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Detection of tick-borne haemoparasites in an isolated buffalo (*Syncerus caffer*) population from the Zambezi delta in Mozambique

by

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A dissertation submitted in partial fulfillment of the requirements for the degree

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Department of Veterinary Tropical Diseases

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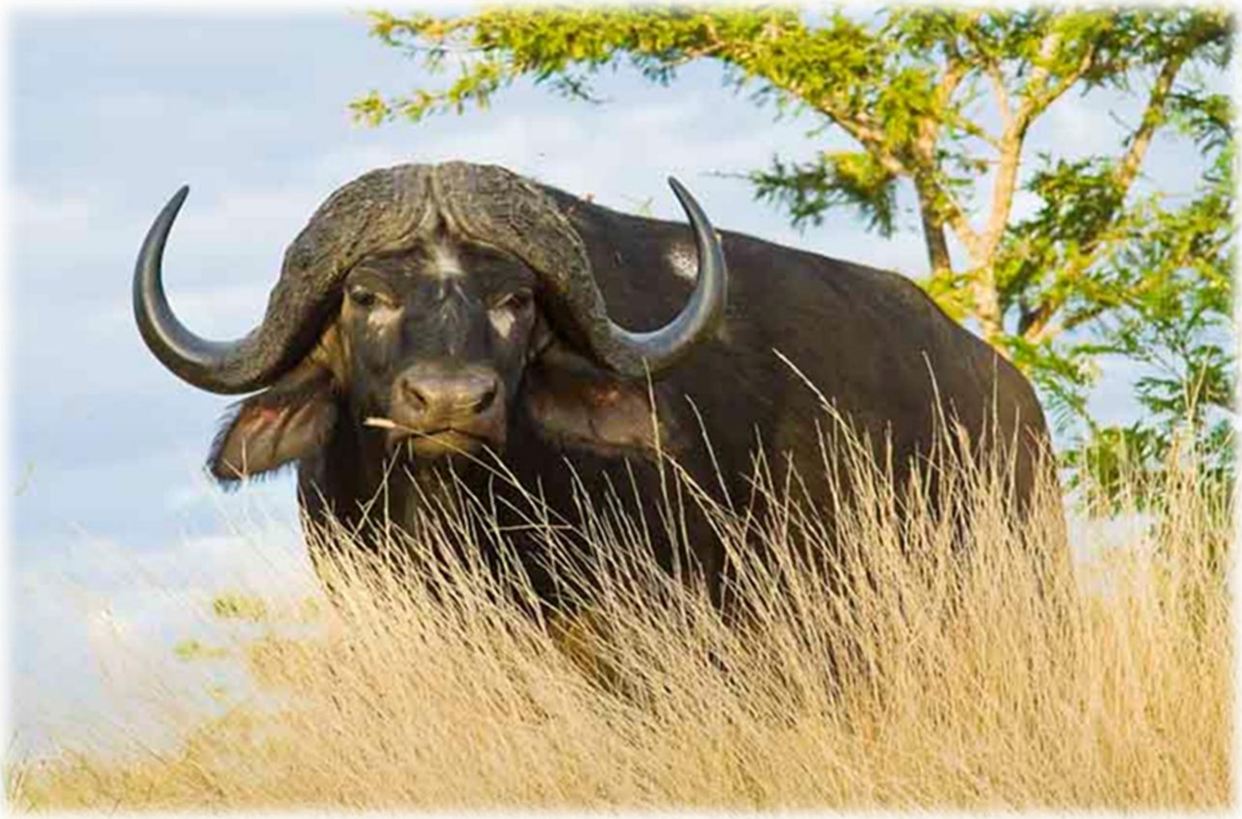

http://www.sa-venues.com/wildlife/wildlife_buffalo.htm

“Host of many parasites”

DECLARATION

I (Simangele Rachel Mkhize) declare that the work presented in this dissertation is my own, and it has been done for the purpose of fulfilling a masters degree in the field of veterinary science. This work has never been submitted for a degree at any institution.

S.R. Mkhize 31 July 2019

DEDICATION

Dedicated to my parents, Gugu and Vukile Mkhize, my sisters Sthokozile, Siwelile, Anele and my beloved daughter Lwenhle Mpathenhle Mkhize, the people who always supported me throughout my masters degree. I'll always be grateful for the love, support, encouragement and confidence in me.

"Let your light shine"

Matt. 5:16

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"The only person who is educated is the one who has learned how to learn and change" **-Carl Rogers**

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LIST OF ABREVIATIONS

ARC	Agricultural Research Council
APH	Animal production and Health subprogram
bp	base pairs
CVRI	Central Veterinary Research Institute
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECF	East Coast fever
ECL	Enhanced chemiluminescence
EDAC	1-ethyl-3-(-3-dimethylamininopropyl) carbodimide
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FMD	Foot-and-mouth disease
IFAT	Indirect fluorescent antibody test
KNP	Kruger National Park
$MgCl_2$	Magnesium chloride
ml	Millilitre
Mm	Millimolar
LMNP	Lake Mburo National Park
µl	Microlitre
µM	Micromolar

PCR	: Polymerase chain reaction
PIM	: Polymorphic immunodominant molecule
%	: Percentage
qPCR	: Quantitative real-time PCR
RFLP	: Restriction fragment length polymorphism
RLB	: Reverse line blot
rRNA	: Ribosomal ribonucleic acid
SDS	: Sodium dodecyl sulphate
SSPE	: Sodium chloride-sodium phosphate EDTA
TBDs	: Tick-borne diseases
UDG	: Uracil DNA glycosylase
OVI	: Onderstepoort Veterinary Institute

Detection of tick-borne haemoparasites in an isolated buffalo (*Syncerus caffer*) population from the Zambezi delta in Mozambique

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SUMMARY

Theileria parva is the most significant tick-borne haemoparasite associated with buffalo and it causes Corridor disease in Southern Africa. African buffalo (*Syncerus caffer*) is known for being the host of many pathogens causing diseases of veterinary importance, resulting in severe economic loss. The objective of this study was to detect haemoparasites species in blood samples obtained from a buffalo herd in the Zambezi delta in Mozambique, using DNA-based diagnostic assays. Several tick-borne blood-parasites namely *Theileria, Babesia, Anaplasma* and *Ehrlichia* species were detected, either as single or mixed infections, with the use of the reverse line blot hybridization assay. Furthermore, *Theileria parva* infections were confirmed by the quantitative real-time PCR (qPCR). This is the first report of tick-borne haemoparasites in the area of Zambezi delta in Mozambique. The results of this study indicate the occurrence of the following tick-borne haemoparasites in the Zambezi delta buffalo population. *Theileria parva* (95%) was the most frequently found species followed by *Theileria* sp. (sable) (85%), *A. marginale* (63%), *T. velifera*, (45%), *T. mutans* (43%), *A. centrale* (10%), *T.* sp. (buffalo) (3%) finally *Anaplasma* sp. (Omatjienne) (3%). These results strongly indicate a high challenge of the buffalo population in this area by tick-borne haemoparasites, especially *T. parva*. The qPCR was the most sensitive technique for detection of *T. parva,* detecting this pathogen in 100% of tested samples compared to 95% by RLB. The high occurrence of *T. parva* and *A. marginale* infections in this herd, the causative

agents of the fatal bovine theileriosis and bovine anaplasmosis may pose a risk to the cattle population in the surrounding areas.

CHAPTER 1

1. GENERAL INTRODUCTION

Tick-borne diseases such as Theileriosis, Babesiosis, Anaplasmosis and Heartwater are the major causes of livestock losses in sub-Saharan Africa, especially where the distribution of host, pathogen and vector species overlap. The spread of these diseases occurs mainly through arthropod vectors such as Ixodid ticks that allow pathogens to circulate between domestic and wild animals (Kock 2005). In the past years, research on tick-borne haemoparasites of buffalo has received little consideration. However, due to the threat they cause to livestock, more attention is now being directed towards these pathogens.

Theileriosis is one of the important and widely distributed disease affecting both wild and domestic ruminants caused by the tick-transmitted protozoan parasite of the genus *Theileria*. The most important *Theileria* species in sub-Saharan Africa is *T. parva*, which arguably evolved with African buffalo (*Syncerus caffer*) before the introduction of domestic livestock in the African continent (Lohr *et al.*, 1974). The vectors associated with *T. parva* in east and southern Africa are *Rhipicephalus appendiculatus*, *R. duttoni* as well as *R. zambeziensis* tick species (Norval *et al.*, 1992 and Uilenberg 1999).

Theileria parva infection is usually subclinical in buffalo, while it is fatal to the cattle populations. In the past, this parasite was classified as *T. parva parva*, *T. parva lawrencei* and *T. parva bovis*, which have been linked with three different clinical and epidemiological conditions named, East Coast fever, Corridor disease, and January disease, respectively (Lawrence *et al*., 2004(a), Lawrence *et al*., 2004(b) and Norval *et al*., 1992). However, due to the lack of molecular evidence to support the sub-speciation, this classification was abandoned (Norval *et al*., 1992 and Allsopp *et al.*, 1993). Presently, *T. parva* isolates are classified based on their host of origin and are commonly referred to as cattle-derived or buffalo-derived strains (Norval *et al.*, 1992).

Other tick-borne diseases important to livestock that occur in buffalo are Babesiosis mainly caused by *Babesia bovis* as well as *B. bigemina*, and Anaplasmosis which is caused by *Anaplasma marginale*, and lastly heartwater caused by *Ehrlichia ruminatium*.

