68				
13	*Theileria orientalis*_MH208639	214	217	217	209	215	210	124	125	124	111	73	70			
14	*Theileria buffeli*_KX115426	214	217	217	209	215	210	124	125	124	111	73	70	0		
15	*Theileria tachyglossi*_KY953258	212	208	213	205	211	205	126	127	124	126	94	71	104	104	

The number of base differences per sequence from between sequences are shown. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1335 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

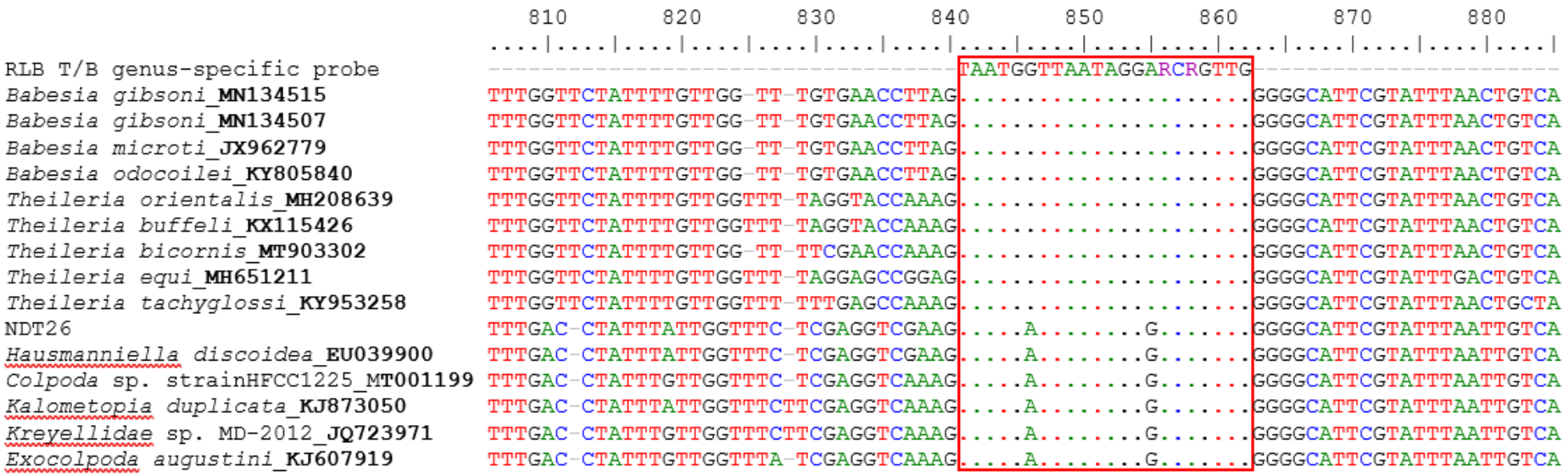


Figure 3.14: Multiple sequence alignment of the 18S rRNA gene. The region enclosed in the red box represents the RLB *Theileria*/*Babesia* genus-specific probe region. The alignment is made up of the 18S rRNA gene sequence obtained from the current study (NDT26), *Babesia* species, *Theileria* species, and various other pathogens detected in the sequence similarity searches. Sequence similarities in the RLB probe region are represented by dots. Nucleotide differences in the probe region are shown with nucleotide letters, i.e. A and/or G.