Babesiosis is a tick-borne disease of economic importance transmitted by *Rhipicephalus (Boophilus) decoloratus* and *R. (Boophilus) microplus*, both one-host ticks (McCosker, 1981, de Vos *et al.*, 2004). Heartwater is a rickettsial disease of domestic and wild ruminants which is transmitted by *Amblyomma* ticks. This disease mainly occurs in sub-saharan Africa, following the distribution pattern of its main vectors (Uilenberg, 1983; Bekker *et al*., 2002; Minjauw and McLeod, 2003). Heartwater is particularly severe in small ruminants, where high mortality is commonly reported (Allsopp *et al.*, 2010). Anaplasmosis is another rickettsial disease that affect both the domestic and wild ruminants mainly in some tropical and subtropical regions worldwide, including Africa (Aubry and Geale, 2011). The tick vectors of the causative agent, *A. marginale*, include *R. (Boophilus) microplus* and *R. evertsi evertsi* (Kocan *et al.,* 2004).

A number of DNA-based assays have been used for decades to diagnose and differentiate haemoparasites. These include conventional PCR, PCR-based RFLP assays, Real-time PCR, and Reverse Line Blot-hybridisation assay (RLB). Sibeko *et al.*, 2008 developed a sensitive and specific technique based on the 18S rRNA gene, which is currently the most sensitive assay available for the detection of *T. parva* in cattle and buffalo in southern Africa.

The current project was initiated in order to identify tick-borne haemoparasites circulating in an isolated buffalo (*Syncerus caffer*) population in the area of the Zambezi delta in Mozambique. Regardless of their significance, not much is known regarding the incidence of tick-borne blood parasites in the area. The project aims to contribute to the existing knowledge on the diversity of haemoparasites occurring in buffalo in the studied area, with emphasis on *Theileria parva*. Furthermore, it provides evidence for the risk of haemoparasite infections to livestock in this area.

Information on tick-borne haemoparasites in Mozambique is scarce and there are no published reports on the presence of *T. parva* in buffalo from the area of Zambezi delta. Thus in this study, the RLB assay was used for the screening and simultaneous detection of tick-borne haemoparasites in buffalo blood samples from the area of Zambezi delta. *Theileria parva* infections were further confirmed, using a *T. parva*-specific qPCR assay.

1.1 Problem statement

Parasite species such as *Babesia, Theileria, Anaplasma* and *Ehrlichia* infect a great range of domestic and wild ruminants. They are considered to be of great veterinary importance in Sub-Saharan Africa and in many areas of the world because of the severe economic losses they cause. These involve disease outbreaks and the decrease of livestock production (Bock *et al*., 2004). Wild animals are reservoir hosts of important haemoparasites. The buffalo population in the area of south wetlands of Zambezi delta in Mozambique has been isolated for many years due to human activities such as poaching activities. However, since buffalo are carriers of different species of haemoparasites, they can be the main source of haemoparasite infections in livestock around the area of Zambezi delta in Mozambique. Therefore, the status of haemoparasites in this area needs to be elucidated.

1.2 Specific objectives:

- To detect haemoparasite species infecting African buffaloes in Zambezi delta, Mozambique using reverse line blot hybridization assay.

- To specifically detect hemoparasites important to livestock, particularly *T. parva* infecting African buffaloes in Zambezi delta, Mozambique, using *T. parva*-specific qPCR

1.3 Hypothesis

It is hypothesized that buffalo residing in the area of Zambezi delta in Mozambique are carriers of tick-borne haemoparasites, especially those that can be infectious to livestock.

CHAPTER 2

2. LITERATURE REVIEW

Africa is probably the continent of the world with the highest diversity of wildlife species, especially large mammals known to be carriers of many pathogens. Carmichael and Hobday, (1975) reported that tick-borne protozoan and bacterial pathogens are widespread in African wildlife species. Actually, it has been postulated that ticks and tick-transmitted haemoparasites have been circulating in African wildlife for thousands of years, before the introduction of domestic animals into the continent (Lohr *et al.*, 1974). Parasites of the genera *Babesia*, *Theileria*, *Anaplasma* and *Ehrlichia* infect a wide range of domestic and wild ruminants. Their veterinary importance in sub-Saharan Africa as a result of production losses they cause has been stressed by many researchers in the past (Bock *et al*., 2004). Several studies have detected vector-borne prokaryotic and eukaryotic haemoparasites associated with buffalo and cattle in Africa, mainly *E. ruminantum*, *B. bovis*, *B. bigemina*, *A. marginale* and *A. centrale* (Andrew and Norval 1989; Eygelaar *et al.*, 2015). Tick-borne diseases associated mortality in wild animals, mostly occurs when both pathogens and their hosts are introduced into a new environment or area where susceptible animals become infected, as frequently occurs with cattle (Lawrence and Norval 1987). The infections can lead to death when the animals are subjected to stress, as suggested by Grootenhuis (1979).

Earlier de Castro (1997) reported that the yearly worldwide costs attributed to ticks and tick-borne diseases in cattle in Zambia has increased between 13.9 billion and 18.7 billion per year. Minjauw & McLeod (2003) estimated the yearly cost of tropical Theileriosis in India to be 384.3 million US dollars, and East Coast Fever (ECF) in dairy systems in Kenya and Tanzania to be between 54.4 million and 4.41 million US dollars. Therefore, tick-borne diseases in Africa are considered to be the most important constraint to animal health and production (Young *et al*., 1988). Among tick-borne diseases, theileriosis caused by *T. parva* poses a major threat to cattle populations in central, eastern and southern Africa. Conversely, other members of this genus are commonly associated with mild or subclinical forms of disease in cattle. The *T. parva* strains which appears to have developed gradually in African buffalo are associated with Corridor disease, and the ones adapted to cattle are associated with ECF and January disease also known as Zimbabwean theileriosis. A number of studies have highlighted the importance of stress for the clinical manifestation of babesiosis in wildlife, including its association with fatal outcomes (McInnes *et al*., 1991; Penzhorn 2006). For example, a sable antelope that was relocated to a zoo in South Africa was reported to have died due to stress related clinical babesiosis (Martinaglia, 1930). Brocklesby (1967), McCulloch and Achard (1969), reported similar cases in Black rhinoceros (*Diceros bicornis*) from Kenya and Tanzania. Several haemoparasites species have been reported by several authors in wildlife species in South Africa. According to Nambota *et al.*, (1994), the first case of theileriosis in cattle in Zambia was reported in the year 1922.

Anaplasmosis caused by *A. marginale* has been reported in young buffalos and giraffe (*Giraffa camelopardalis)* resulting in death (Lightfoot and Norval, 1981). Chris *et al* (2011) reported the presence of various tick borne haemoparasites in the buffalo population in the Lake Mburo National Park, Uganda with a high carrier percentage of *Theileria* and *Anaplasma* spp*.* in buffaloes and *Erhlichia bovis* in bushbucks*.* Young and Basson (1973); Grootenhuis (1979) reported fatal heartwater in eland (*Taurotragus oryx*) caused by *E. ruminantium*.

Buffalo (*Syncerus caffer*) is one of the largest wild ruminant species. In the past, African buffalo were found in most of sub-Saharan Africa though its populations have decreased substantially and are now mostly limited to conservation areas (Winterbach 1998). The Cape buffalo is the most popular type among the three sub-species (*Syncerus caffer. nanus, Syncerus caffer. brachyceros* and *Syncerus caffer. aequinoctialis*) which are found in eastern and southern Africa. It is a carrier of many pathogens, including tick-borne haemoparasites, without showing any clinical signs.

Buffaloes are reservoir host for many diseases, which include Corridor disease, bovine tuberculosis, foot and mouth disease, and brucellosis in Africa Michel *et al*. (2006), Laubscher and Hoffman (2012), Norval, Perry and Young (1992). Studies conducted in Hluhluwe-imfolozi Park, reported that roughly 80% of the total buffalo population are carriers of these pathogens Debeila (2011); Pienaar *et al*. (2011). Ticks and tick-borne pathogens affect animals in game reserves and commercial game farms (Jolles, 2007). In South Africa, highly infected buffalo are found in the Kruger National Park in Mpumalanga Province, the game parks in the KwaZulu-Natal and Mabalingwe Game Reserve in Limpopo (Chaisi, 2012). Buffalo is also distributed in other parts of eastern, central and southern Africa for example in Kenya, Zambia, Botswana, and Zimbabwe (Mans *et al.*, 2011).

2.1. Haemoparasites of buffalo

2.1.1. *Anaplasma* species

Anaplasma marginale is a gram-negative bacterium from the family *Ehrlichiaceae,* class Alphaproteobacteria, order Rickettsiales and the phylum Proteobacteria. The parasite infects the red blood cells of its host causing bovine anaplasmosis, a disease known to affect cattle and lead to substantial morbidity and mortality. The disease is endemic in temperate, tropical and subtropical regions worldwide (Aubry and Geale, 2011). *Anaplasma centrale* formerly known as *A. marginale* subspecies *centrale* is a less virulent organism of cattle that causes a moderate form of anaplasmosis, and is mainly used as a vaccine to overcome the severe form of disease due to *A. marginale. Anaplasma centrale,* is commonly reported as a mixed infection with *A. marginale* (Bell-Sakyi *et al.*, 2015). Different kind of tick-borne blood parasites of veterinary importance belonging to the genus *Anaplasma*, namely: *A. marginale*, *A. centrale* and *A. phagocytophilum* have been reported recently in the South African buffalo population (Debeila, 2012; 2016; Khumalo *et al*., 2016; Sisson *et al*., 2017)

It has also been reported by Eygelaar *et al*., (2015) that about 74% of the buffalo were found to be carriers of *A. marginale*, in Lake Mburo National Park. In addition to buffalo, *Anaplasma* species infect cattle and other domestic and wild ruminants. *Anaplasma marginale* generally has no clinical effect on wild ruminants. These ruminants are persistently infected with *Anaplasma* parasites and play an important role as reservoirs (Kocan *et al.*, 2003). *A. marginale* is biologically transmitted by tick vectors of the genus *Rhipicephalus* in South Africa and can also be mechanically transmitted by biting flies (Potgieter *et al*., 2004). *A. marginale* and *A. centrale* have been reported to be prevalent in several countries in Africa including Uganda (Oura et al., 2011;

Byaruhanga et al., 2016), Botswana (Eygelaar et al., 2015), Mozambique (Machado et al., 2016) and in South Africa (Khumalo et al., 2016; Sisson et al., 2017).

2.1.2. *Babesia* species

The genus *Babesia* consists of prokaryotic blood parasites that represent an important health risk for cattle (Hunfeld *et al.*, 2008). It belongs to the Babesiidae family, from the order Piroplasmida, and Aconoidasida class as well as the Phylum Apicomplexa. *Babesia bigemina* and *B. bovis* are the main cause of bovine babesiosis also known as redwater fever. The tick vectors Rhipicephus (*Boophilus) decoloratus*, transmits only *B. bigemina*, while R. (*Boophilus) microplus* transmit both *B. bigemina* and B.bovis. This disease is mostly endemic in Africa and in Southern parts of Europe (de Vos, *et al.*, 2004). Eygelaar *et al.*, 2016 reported the absence of *B. bovis* in buffalo from various parts of Botswana. Recently, Penzhorn (2006) reported *B. bovis* to be present in low percentage in buffalo from Okavango delta. *B. bigemina* has been reported in every province of Zambia and also in eastern, southern and central Africa in buffalo and cattle (Eygelaar *et al*., 2015). Generally, these parasites are not associated with the clinical manifestation of disease in wildlife species, which are commonly asymptomatic carriers.

Rhipicephalus (Boophilus) microplus, originated in south East Asia and presently its distribution covers all major tropical regions (Potgieter, 1994). Historically, in Africa, this tick was restricted mainly to the Malagasy region, South and East Africa, except for very cold and dry areas (Walker *et al*., 1987, Estrada-Peña 2003). *Rhipicephalus (Boophilus) microplus* currently has a wide distribution range in the African continent; throughout Mpumalanga, Zambia and Limpopo provinces. It is currently present in the northern Tanzania region, as well as in Kenya and Mozambique (Lynen *et al.*, 2008 and Madder *et al*., 2007), in South Africa and Central Africa. *Rhipicephalus (Boophilus) microplus* has also been reported in West Africa, where it occurs in Ivory Coast, Benin, Burkina Faso and Mali. The cause of the re-emergence of this ectoparasite is still unknown, but believed to be linked with the importation of cattle (Madder *et al*., 2007)

Rhipicephalus (Boophilus) decoloratus is the mostly distributed cattle tick species in Africa, and it is widely distributed in suitable environments throughout the continent, including most of the regions of south of Sahara. However, its presence has not been documented in some southern African regions, mainly in Botswana, due to the dryness of the area. In West Africa, *R. (Boophilus) decoloratus* occurs alongside *R. (Boophilus) annulatus*, *R. (Boophilus) geigyi* and recently *R. (Boophilus) microplus*. While, in East Africa and southern Africa it occurs together with *R. (Boophilus) microplus* (Lynen *et al.*, 2008).

2.1.3. *Ehrlichia* species

Heartwater is a non-contagious but infectious disease infecting ruminants, caused by the rickettsial organism *Ehrlichia ruminantium*. Most African species of the genus *Amblyomma* are responsible for the transmission of the parasite, mainly *A. hebraeum* and *A. variegatum* (Allsopp, 2010). *Ehrlichia ruminatum* has been reported in the African buffalo population in Chobe National Park, as well as in the Okavango delta in low percentages (Penzhorn, 2006; Eygelaar *et al*., 2016). Debeila (2011) and Oura *et al*., 2011 reported *E. ruminatium* in buffalo from Hluhluwe-imfolozi, as well as Kruger National Park. The pathogen also occurs in eastern and southern parts of Africa, including in Kenya (Ngumi *et al*., 1997).

2.1.4. *Theileria* species

The genus *Theileria* consist of tick-transmitted, apicomplexan parasites. *Theileria* species are known to infect wild and domestic ruminants. Mixed infections with pathogenic, to less pathogenic *Theileria* spp. are common in Africa, especially in the regions where these species overlap (Allsopp *et al*., 1993).These includes *T. parva, T. mutans, T. velifera, T. buffeli, Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei). January disease, Corridor disease and ECF are disease syndromes resulting from infection with *T. parva*, the most pathogenic haemoparasite species of cattle and buffalo occurring in eastern, central and southern Africa (Gubbels *et al*., 1999). *Theileria mutans*, *T. velifera*, *T. buffeli, Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) have moderate to low pathogenicity, which makes them of little economic importance. However, they can lead to serious disease under poor nutrition or stress due to relocation (Lawrence *et al.*, 1994). Different tick vectors transmit these species depending on their geographical distribution. (**Table 2.1**)

Table 2.1: Summary of the pathogenicity, host specificity and geographic distribution for *Theileria* species

Pathogenicity	***Theileria spp.***	**Host**	**Vector**	**Distribution**
High	*T. parva*	Cattle and buffalo	*Rhipicephalus appendiculatus,* *R. zambeziensis, R. duttoni,*	Sub-Saharan east Africa from South Sudan to Republic of South Africa, including eastern Democratic Republic of Congo, Rwanda, Kenya, Uganda, Tanzania, Swaziland, Mozambique, Zambia, Zimbabwe Botswana and Angola
Benign - Low to Moderate	*T. mutans, T. velifera, T. buffeli, Theileria* sp. *(*buffalo*) and Theileria* sp. *(*bougasvlei*).*	Cattle, buffalo	*Amblyomma* spp.	Sub-Saharan east Africa

Source: Darghouth *et al.*, 2010

2.2. Classification of *Theileria* species

Theileria species are classified as follows:

Kingdom : Animalia

Phylum : Apicomplexa

Class : Aconoidasida

Order : Piroplasmida

Family : Theileriidae

Genus : *Theileria*

Species : *Theileria parva*

2.2.1 Pathogenic *Theileria* species found in southern Africa

2.2.1.1. *Theileria parva*

The haemoprotozoan parasite*, Theileria parva,* is widespread and infects mostly buffalo, and appears to cause no clinical signs (Musoke *et al.*, 1997). The first outbreaks of Theileriosis in southern Africa were recorded in 1901 from southern Mozambique, following the importation of infected cattle from Tanzania by Beira and Lourenco Marques. Subsequently, the disease was also reported from Zimbabwe and South Africa (Dias *et al.*, 1977)

In Zimbabwe, high motality attributed to ECF, was reported after its introduction. In the year 1900s ECF was introduced in South Africa and eradicated in 1955 (Norval, 1992). In Mozambique, the disease was cleared in 1917 due to the right control measures, but the disease was again recorded in 1960 in Mapai a south-western district of Gaza Province, Mozambique (Dias, 1977). This latter outbreak continued in 1970 and 1971 in Mossurize, Manica and Chimoio (Dias, 1977). To date, the disease is still endemic in Malawi and Zambia since its appearence between in 1912 and 1922 (Lawrence *et al.*, 1994).

Theileria parva is of great importance in eastern, central and southern Africa as it is a major threat to livestock causing cattle morbidity, mortality and production losses. It is also important economically, due to the costs associated with disease management (Mukhebi *et al.*, 1992). Wild animals such as waterbuck, giraffe, zebras and buffalo are the hosts of many *Theileria* species (Oura *et al.*, 2011). Wildlife animals that are infected by *T. parva,* usually show no clinical signs (Lightfoot and Norval 1981). However, like many pathogenic micro-orgamisms of wildlife, *T. parva* infections can result in lethal disease due to stress caused by change of location (Nijhof *et al*., 2003)*. Rhipicephalus appendiculatus, R. zambeziensis* and *R. duttoni* have been incriminated as vectors of *T. parva* (Uilenberg *et al.*, 1999). *Theileria parva* and its tick vectors occur in 11 African countries, expanding from Southern Sudan to Northern KwaZulu-Natal in South Africa (**Figure 2.1**). These vectors can transmit the parasite from *T. parva*-infected buffalo to naïve cattle causing Corridor disease. The disease syndromes caused by *T. parva* infections are discussed further in subsequent sections.

2.2.2. Benign and mildly pathogenic *Theileria* species of buffalo in Southern Africa

African buffalo are associated with less harmful to non-harmful *Theileria* species reported in Africa such as *Theileria* sp. (buffalo), *T. mutans, T. taurotragi, T. sergenti/buffeli/orientalis and T. velifera.* These species may cause only mild or subclinical disease, mostly in cattle.