CHAPTER 4

DISCUSSION

The current study investigated the occurrence of haemoparasite infections in cattle from Hurungwe district in Mashonaland West Province and buffalo from Gonarezhou National Park, Zimbabwe. Tick-transmitted haemoparasitic diseases have a major economic impact on the cattle industry worldwide. Haemoparasitic diseases such as theileriosis, babesiosis, anaplasmosis and heartwater have a significant impact on the livelihood of both commercial and resource-poor farmers in Zimbabwe (Asiimwe et al., 2013). Ticks and tick-borne diseases (TBDs) were successfully brought under control and eradicated from Zimbabwe following the introduction of compulsory dipping of cattle in 1914 (Norval, 1979). However, the disruption of regular dipping that took place during the years 1972 to 1980 resulted in increased tick populations, and consequently the associated TBDs (Norval, 1979; Norval et al., 1983; 1984; 1985). Furthermore, the subsequent land reform programme which resulted in the movement of farmers and their livestock, led to the introduction of ticks and TBDs to areas where they previously did not occur (Sungirai et al., 2015; 2017). Due to the lack of infrastructure and resources for disease surveillance, disease control remains a problem, and data on the current status of tick-borne haemoparasites occurring in cattle at Hurungwe district in Mashonaland West Province is lacking.

The current study identified pathogenic and non-pathogenic tick-borne haemoparasites, using molecular diagnostic techniques. Discrepancies between the results obtained using the RLB and the qPCR assays highlighted the reduced sensitivity of the RLB assay in detecting these haemoparasites, especially in low infection levels. Thirty-seven (42.5 %) samples tested positive for haemoparasite infections using various qPCR assays, while the RLB assay only detected parasites in ten (11.5 %) samples. These finding were consistent with observations from other studies where comparison between RLB and qPCR was performed (Decaro et al., 2008; Chaisi et al., 2017; Hove et al., 2018). Although the RLB assay has lower sensitivity, it still remains a valuable screening tool in epidemiological studies as it is the only assay that

allows simultaneous detection of a wide range of known and novel tick-borne haemoparasite infections (Gubbels et al., 1999; Bekker et al., 2002).

Attempts at amplifying and sequencing the complete 18S and/or 16S rRNA genes from all samples that hybridized exclusively to the RLB genus-specific probes; *Theileria*/*Babesia* (n=8) and *Ehrlichia*/*Anaplasma* (n=9) were unsuccessful. The low parasitaemia could explain the inability to amplify the complete gene fragments from all samples. Only one complete 18S rRNA gene and nine 16S rRNA gene fragments were cloned and sequenced. Although good-quality sequence data was obtained, BLASTn analysis failed to provide hits to any haemoparasites expected to occur in cattle and buffalo. Further analysis of the sequence data indicated the absence of the RLB probe sequences, confirming that the sequence results obtained were the result of a lab contamination. However, this possibility was not confirmed as the positive control was not included in the sequence analysis. Due to time and financial constraints, this objective could not be fulfilled, however based on the haemoparasites that currently occur in Zimbabwe (Norval et al., 1983; 1984; 1985; Peter et al., 1998; Katsande et al., 1999; Smeenk et al., 2000; Moyo et al., 2017; Manyenyeka et al., 2021), we can assume that the sequences would have confirmed either the same infections or variants of these infections.

The RLB hybridization assay detected infections with *T. mutans* (4.6 %), *T. velifera* (4.6 %) and *T.* sp. sable (1 %) from 10 samples. However, it should be noted that the oligonucleotide probes of *T. velifera* and *T.* sp. sable used in the RLB hybridization assay, have been reported to cross-react (Mans et al., 2015). Therefore, the results for *T. velifera* and *T*. sp. sable should be confirmed by sequencing. The *Theileria* species detected in the current study are known to infect both cattle and buffalo and are considered less pathogenic to non-pathogenic causing moderate to asymptomatic infections in cattle (Theiler, 1906; Schreuder et al., 1977; Nijhof et al., 2005; Yusufmia et al., 2010; Mans et al., 2015; Moumouni et al., 2015; Njiiri et al., 2015). However, some strains of *T. mutans* can cause severe clinical illness in cattle, which may lead to death (Saidu, 1981; Lawrence and Williamson, 2004). Irvin et al. (1972) described fatal infections of *T. mutans* in cattle during a study conducted at Narok District of Kenya. Other benign or non-pathogenic *Theileria* species infecting cattle and buffalo include *T. taurotragi*, *T. buffeli/T. orientalis*/*T. sergeti* group and *T*. sp. buffalo (Norval et al., 1992b;