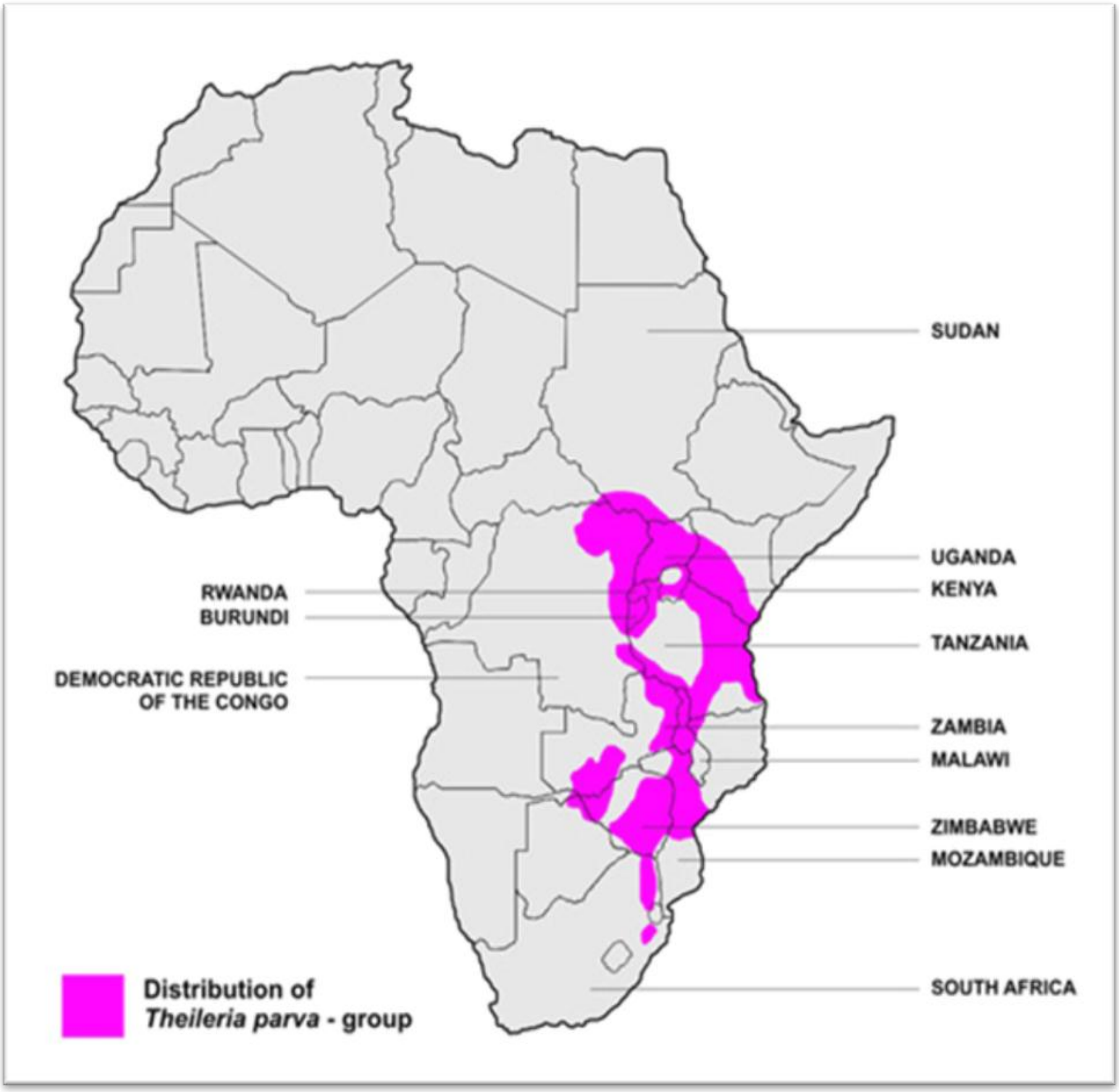


Figure 2.1: The map showing the distribution of *Theileria parva* in Africa (ILRAD, 1990).

2.2.2.1 *Theileria* sp. (buffalo)

This parasite was reported in buffalo from Kenya and East Africa by Conrad *et al.*, 1987, and in Uganda by Oura *et al.*, 2011 as well as in South Africa (Chaisi *et al*, 2011; Piennar *et al.*, 2011). Moreover, it is genetically closely related to *T. parva* and have been frequently reported in buffalo (Chaisi *et al.,* 2011; Mans *et al.*, 2011). This pathogen is not known to occur in cattle and it was not yet formally named because of it unknown vectors (Chaisi *et al.,* 2011).

2.2.2.2*. Theileria mutans*

Theileria mutans was first described in 1906 in South Africa. It is considered the parasite of African buffalo, but also infect cattle (Uilenberg 1982).

2.2.2.3*. Theileria velifera*

This parasite is considered non-pathogenic and has no economic importance. The piroplasms are usually observed in mixed *Theileria* infections, but it can be easily distinguished from the others by Giemsa-stained blood and lymph node smears, as well as by PCR based assays (Uilenberg *et al*., 1982) The parasite was first reported from cattle in South Africa in 1979 (Berger, 1979).

2.2.2.4. *Theileria buffeli/sergenti* and *orientalis*

These species causes benign infections in both buffalo and cattle in South Africa, Australia, Europe and Asia (Chae *et al.*, 1999; Chaisi *et al.*, 2012). Fujisaki *et al* 1994 described that the first report of *Theileria buffeli* was from the Asian water buffalo in 1908, while *T. sergenti* and *T. orientalis* reported in eastern Siberia in 1903 by Yakimov and Dekhterev.

2.2.2.5*. Theileria tauratragi*

The presence of this parasite was first reported in 1909 in South Africa, and it was detected both in eland and buffalos in Kenya. The tick vectors that transmit this parasite are *R. appendiculatus* and *R. zambeziensis* (Uilenberg *et al*., 1982; Lawrence *et al*., 1983).

2.3. Diseases resulting from *Theileria parva* infections

2.3.1. East Coast fever

East Coast fever is a fatal protozoan disease of cattle and it caused by *Theileria parva* parasites. With 28 million cattle at risk of theileriosis in Kenya, this can result in major economic loss (Minjauw *et al.*, 2003). Furthermore, it is considered as the main impediment to the introduction of productive exotic (*Bos taurus*) cattle breeds in eastern, central and southern Africa. *Theileria parva* is transmitted through infections by tick from infected cattle to uninfected cattle, also known as cattle-to-cattle transmission. The characterisation of the disease is by high fever and lymphadenopathy (Lawrence *et al.*, 1994). A number of animals may recover from the disease, but the infected animals become carriers of the parasite, and consequently a source for tick infection.

2.3.2. Corridor disease

Corridor disease is the disease of cattle with similar features as ECF. The disease is mainly caused by strains of *T. parva* derived from buffalo previously called *T. parva lawrencei* (Lawrence, 1994). Disease transmission occur when cattle and carrier buffalo share grazing environments with the tick vector *R. appendiculatus*, *R. zambeziensis* or *R. duttoni*, resulting in buffalo-to-cattle transmission (Neitz, 1957). The symptoms of Corridor disease are the similar to those of ECF, except that the course is usually shorter and the fatality rate is much higher.

2.3.3. Zimbabwean theileriosis (also known as January disease)

January disease is a serious disease of cattle, also caused by *Theileria parva* infection formerly known as *T. parva bovis*. The disease was first recognised and distinguished from East Coast Fever in 1936 in Zimbabwe (Lawrence *et al*., 1994). January disease symptoms are the same as the ones of ECF (Lawrence *et al.*, 1994). It is known as January disease because of its seasonal occurrence, which is between December and March (Matson, 1967).

2.4. Life cycle of *Theileria parva*

The life cycle of *Theileria* parasites consists of many different stages which occur in the mammalian host and in the tick vector (**Figure 2.2**). The ability of the parasite to transmit or survive depends on the developmental stages, such as the schizont and merozoite in the mammalian host, as well as the zygote and the sporozoite in the tick vector. These developmental stages have the potential to identify and occupy specific host cells (Dobbelaere and Rottenberg, 2003).

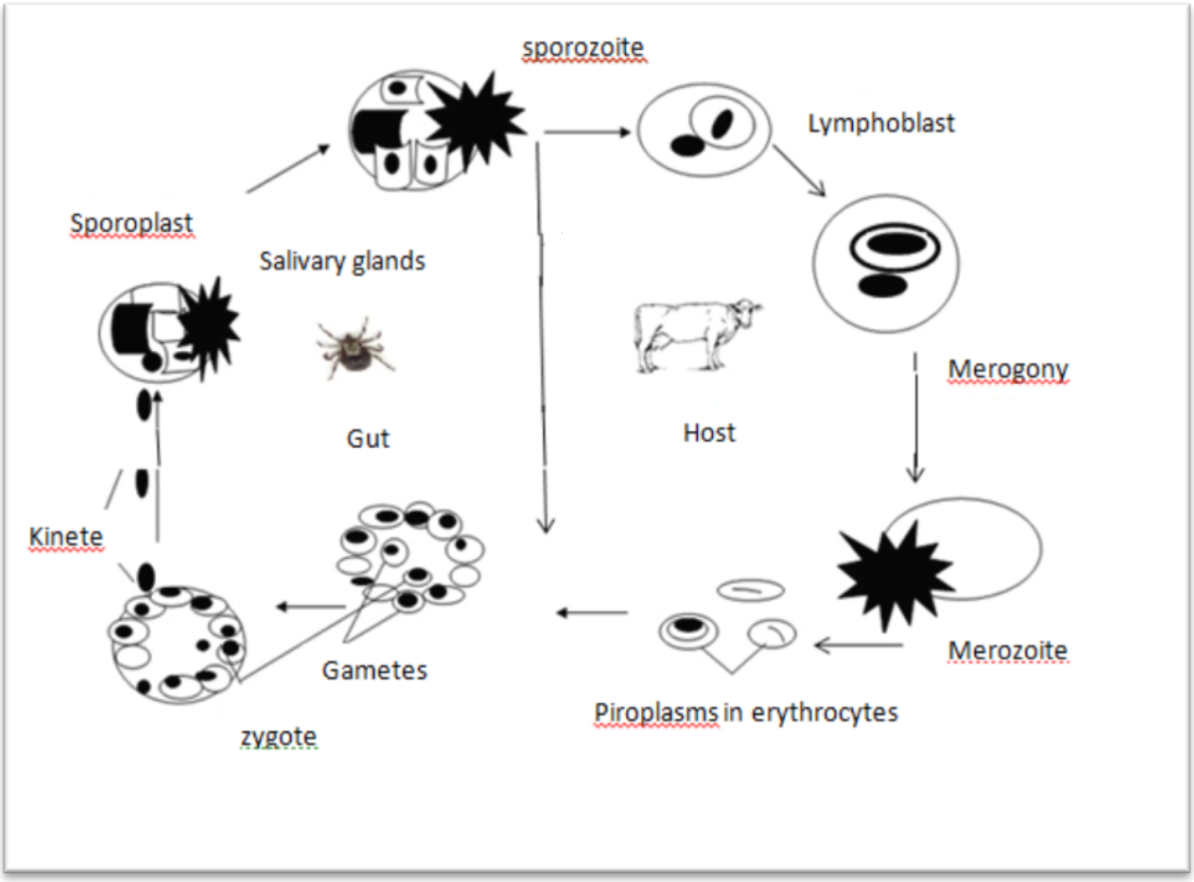


Figure 1.2: Life cycle of *T. parva* in buffalo and the ixodid tick vector *Rhipicephalus appendiculatus.*

2.4.1 *Theileria* life cycle in the tick vector

During tick feeding, *Theileria* piroplasms, the infective stage to the tick, are acquired from the infected red blood cells of the host. Inside the tick gut, the piroplasms separate into male and female gametes, in which they merge to form zygotes, which invade the gut epithelial cells where they remain in every part of the tick shedding cycle and develop into one kinete (Melhorn and Schein, 1985). Subsequently, the kinete enters the haemolymph and infects the cells of the salivary glands where sporoblast occurs and produces sporozoites. The sporozoites remains in the salivary glands of the infected tick for maturity, until suitable environmental conditions are met for transmission of infection to another animal host during feeding (Norval *et al.*, 1992).

2.4.2 *Theileria* life cycle in the mammalian host

The sporozoites, (the infective stage), enter and infect the lymphocytes where they differentiate into schizonts, which eventually undergo schizogony. The infected lymphocytes grow and start dividing syncrhrounsly with the schizonts. The schizonts stimulate the host cells to divide. Lymphocytes that are infected expand and spread rapidly into the lymphoid of the host, causing a great destruction of the host cells. In the lymphocytes, the schizonts differentiate into merozoites, invading the erythrocyte and transforming into piroplasms, stages infective to the tick. (Norval *et al.*, 1991).

2.5. Diagnosis of haemoparasites

Direct and indirect methods have been employed for diagnosis of tick-borne haemoparasites. Direct parasitological methods involve the identification of the parasite from the blood or tissue samples. However, microscopic examination of blood and/or tissue smears is less sensitive, especially during the early and later stages of infection (Terkawi *et al.*, 2011). The indirect methods involve the use of serological methods to screen for antibodies against the tick-bone parasites. A common drawback of these methods is low specificity.

The molecular diagnostic techniques for the characterisation and identification of haemoparasites have been developed, such as polymerase chain reaction-Reverse Line Blot hybridization (PCR-RLB) and quantitative real-time PCR (qPCR). Although classical methods can detect parasites, they require some time of about 10-15 min, meaning they are less specific, sensitive and reliable when compared to the molecular techniques.

2.5.1 Serological methods

2.5.1.1 Immunofluorescent antibody test

The IFA test is one of the serological assays that have been employed for diagnosis of *Theileria, Babesia, Anaplasma* and *Ehrlichia*. It is used for screening of antibodies against *T. parva* with the use of infected lymphoblasts as the antigen (Lawrence *et al.*, 2004). Moreover, this test can detect *T. parva* antibodies from the animal after it has recovered from the infection (OIE, 2004).

2.5.1.2 Enzyme linked immunosorbent assay

The ELISA test is employed for detecting *T. parva* antibodies with the use of recombinant polymorphic immonodominant molecules (PIM), a surface protein expressed by sporozoite and schizont. It is the immunogenic *T. parva* antigen which result in antibody production. Although the test is sensitive and has a long shelf life, it does not distinguish between current and past infections (Katende *et al.*, 1998). Enzyme-linked immunosorbent assays are used also to detect *B. bovis*, *A. marginale* and *Ehrlichia ruminatium* antibodies, in animals already exposed to infections, however, as previously mentioned, the presence of antibodies does not neccessarily correlate with current infections. Until now, application of ELISA for B. bigemina is still unreliable until a more purified Babesia-specific antigen or specific monoclonal antibodies are available(de Vos *et al*., 2004).

2.5.1.3 Complement fixation test

CFT is based on the use of complement to detect the presence of specific antibodies in the serum. These assay have been used for diagnosis of *Theileria*, *Babesia*, but it is not a commonly used for the diagnosis of these diseases (Herr *et al.*, 1985 and Bose *et al.*, 1995).

2.6 Molecular methods

Molecular techniques created the possibility of earlier and more accurate diagnosis. They are able to specifically detect parasites from blood or other tissue samples with high levels of sensitivity (Caccio *et al.*, 2000). Detecting haemoparasites infections in animals that are carriers has been always difficult when using parasitological and serological methods. However, molecular techniques are considered as the most suitable alternative for blood parasites detection due tor their higher sensitivity and specificity (Oura, *et al*., 2004; Pienaar, *et al*., 2011; Chaisi, *et al*., 2011). These include PCR-based RFLP assay, Real-time PCR, Conventional PCR assays and RLB-hybridisation assay.

2.6.1 Conventional PCR assays

The conventional PCR include the use of a *Taq* polymerase enyzme to makes new strands of DNA, using existing strands as templates, as well as primers that bind to the target DNA (Mullis, 1990). The agarose gel is used to visualize DNA segments according to their size by comparing to DNA fragments of known length. The use of primers specific for *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* species in a PCR, allows for the successful amplification of their DNA.

Problems with PCR for the diagnosis may sometimes arise when primers are less specific, resulting to amplification of unwanted parasites but of the related species (Bishop *et al.*, 1992; Watt *et al.*, 1998). Moreover, conventional PCR can encounter cross-contamination and false-positives.

2.6.2 PCR-based RFLP assays

A number of PCR-based restriction fragment length polymorphism (RFLP) assays have been used for screening and characterization of *T parva* isolates according to their polymorphic profiles. Other PCR-RLFP assays are used in mixed infections to differentiate between different *Theileria* spp, and *T. parva* stocks with the use of 18S rRNA, polymorphic immunodominant molecule (PIM) and p l04 genes (Geysen *et al.*, 1999; Bishop *et al*., 2001 and Sibeko *et al.*, 2010).These assays are easy to perform but they require the restriction enzyme digestion which is time consuming (Sibeko *et al.*, 2010).

2.6.3 PCR-RLB hybridization

The reverse line blot hybridization assay allows combined detection of multiple pathogens from one sample. (Gubbels *et al.*, 1999). This method has been used for the detection of various species in the genus *Anaplasma*, *Babesia*, *Ehrlichia* and *Theileria* in different hosts and vectors from different geographical areas (Gubbels *et al.*, 1999; Bekker *et al.*, 2002). Although this assay is very important in detecting parasites in samples with mixed infections, it is less sensitive in detecting parasites that occur at low level. Moreover, some probes lack specificity, because of the competition for primers, in case where there are mixed infections with the parasites of the same genus (Bishop *et al.*, 1992; Watt *et al.*, 1998; Sibeko *et al*., 2008).

2.6.4 Real-time PCR

The real-time PCR enables the specific detection of tick-borne haemoparasites and also allows for the quantification of the parasitic load (Katzer *et al.*, 1998). It is also used for the monitoring of the results in real-time, while the run is in progress. Moreover, *T. parva* real-time PCR assay is considered time efficient, when compared to other assays that were used previously and it’s highly sensitive (Sibeko *et al.*, 2008).

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Study area

Zambezi delta in Mozambique is located on the east coast of southern Africa (**Figure 3.1**), in which a buffalo population have been isolated for many years. The area is surrounded by a Tsetse fly belt where the trypanosome challenge is very high. The delta is characterized by extensive mangrove forest, one of the largest on the east coast of Africa. The grassland is very productive and supports a diversity of wildlife, including the African buffalo (*Syncerus caffer*), African elephant and other animals. It covers an approximate area of 1.2 million hectares on the central coast of Mozambique (Warren *et al.*, 2012).

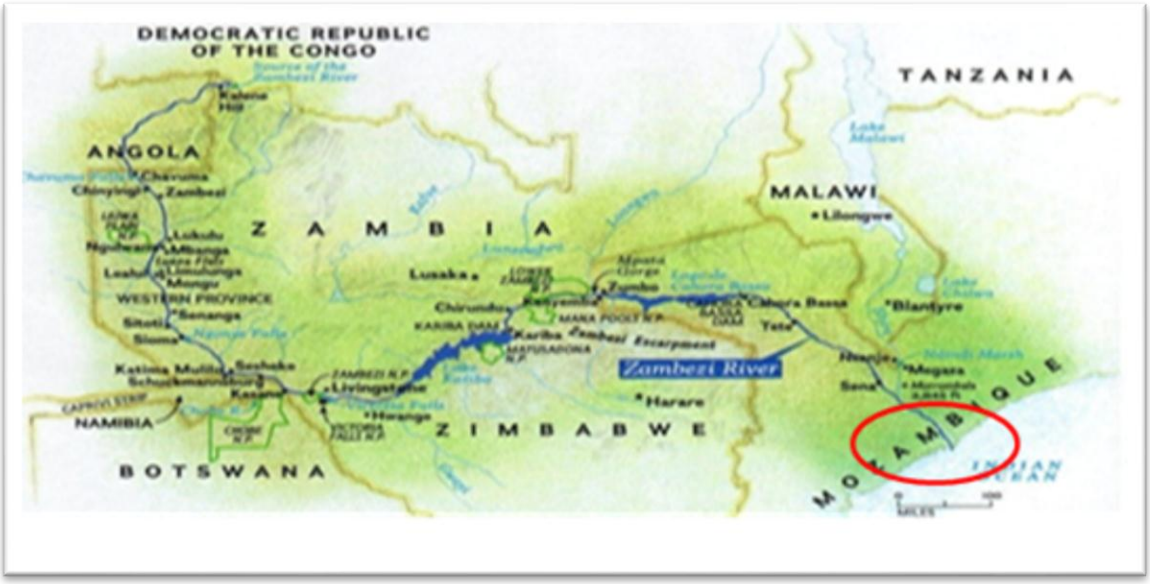


Figure 2.1: Map of the location in the area of Zambezi delta in Mozambique, where the buffalo population investigated in this study is located.