Gubbels et al., 1999; 2000; Eygelaar, 2015). Although these *Theileria* spp. were not detected in the current study, infections with these species might interfere with the diagnosis of the pathogenic *T. parva*. The *T. buffeli*/*T. orientalis*/*T. sergeti* group may cause disease in cattle and loss of production (Norval et al., 1992a; Gubbels et al., 2000; Jeong et al., 2010; Kamau et al., 2011). *Theileria taurotragi* is considered non-pathogenic or mild (Uilenberg et al., 1982) and it has been incriminated as the causative agent of bovine cerebral theileriosis (De Vos et al., 1981; Jongejan et al., 1986; Binta et al., 1998). The morphology of microschizonts of *T. taurotragi* in cattle is similar to that of *T. parva*, which may cause complications in the diagnosis of both these *Theileria* species (Jongejan et al., 1986), especially when using traditional parasitological diagnostic methods such as microscopic examination of blood smears.

The most pathogenic *Theileria* species infecting cattle in sub-Saharan Africa, including Zimbabwe, is *T. parva*. *Theileria parva* infections were not detected in the samples analysed in this study. This finding was unexpected as January disease, the common form of bovine theileriosis caused by *T. parva* in Zimbabwe, has been reported to cause a substantial number of cattle mortalities each year in the country (Norval et al., 1985; Latif et al., 2001; Moyo et al., 2017; Manyenyeka et al., 2021). The disease also limits livestock production and improvement of existing livestock in Zimbabwe (Simuunza et al., 2011). January disease usually occurs from December to May, coinciding with the distribution of its tick vectors (Latif et al., 2001). Recently, the cases of January disease have been reported to be on the increase throughout Zimbabwe, with changes in seasonal occurrence of the disease (Manyenyeka et al., 2021). The recent increase in cases of theileriosis in Zimbabwe can be attributed to the collapse of tick control programs resulting from reduced financial support from the government (Sungirai et al., 2016; Moyo et al., 2017). Moreover, unauthorized animal movements and changes in environmental conditions also contribute to the expanding distribution of ticks and associated tick-borne diseases (Sungirai et al., 2015; 2017).

It is unclear why *T. parva* infections were not detected from sampled animals; however, factors such as the climate, vegetation, acaricide usage, and host availability are reported to have a direct influence on the abundance and infection rates of transmitting tick vectors and the diseases they transmit (Moyo et al., 2017). Moreover, the blood samples used in this study

were collected from cattle that were brought to the diptanks for application of acaricide to control tick burden. It is possible that due to the tick control programme and the subsequent reduced exposure to infected ticks, these animals were protected from some of the tick-transmitted pathogens and if infected, the parasitaemia was reduced to extremely low levels, below the detection limits of the diagnostic tools used in this study.

The African buffalo (*Syncerus caffer*) are important reservoirs of various tick-borne haemoparasites. Previous reports have detected *T. parva* and other *Theileria* spp such as *T. mutans*, *T. velifera*, *T. buffeli*, *T.* sp. buffalo and *T*. sp. sable in the buffalo populations from several countries, including Zimbabwe (Chaisi et al., 2011; Latif and Hove, 2011; Oura et al., 2011; Pienaar et al., 2011; Caron et al., 2013; Eygelaar, 2015). In the current study, one buffalo was positive for *T. mutans*. *Theileria parva* was not detected in the buffalo sampled. The buffalo at Gonarezhou National Park are free ranging and usually expected to be positive with quite a number of tick-borne haemoparasites; however, only seven buffalo samples were available for analysis in our study, a larger sample size should be analysed to determine the true status of *T. parva* infections among the buffalo population in the Gonarezhou National Park. A preliminary study conducted inside and at the periphery of Gonarezhou National Park, tested a larger sample size (n=38) and found a higher prevalence of theileriosis in the buffalo samples (De Garine-Wichatitsky, 2009). Another study conducted at the same location (Gonarezhou National Park) in Zimbabwe reported 19/40 (using RLB assay) and 28/40 (using qPCR assay) *T. parva* positive buffalo (Eygelaar, 2015).