3.2. Sample collection

Blood samples used in the study were made available by the Biotechnology Center of Eduardo Mondlane University, Maputo Mozambique. The samples were originally collected from 40 buffalo on the 23th and 24th November 2015. During the capture process, 5 ml of blood were collected from the jugular vein, using 18G needles and ethylene diamine tetra-acetic acid (EDTA) coated vaccutainer tubes. Blood samples were transported on cooler pack and maintained at -20°C at the the Biotechnology Center.

3.3. DNA extraction

DNA was extracted from buffalo whole blood using a Qiagen QIAmp® DNA extraction kit (Qiagen, Germany) with slight modifications. The blood samples were incubated for 30 minutes at 56°C incubation, instead of the recommended 10 min. The integrity of DNA was determined by electrophoresis on a 2% agarose gel and the concentration was confirmed using the Qubit fluorometer 2.0 (Invitrogen, Thermo Scientific^{TM}, Randburg, South Africa).

3.4. Reverse line blot (RLB) hybridization assay

3.4.1. Membrane and oligonucleotide preparation

A Biodyne C membrane (ThermoFisher Scientific, United States of America) was prepared by placing a membrane in the miniblotter. The membrane was incubated in 10 ml of 16% Ethylene diamine tetra-acetic acid **(**EDAC) (ThermoFisher Scientific, United States of America) for 10 min.

3.4.2. Preparation of oligonucleotide probes

The genus- and species-specific oligonucleotides probes, designed based on the variable region of the 16S and 18S rRNA genes, were used for specific detection of *Babesia/Theileria* and *Anaplasma/Ehrlichia* species, respectively. The probe sequences for tick-borne haemoparasites investigated in this study are shown in **table 3.1.** The genus-specific probes were diluted to 40 pmol/140 µl. for *Babesia/Theileria* (B/T) and 100 pmol/140 µl. for the *Anaplasma/Ehrlichia* (A/E), in

400 mM sodium hydrogen carbonate (pH of 8.4). The probes for detection of *Babesia bigemina* and *Theileria parva* were prepared at 100 pmol/140 µl. and the rest of the species-specific probes were prepared at 400 pmol/140µl. For the working stock, each probe was diluted in 0.5 mM of sodium hydrogen carbonate to a final concentration of 2 pmol/µl. Two hundred µl was loaded on the membrane. At room temperature, the membrane was incubated for 2 min. It was later inactivated with 100 mM of NaOH for 8 min on a shaker.

Table: 3.1. Sequences of the genus- and species-specific oligonucleotide probes used for the detection of tick-borne haemoparasites using the RLB hybridization assay.

Oligonucleotide probe name	**Sequence (5' to 3')**
Ehrlichia/*Anaplasma* genus group-specific	GGG GGA A AG ATT TAT CGC TA
Anaplasma centrale	TCG AAC GGA CCA TAC GC
Anaplasma marginale	GAC CGT ATA CGC AGC TTG
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG
Anaplasma bovis	GTA GCT TGC TAT GAG AAC A
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT
Anaplasma sp. (Omatjenne)	CGG ATT TTT ATC ATA GCT TGC
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA
Theileria/*Babesia* genus group-specific	TAA TGG TTA ATA GGA RCR GTT G
Theileria genus-specific	ATT AGA GTG TTT CAA GCA GAC
Babesia genus-specific 1	ATT AGA GTG TTT CAA GCA GAC
Babesia genus-specific 2	ACT AGA GTG TTT CAA ACA GGC
Babesia felis	TTA TGC TTT TCC GAC TGG C
Babesia divergens	ACT RAT GTC GAG ATT GCA C
Babesia microti	GRC TTG GCA TCW TCT GGA
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG
Babesia rossi	CGG TTT GTT GCC TTT GTG
Babesia canis	TGC GTT GAC GGT TTG AC
Babesia vogeli	AGC GTG TTC GAG TTT GCC

Babesia bicornis	TTG GTA AAT CGC CTT GGT C
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT
Babesia leo	ATC TTG TTG CTT GCA GCT T
Babesia gibsoni	CAT CCC TCT GGT TAA TTT G
Babesia sp. (sable)	GCG TTG ACT TTG TGT CTT TAG C
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C
Theileria bicornis	GCG TTG TGG CTT TTT TCT G
Theileria annulate	CCT CTG GGG TCT GTG CA
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
Theileria mutans	CTT GCG TCT CCG AAT GTT
Theileria parva	GGA CGG AGT TCG CTT TG
Theileria taurotragi	TCT TGG CAC GTG GCT TTT
Theileria velifera	CCT ATT CTC CTT TAC GAG T
Theileria equi	TTC GTT GAC TGC GYT TGG
Theileria lestoquardi	CTT GTG TCC CTC CGG G
Theileria ovis	TTG CTT TTG CTC CTT TAC GAG
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG
Theileria separate	GGT CGT GGT TTT CCT CGT

The degenerate position R denotes either A or G, W denotes either A or T and Y denotes C or T.

3.4.3. PCR amplification of the 16S and 18S rRNA variable region

The PCR was performed using primer sets RLB-F2 and RLB-R2 for *Theileria* and *Babesia* species (Nijhof, *et al.*, 2003; Table 3.2) and EHR-F and EHR-R for *Anaplasma* and *Ehrlichia* species (Bekker *et al*., 2002; **Table 3.2**), targeting the V4 and V1 regions of the parasite' small subunit 18S and 16S rRNA genes, respectively. Reverse primer in each case, was marked with biotin for easy detection of the PCR product at the end of hybridization. The PCR reaction mixture was prepared using 1X of Platinum® Quantitative PCR SuperMix-UDG (ThermoFisher Scientific Massachusetts, United States of America), 8 pmol of each primer, 9.5 µl of nuclease-free water and 2.5 µl of DNA to make a total reaction volume of 25 µl.

Table 3.2: Sequences of primers used foramplification of *Theileria* / *Babesia* and *Anaplasma* / *Ehrlichia* species

Target genus	Primer name	Primer sequence	Reference
Theileria &	RLB-F2	(5′-GAC ACA GGG AGG TAG TGA CAA G-3′)	Nijhof, *et al.*, 2003
Babesia	RLB-R2	(biotin-5′-CTA AGA ATT TCA CCT CTG ACA GT-3′)	
Anaplasma &	EHR-F	(5′-CCC TAG TCA CTR ACC CAA CCT TA-3′)	Bekker *et al.*, 2002
Ehrlichia	EHR-R	(5′-GAG TTW GCC GGG RCT TYT TCT-3′).	

Two sets of PCR master mix were prepared for *Theileria* and *Babesia* species amplification, as well as for *Ehrlichia* and *Anaplasma* species. A touch-down PCR programme was used as previously described (Nijhof *et al.*, 2003) and the conditions of the PCR are shown in **Table 3.3**. The positive controls used were *A. centrale* and *Babesia bovis* DNA for the 16S rRNA and 18S rRNA PCRs, respectively, while nuclease free water was used as a template for the negative control reaction. The amplicons were observed on a 2% agarose gel and then prepared for hybridization as described in the following section.

Table 3.3: Thermocycling programme for the touchdown PCR

Cycle	*Time	Temperature	Reaction
1 cycle	3 min	37^oC	UDG activation
1 cycle	10 min	94^oC	UDG inactivation & Taq polymerase activation
2 cycles	20 sec 30 sec 30 sec	94^oC 67^oC 72^oC	DNA template denaturing Primer annealing Amplicon extension
2 cycles	20 sec 30 sec 30 sec	94^oC 65^oC 72^oC	DNA template denaturing Primer annealing Amplicon extension
2 cycles	20 sec 30 sec 30 sec	94^oC 63^oC 72^oC	DNA template denaturing Primer annealing Amplicon extension
2 cycles	20 sec 30 sec 30 sec	94^oC 61^oC 72^oC	DNA template denaturing Primer annealing Amplicon extension
2 cycles	20 sec 30 sec 30 sec	94^oC 59^oC 72^oC	DNA template denaturing Primer annealing Amplicon extension
40 cycles	20 sec 30 sec 30 sec	94^oC 57^oC 72^oC	DNA template denaturing Primer annealing Amplicon extension
1 cycle	7 min	72^oC	Final extension

*Min=Minutes, Sec= Seconds

3.4.4. Hybridization

The hybridization of PCR products to the probes was performed as described by (Nijhof *et al*, 2005). Briefly, at room temperature the Biodyne C membrane including the genus- and species-specific probes was made active in 50 ml of 2X SSPE/0.1% SDS for 5 min. For hybridization, the PCR products were made ready for use by adding 130 µl of 2 X SSPE and 0.1% SDS to an aliquot of 2.5 µl in each PCR product, denatured for 10 min at 98^o C on a thermal cycler machine and immediately cooled on ice. The PCR products were denatured and hybridized to a genus- and species-specific probes on the membrane. Hybridization was done at 42°C for 60 min.

The membrane was washed two times and heated beforehand in 2 X SSPE and 0.5% SDS for about 10 min at 50°C, was also incubated in 10 ml of 2 X SSPE/0.5% SDS and 12.5 µl streptavidin-POD (peroxidase labelled) conjugate (1.25 U) for 30 min at 42°C. For detection of hybridized PCR products, 10 ml of enhanced chemiluminescence (ECL) (5 ml ECL1 + 5 ml ECL2) at room temperature it was added onto the membrane for 1 min. After that the membrane was uncovered for easy X-ray filming about 5 to 10 minutes by actually placing the membrane on the X-ray film in a sealed container in the room with light turned off. The X-ray film was then sequentially dipped into a fixer and developer fluid and finally rinsed with clean water. After the development of the X-ray film, dark spots resulting from a chemiluminescence reaction indicate hybridisation between the amplicon and the respective probe (Gubbels *et al*, 1999).

3.5. *Theileria parva*-specific real-time PCR

For confirmation of *T. parva* infection, DNA extracted from buffalo blood samples was subjected to the hybridization probe-based real-time PCR, as described by Sibeko *et al.* (2008). The primers and probes used to amplify the 167 bp fragment from the V4 variable region of the 18S rRNA gene are shown in **Table 3.4**. The reaction mix was prepared using the 10 x LightCycler FastStart DNA Master^{Plus} Hybridization Probes mix (Roche Diagnostics, Mannheim, Germany). The reaction mix for the positive control consisted of DNA from a *T. parva* positive buffalo sample, previously described by Sibeko *et al* (2018), and the DNA template was replaced with water in the negative control.

Table 3.4: Primers and probe sequences for the *Theileria parva* real-time PCR assay

Primer/Probe	Sequence 5'-3'	Reference
Forward primer	CTG CAT CGC TGT GTC CCT T	Sibeko *et al*. (2008)
Reverse primer	ACC AAC AAA ATA GAA CCA AAG TC	
Anchor probe	GGG TCT CTG CAT GTG GCT TAT-FL AGA AAA TTA GAG TGC TCA AAG CAG GCT TT-FL	
Sensor probe	LCRed640-TCG GAC GGA GTT CGCT-PH LCRed705-GCC TTG AAT AGT TTA GCA TGG AAT-PH	

3.6. Cloning and sequencing of the full-length 16 and 18S rRNA genes

Sequence analysis was performed to identify the *Anaplasma* or *Erhlichia* species for DNA samples that only hybridized to the genus-specific probe, A/E, and did not hybridize to any species-specific probe from the RLB hybridization assay analysis. Furthermore, to confirm *Theileria* sp (buffalo) infection since this haemoparasite was detected in only one sample. Thus, for the confirmation of *Anaplasma* or *Ehrlichia* species fD1 and rP2 were used to amplify the full-length 16S rRNA gene (1 500bp) as previously described by Weisburg *et al*. (1991). The forward and reverse primers, Nbab-1F and TB 18S-Rev, respectively, was used to amplify the full-length 18S rRNA gene (1 700bp) using PCR conditions described by Chaisi *et al*. (2011), in order to confirm *Theileria* sp. (buffalo) infection. The PCR reaction mixtures consisting of 2 µl DNA, 0.16 µM of each primer, and 1 x Phusion flash-high fidelity Master mix (ThermoFisher Scientific, United States of America) and water to make a 25 µl total volume, were used. The PCR conditions of 1 cycle of 98°C for 10 sec, and 32 cycles of 98°C for 1 sec, 55°C for 5 sec, 72°C for 15 sec and the final temperature at 72°C for 1 min was applied. The amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. Purified samples were ligated onto the pGEM-T Easy vector and used to transform competent cells (JM109) (Promega, Madison, WI). At least ten positive white colonies were picked from each sample and screened by colony PCR using primers RLB-F2 and RLB-R2 to confirm recombinants. Five microliters of the PCR product was analyzed by electrophoresis on a 2% agarose gel. PCR products were sent to Inqaba Biotec (South Africa) for DNA sequencing.

3.7. Sequence and phylogenetic analyses

The quality of DNA sequences obtained from the 16S rRNA and 18S rRNA gene recombinant clones was assessed and edited using the CLC main workbench version 7.5.1 (CLC Bio, Boston, MA, USA). The nucleotide Basic Local Alignment Search Tool [BLASTn] (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm if the correct gene was sequenced, by searching for homologous reference sequences, using the blastn algorithm. Subsequently, the multiple sequence alignments of the near full-length 16S rRNA gene (1,113 bp) and 18S rRNA gene (1,470 bp) sequences, with published sequences for the same genes, were constructed using the Multiple Alignment using Fast Fourier Transform (MAFFT) [version 7] program (Katoh and Standley, 2013) and manually edited on BioEdit (version 7.2.5) (Hall, 1999). Sequence identities were calculated by pairwise comparison using MEGA7 (Kumar *et al*., 2015). The best-fit nucleotide substitution model, GTR + I + G, was used for both the 16S rRNA and 18S rRNA sequences to estimate genetic variation as a basis for evolutionary analysis for the phylogenetic trees. Phylogenetic trees for the 16S rRNA gene and 18S rRNA gene were reconstructed using maximum likelihood (ML) as implemented in MEGA7 (Kumar *et al*., 2016). The reliability for the internal branches for ML and parsimony methods was assessed using bootstrapping [1000 bootstrap replicates] (Felsenstein, 1985). Graphical representation and editing of the phylogenetic trees were performed with MEGA7 and Paint Tool for Windows 10.0.

CHAPTER 4

3. RESULTS

4.1. Haemoparasite detection by reverse line blot hybridization assay

The simultaneous screening for *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species infection in DNA prepared from 40 buffalo blood samples resulted in the detection of five *Theileria* [*Theileria* sp. (sable), *T. mutans*, *T. parva*, *T. velifera*, *Theileria* sp. (buffalo)] and three *Anaplasma* [*Anaplasma* sp. (Omatjenne), *A. marginale,* and *A. centrale*] species. **Figure 4.1** shows an x-ray film of the RLB results obtained from the analysis of some of the samples investigated in this study.

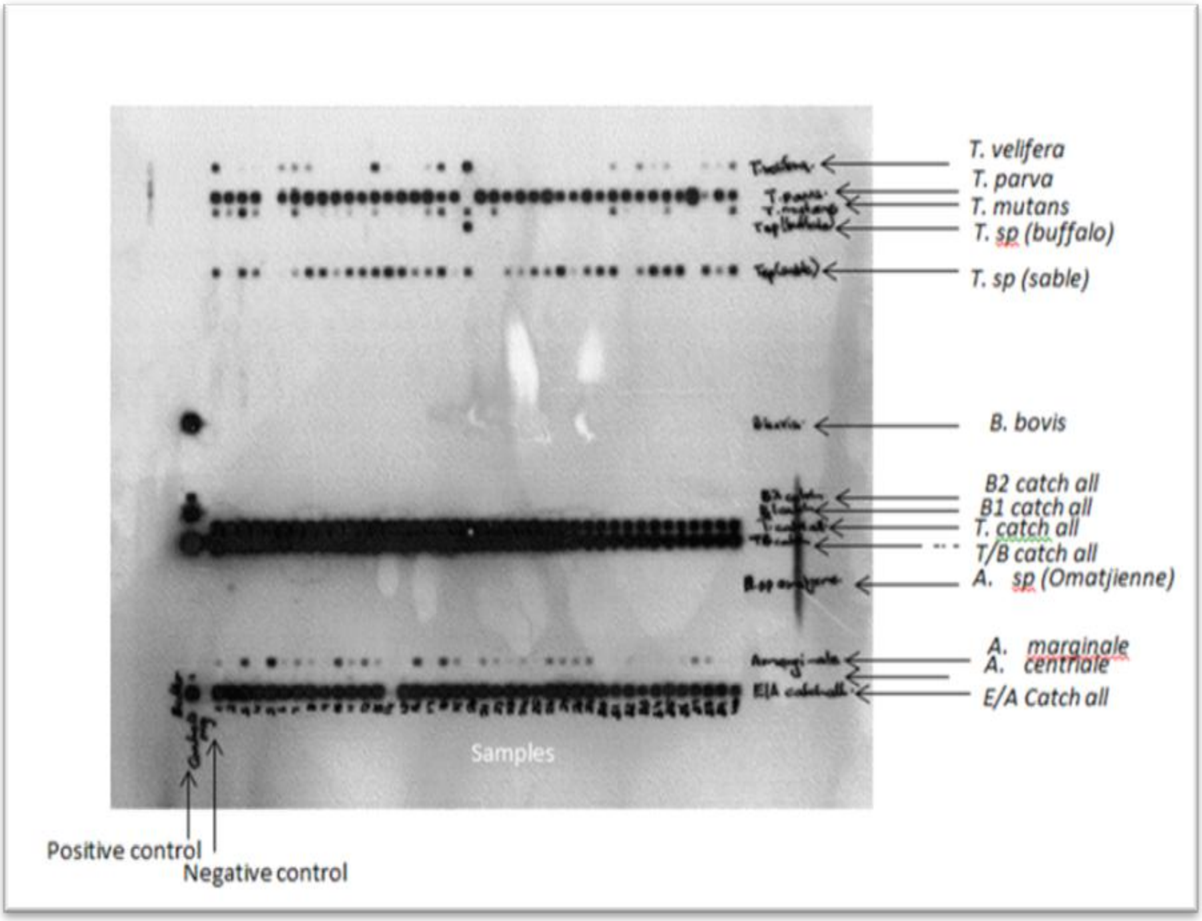


Figure 4.1: The representative reverse line blot (RLB) x-ray film showing results of the simultaneous detection of *Ehrlichia, Anaplasma, Theileria* and *Babesia* species DNA in the African buffalo (*Syncerus caffer*) blood samples investigated in this study. The RLB results demonstrated that infections of the detected haemoparasites occurred as either single or mixed infections. Many of the samples examined, except one, showed mixed populations of either *Theileria* speies or the combination of *Theileria* and *Anaplasma* species, with the minimum of two and maximum of five parasites per sample (**Figure 4.2**). The most represented mixed infection included *A. marginale, Theileria* sp. (sable) and *T. parva.*