The two economically important causative agents of bovine babesiosis (also known as redwater) in Africa are *Babesia bigemina* and *B. bovis*. These *Babesia* species also occur in Zimbabwe (Norval et al., 1983). In our study, both *B. bigemina* (1 % using RLB; 8 % using qPCR) and *B. bovis* (2 % using qPCR) were detected in cattle samples. *Babesia bovis* is the more virulent organism, however, *B. bigemina* is widely spread (Bock et al., 2004). *Babesia bigemina* has been present throughout Zimbabwe since the 1890s, causing morbidity and mortality in imported cattle; while *B. bovis* was found to be restricted to the eastern regions of Zimbabwe (Lawrence and Norval, 1979). A countrywide survey of *B. bigemina* and *B. bovis* conducted in one- to three-year-old calves between April 1980 and April 1981, confirmed the wider distribution of *B. bigemina* while *B. bovis* was limited to the eastern parts of Zimbabwe

(Norval et al., 1983). However, in the regions where both species occur, there are contradictory reports on the prevalence of the two species. In a study conducted in the northern and eastern regions of Zimbabwe, Katsande et al. (1999) reported a higher occurrence of *B. bigemina* [52.4 %] in the eastern region, close to the border with Mozambique, compared to *B. bovis* [32.3 %]. In contrast, Smeenk et al. (2000) reported a higher prevalence of *B. bovis* (47 %) than *B. bigemina* (35 %) in a study conducted in the eastern and north-eastern regions in Zimbabwe. The results of the current study confirmed the presence of *B. bigemina* and *B. bovis* at Hurungwe district in Mashonaland West Province, Zimbabwe, consistent with the findings of Norval et al. (1983) and Katsande et al. (1999) with regard to the wide distribution of *B. bigemina* in Zimbabwe. Since *B. bovis* is reported to be restricted in the eastern parts of Zimbabwe, this explains the lower detection of this species in the study area, which is part of the Western province. . The buffalo sampled in the current study tested negative for both *B. bigemina* and *B. bovis*. The absence of these species in buffalo is consistent with reports from other studies, which investigated the presence of tick-transmitted haemoparasites in African buffalo from the same game park (Gonarezhou National Park) in Zimbabwe; two National Parks in northern Botswana (Chobe National Park and Okavango Delta National Park) and four provinces in South Africa (Free State, Mpumalanga, Limpopo and Gauteng provinces) (Berggoetz et al., 2014; Eygelaar et al., 2015).

Anaplasma marginale was the pathogen most detected in the current study, from both cattle (28%) and buffalo (86%) (duplex qPCR). *Anaplasma centrale* was also detected in both cattle (4 % using RLB; 8% using qPCR) and buffalo (14% using qPCR). Bovine anaplasmosis caused by *A. marginale* was reported to be responsible for the majority of cattle deaths in Zimbabwe during 1965 (Matson, 1965). Two decades later, samples collected throughout Zimbabwe from various hosts, which included cattle, African buffalo and impala, were tested for the presence of *A. marginale* antibodies (Norval et al., 1984). The findings of their study indicated that *A. marginale* was present throughout Zimbabwe. There are no recent reports on molecular detection of bovine anaplasmosis in Hurungwe district, making the current study - the first to report of molecular data on bovine anaplasmosis in this district. Studies in Zimbabwe and other countries have also reported the presence of *A. marginale* and *A. centrale* in wildlife spp. including the African buffalo (*Syncerus caffer*) (Norval et al., 1984; Kocan et al., 2003; Debeila, 2013; Eygelaar et al., 2015; Sisson et al., 2017). *Anaplasma*

marginale is endemic worldwide causing clinical disease often in cattle while *A. centrale* causes a less virulent type of anaplasmosis. The latter is often used as a live vaccine to control anaplasmosis caused by *A. marginale* (Kocan et al., 2003; 2004; 2010; Hove et al., 2018).

Ehrlichia ruminantium was not detected in the current study. *Ehrlichia ruminantium* (previously known as *Cowdria ruminantium*), transmitted by *Amblyomma* ticks, is widely distributed in sub-Saharan Africa and causes heartwater or cowdriosis in domestic and wild ruminants (Allsopp, 2010). The distribution of *Amblyomma* ticks (*A. hebraeum* and *A. variegatum*) were reported in the southern and northern regions in Zimbabwe (Norval et al., 1994), spreading towards the eastern and the North-central regions (Peter et al., 1998). Recent reports found *A. variegatum* in the North-central, North-eastern and the Zambezi valley, while *A. hebraeum* was found in the eastern regions and Hwange National Park in the western Lowveld region in Zimbabwe (Sungirai et al., 2015; 2018). *Amblyomma gemma*, another tick species that transmits *E. ruminantium* in wild and domestic ruminants (Wesonga et al., 2001) was reported in the Mazowe and Shurugwi districts, in Mashonaland central and Midlands provinces of Zimbabwe (Mandara, 2018). Based on the known distribution of the tick vectors in Zimbabwe, and the findings of the current study, we can hypothesize that the failure to detect *E. ruminantium* in Hurungwe district in Zimbabwe could be related to the absence of the ticks that transmit this parasite. De Garine-Wichatitsky (2009) reported a lower prevalence of heartwater in buffalo from inside and at the periphery of Gonarezhou National Park, while a study conducted a few years later at the same location, failed to detect *E. ruminantium* in buffalo (Eygelaar et al., 2015). Although our study confirms this observation, only seven buffalo samples were available for screening and further investigations targeting a larger sample population are required.

Amblyomma hebraeum, *A. variegatum* and *A. lepidum* also transmit *T. mutans* and *T. velifera*; while *T. parva* is transmitted by *Rhipicephalus appendiculatus* and *R. zambeziensis*. The distribution of these ticks is higher in the Highveld than in the Lowveld regions of Zimbabwe (Walker, 2003; Ndhlovu et al., 2009; Sungirai et al., 2015; 2017; Moyo et al., 2018). A study by Moyo et al. (2018) conducted in the wildlife parks of the Zambezi valley in the northern part of Zimbabwe, reported that buffalo were parasitized by four tick species (*R. decoloratus*, *R. appendiculatus*, *R. zambeziensis* and *A. hebraeum*).

Rhipicephalus simus has been shown to transmit *A. centrale* (Potgieter and van Rensburg, 1987), compared to 20 different tick species implicated in the transmission of *A. marginale* (Kocan et al., 2004). *Rhipicephalus simus* was not identified in Hurungwe district (Sungirai et al., 2015; 2017). It is possible that *A. centrale* detected in the current study was transmitted by other tick species found in Zimbabwe, since this haemoparasite can also be transmitted by Ixodes and Haemophysalis ticks (Rymaszewska and Grenda, 2008). The transmission of anaplasmosis can also be mechanical through biting flies and blood contaminated fomites (Kocan et al., 2003; 2004; 2010). *Rhipicephalus decoloratus* ticks are the primary vectors of *A. marginale* in Zimbabwe (Norval, 1979). These ticks also transmit *B. bigemina* (Bock et al., 2004). *Rhipicephalus decoloratus* ticks are widespread in Zimbabwe and have been reported in Hurungwe district (Sungirai et al., 2017; 2018; Moyo et al., 2018). The distribution of *R. microplus* ticks, which transmit *B. bigemina* and *B. bovis*, has been reported in the south-eastern Lowveld, northern Highveld and in the northern Lowveld regions of Zimbabwe. However, *R. decoloratus* was found to be more abundant in this district (Sungirai et al., 2017; 2018).

CONCLUSION

Collectively, the objectives of the current study were to determine the occurrence of tick-transmitted haemoparasites in cattle from Hurungwe district at Mashonaland West Province and buffalo from Gonarezhou National Park at Masvingo Province in Zimbabwe, using molecular diagnostic tools. This study provides an update of the occurrence of haemoparasites circulating in cattle and buffalo from the study locations.

Our results revealed the presence of important tick-borne haemoparasites of cattle, however, the occurrence of these tick-borne haemoparasites did not follow the reported trend as *T. parva* and *E. ruminantum* infections were not detected. Recent reports in Zimbabwe indicate that cattle theileriosis is the major cause of cattle mortalities, followed by babesiosis, heartwater, and then anaplasmosis (Norval, 1979; Norval et al., 1983; 1984; 1985; Manyenyeka et al., 2021). Our findings suggest that the occurrence of TBDs in various provinces may vary depending on specific vector-parasite-host-environment dynamics in each province. The low occurrence of haemoparasite infections in buffalo was noted, however, a larger number of buffalo samples will have to be investigated for conclusive results. The data presented in the current study highlights the importance of the African buffalo as reservoir hosts of various tick-borne haemoparasites, and the possible role they could play in the epidemiology of TBDs.

STUDY LIMITATIONS AND RECOMMENDATIONS

A major limitation to the study was the lack of sample collection data. Convenient sampling was conducted at diptanks in Hurungwe district at Mashonaland West Province and Gonarezhou National Park at Masvingo Province in Zimbabwe. Future studies could be improved by capturing data which includes the age of the cattle sampled, a month or season when the samples were collected, the exact location of diptanks and clinical information. In addition, collection of ticks on sampled animals could assist with correlation of the tick-borne haemoparasites detected and the ticks found on the sampled animals.

Another limitation to the study was that a small sample size for buffalo was available for this study, which likely affected the detection of tick-borne haemoparasites circulating in buffalo population at Gonarezhou National Park. An increased sample size would provide a true reflection of tick-borne haemoparasites circulating in this buffalo population and allow the evaluation of the threat these may pose to the cattle population around this area.

In addition, failure to characterize haemoparasite infections and the lack of sampling of other wildlife that harbour tick-borne pathogens of economic importance at Gonarezhou National Park was a limitation to drawing reasonable conclusions from the findings of the study. This could have benefited the study by determining the disease dynamics in the study area.

The results presented in the current study emphasize the need to continuously update the data on the distribution of ticks and tick-borne diseases, in order to assist with the development of adequate control measures. We recommend a more intensive study on the current tick distribution as well as associated tick-borne diseases in the Hurungwe district of Mashonaland West Province. Such information will assist with the design of effective disease management measures.

CHAPTER 5

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RESEARCH OUTPUTS

CONFERENCES

A.A.R. Modirwa, R.V. Bhoora, K.P. Sibeko-Matjila, 2019. Molecular detection of tick-borne haemoparasites in cattle and buffalo samples from Mashonaland West and Masvingo Provinces, Zimbabwe. University of Pretoria Faculty Day at the Faculty of Veterinary Science, 2019. (Poster).

A.A.R. Modirwa, R.V. Bhoora, K.P. Sibeko-Matjila, 2019. Molecular detection of tick-borne haemoparasites in cattle and buffalo samples from Mashonaland West and Masvingo Provinces, Zimbabwe. 48th Annual Conference of the Parasitological Society of Southern Africa (PARSA), 15 – 17 September 2019, Windhoek, Namibia (Poster).
This poster won 1st place for 'Best First-time Poster Presentation'.

APPENDICES

Appendix 1: University of Pretoria Animal Ethics Committee approval for the MSc study: Molecular detection of tick-borne haemoparasites in cattle and buffalo samples from Mashonaland West and Masvingo Provinces, Zimbabwe.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

7 December 2020

Approval Certificate
New Application

AEC Reference No.:	REC086-20
Title:	Molecular detection of tick-borne haemoparasites in cattle and buffalo samples from Mashonaland West and Masvingo Provinces, Zimbabwe
Researcher:	Miss AAR Modirwa
Student's Supervisor:	Prof KP Sibeko-Matjila

Dear Miss AAR Modirwa,

The **New Application** as supported by documents received between 2020-09-03 and 2020-12-04 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-12-04.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Buffalo	
Cattle	
Samples Archived dried blood (Buffalo)	7
Archived dried blood (Cattle)	80

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-12-07.
3. Please remember to use your protocol number (REC086-20) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. As part of your approval, the committee requires that you record a **short video footage** of major animal procedures approved in your study. The committee may request them for monitoring purposes at any later point.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Appendix 2: Section 20 approval to conduct research in terms of the Animal Disease Act, 1984 (Act No. 35 of 1984)

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

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

Dr Kgomotso Penelope Sibeko-Matjila
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Old Soutpan Road
Onderstepoort

Email: kgomotso.sibeko@up.ac.za

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

Dear Dr Sibeko-Matjila

Your application, submitted 2 July 2018, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

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

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him/her by any other Act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. A South African veterinary import permit must be obtained prior to the importation of the cattle and buffalo blood-spotted FTA cards from Chinhoyi University of Technology, Zimbabwe;

4. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act No. 93 of 1996);
5. Only a registered waste disposal company may be utilised for the removal of waste generated during the study;

Title of research/study: "Molecular detection of haemoparasites in cattle and buffalo at Manicaland province in Zimbabwe"
Researcher: Dr Kgomotso Penelope Sibeko-Matjila
Institution: Department of Veterinary Tropical Diseases, University of Pretoria
Reference: 12/11/1/1/6
Expiry: 31 December 2020

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2018 -08- 0 8

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

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

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

Dr Kgomotso Penelope Sibeko-Matjila
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Old Soutpan Road
Onderstepoort
Email: kgomotso.sibeko@up.ac.za

Dear Dr Sibeko-Matjila

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "MOLECULAR DETECTION OF HAEMOPARASITES IN CATTLE AND BUFFALO AT MANICALAND PROVINCE IN ZIMBABWE"

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

i) Blood and DNA samples, obtained from this study, may be stored in long-term storage at -80°C in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science of the University of Pretoria;

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

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2018 -08- 08

Appendix 3: Department of Agriculture, Forestry and Fisheries import permit of animal samples for research

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate of Animal Health
Import-Export Policy Unit
Private Bag X138
Pretoria, 0001
Republic of South Africa

Tel: (27)-012-3197514
Fax: (27)-012-3298292

DEPARTMENT OF AGRICULTURE
DIRECTORATE: ANIMAL HEALTH
PRIVATE BAG X138
2018 -09- 25
PRETORIA 0001
FORESTRY AND FISHERIES

IMPORTER:
DR KGOMOTSO P.SIBEKO-MATJILA
DEPARTMENT VETERINARY TROPICAL DISEASES
FACULTY OF VETERINARY SCIENCE
UNIVERSITY OF PRETORIA

DAH NO: 13/1/1/30/2/0
PERMIT NO: 201809002588
Valid from: 2018-09-25
Expiry date: 2018-12-25

VETERINARY IMPORT PERMIT FOR ANIMAL SAMPLES IMPRINTED ONTO FTA PAPER FOR RESEARCH OR DIAGNOSTIC TESTING

[Issued in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984)]

Authority is hereby granted for you to import the following into Republic of South Africa:
80 X CATTLE AND 80 X BUFFALO BLOOD SAMPLES IMPRINTED ONTO FTA PAPER FOR RESEARCH PURPOSES

from: **CHINHOYI UNIVERSITY OF TECHNOLOGY** (company) **in ZIMBABWE** (country)
subject to the following conditions:

1. The consignment must be accompanied by:
   1.1 this original veterinary import permit and
   1.2 a veterinary health certificate issued by a veterinarian authorised thereto by the Veterinary Authorities of the exporting country to the effect that:
      i. He/she is not aware of any animal or human health or infectious risks associated with the import of these samples
      ii. These samples do not originate from an area under restrictions for any disease the species is susceptible to.

1. The impression samples/blood spots on the FTA paper must be completely dry, and securely packed in leakproof containers and sealed by an authorised official of the Veterinary Authorities of the exporting country. No fresh samples may be imported using this permit.

2. The consignment to be airfreighted through port of entryOR TAMBO INTERNATIONAL AIRPORT. **Samples may only be imported as manifest cargo under an airwaybill number and may not be imported as personal luggage.**

3. The consignment must be accompanied by this permit and its arrival reported immediately to the inspecting veterinary official: KEMPTON PARKTel:011 973 2827, and may not be released without his/her written permission.

4. Upon arrival the inspecting veterinary official will inspect the consignment and release it to the importer only after he/she is satisfied that all the import conditions have been complied with in full.

5. The specimens must be kept and used at the MOLECULAR BIOLOGY LABORATORY UNIVERSITY OF PRETORIA, under the personal supervision of DR KGOMOTSO SIBEKO-MATJILA

6. On completion of tests/research the specimens, including all contaminated/infectious things or animal products (as defined by the Animal Diseases Act, 1984 [Act No. 35 of 1984]) derived/produced from or that came into contact with the above-mentioned specimens, must be destroyed by incineration. Records of the incinerations must be maintained for a period of 5 years, and made available for auditing to the Veterinary Authority upon request.

Signature_____

Page 1 of 2

7. This permit does not absolve the importer from compliance with the provisions of any other legislation relating to this import

Signature_____

8. This permit is subject to amendment or cancellation by the Director Animal Health at any time and without prior notice being given.

9. This permit is valid for three (3) months from date of issue and FOR ONE CONSIGNMENT ONLY.

SPECIAL CONDITIONS:

DIRECTOR: ANIMAL HEALTH

NOTE:

- All imports for research purposes require Section 20 permission in compliance with the Animal Diseases Act.
- Any consignment imported into South Africa packed with either wood packaging material or dunnage, will require treatment to remove any pests present (by heat or methyl bromide fumigation). Treatment must be indicated as per IPPC prescript on wood packaging material. [Directorate: Inspection Services Tel: 012 309 8754 or Fax 086 732 4768 or www.daff.gov.za]

Appendix 4: Letter of permission to use cattle and buffalo samples from Mashonaland and Masvingo provinces, Zimbabwe

CHINHOYI UNIVERSITY OF TECHNOLOGY
DEPARTMENT OF ANIMAL PRODUCTION AND TECHNOLOGY
P. Bag 7724, CHINHOYI, ZIMBABWE
☎ +263 (0)67 22203-5 ext 302 /+263 774 160 715
Website: www.cut.ac.zw
E-mail: cgomo@cut.ac.zw

30 November 2020

To: Principal Investigator
Cc Animal welfare officer

Re: Cattle and African Buffalo blood samples were collected following University Animal Welfare and Bioethics procedures.

This letter confirms that the samples listed below were collected following animal welfare procedures recommended by the University Animal Welfare and Bioethics committee.

Cattle: 80 blood samples on Filter papers (Mashonaland West province, Zimbabwe)

African Buffaloes samples: 40 blood samples on Filter papers (Gonarezhou National park, Masvingo province, Zimbabwe)

Thank you

Sincerely,

Animal health Management
Dr Calvin Gomo
Chinhoyi University of Technology, Department of Animal Production and Technology, School of Agricultural Sciences and Technology Agriculture
72 off Harare -Chirundu Road, Chinhoyi Zimbabwe
+263774160715
Geesdr1@gmail.com