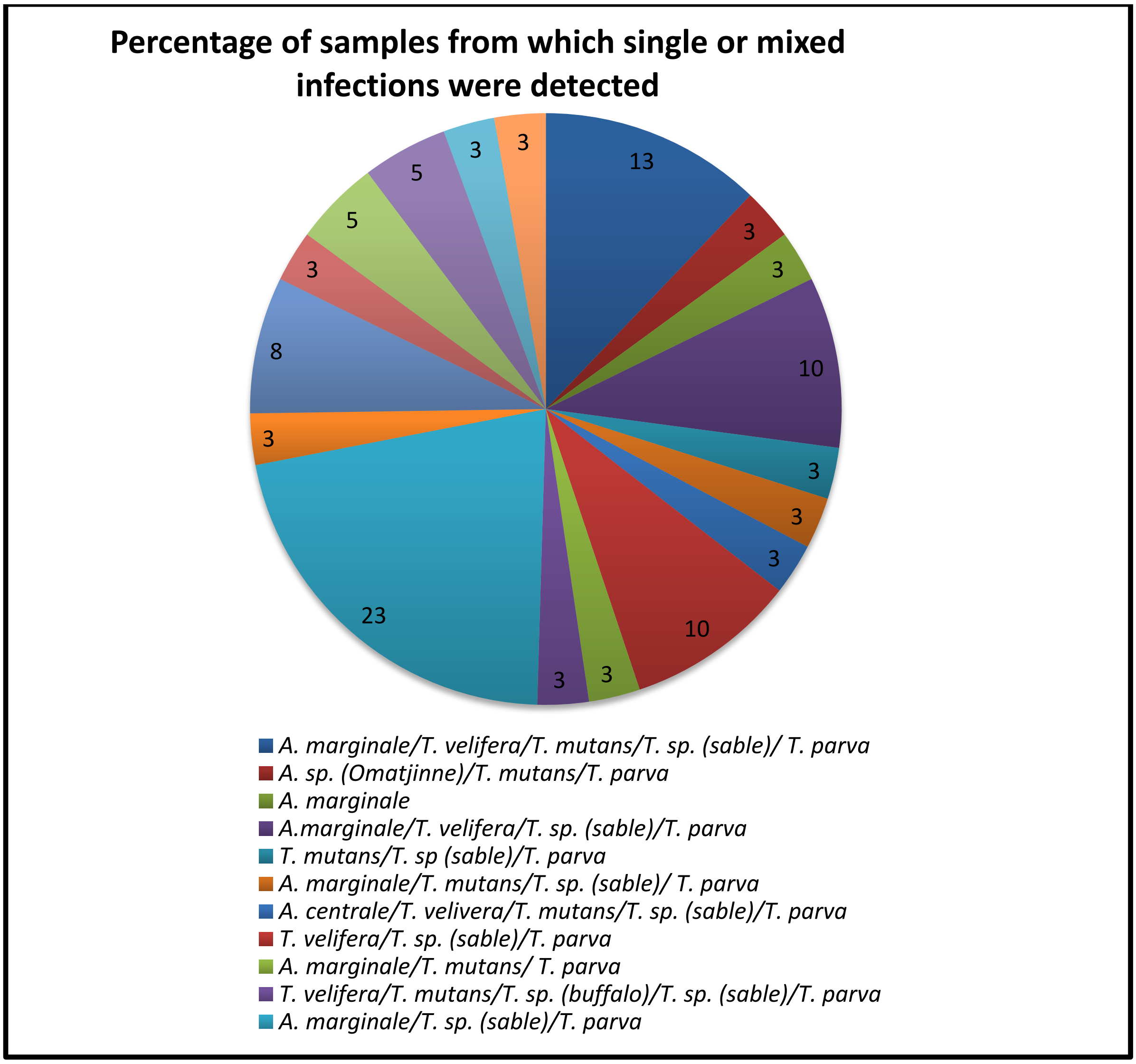


Figure 4.2: Detection of single and mixed infections of *Theileria* and *Anaplasma* species in blood samples of buffalo from the Zambezi delta area in Mozambique, as determined by the RLB hybridization assay.

The analysis of the representation of each haemoparasite species showed that the most frequently occurring was *Anaplasma marginale* (63%) for the *Anaplasma* species, followed by *A. centrale* (10%) and *Anaplasma* sp*. omatjienne* (3%), with regard to *Theileria* species, the most common piroplasms identified were *T. parva* (95%), and *Theileria* sp. *(sable)* (85%), followed by *T. velifera* (45%), *T. mutans* (43%), and *Theilera sp. (buffalo)* (3%) (**Figure 4.3**). No *Babesia* or *Ehrlichia* infections were detected from any of the 40 DNA samples analysed.

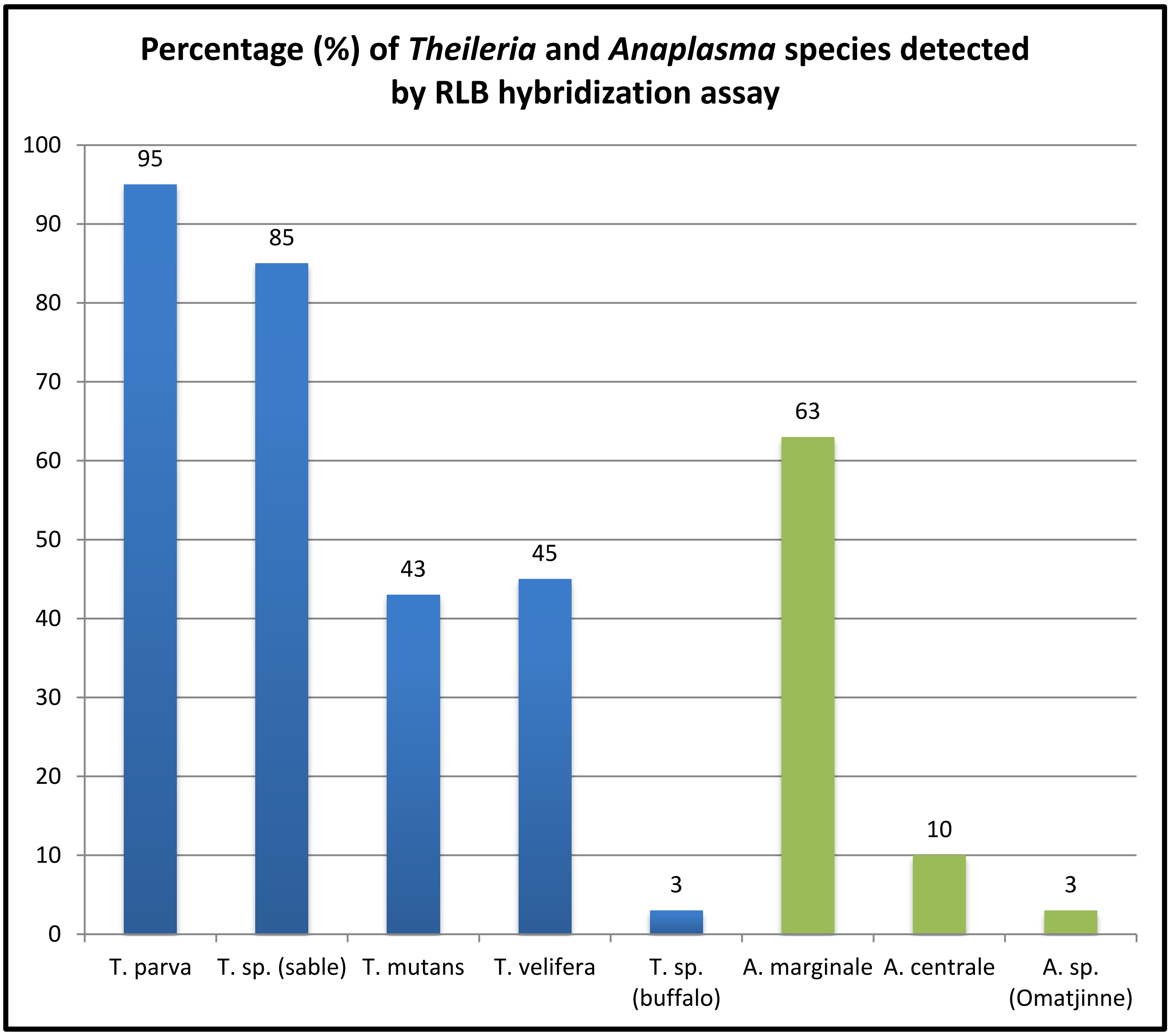


Figure 4.3 The frequency of the occurrence of *Theileria* **(shown in blue bars)** and *Anaplasma* (**shown in green bars**) spp. detected in blood samples from buffalo of the Zambezi delta area in Mozambique, as determined by the RLB hybridization assay.

4.2. Specific detection of *Theileria parva* using real time PCR

To confirm *T. parva* infections, a hybridization probe-based real-time PCR assay (Sibeko *et al.,* 2008), was used. The specific detection of *T. parva* is shown by a melting peak at 63 ± 0.62°C (**Figure: 4.4**). Thus, the presence of *T. parva* infection was confirmed in 100% of the 40 samples tested.

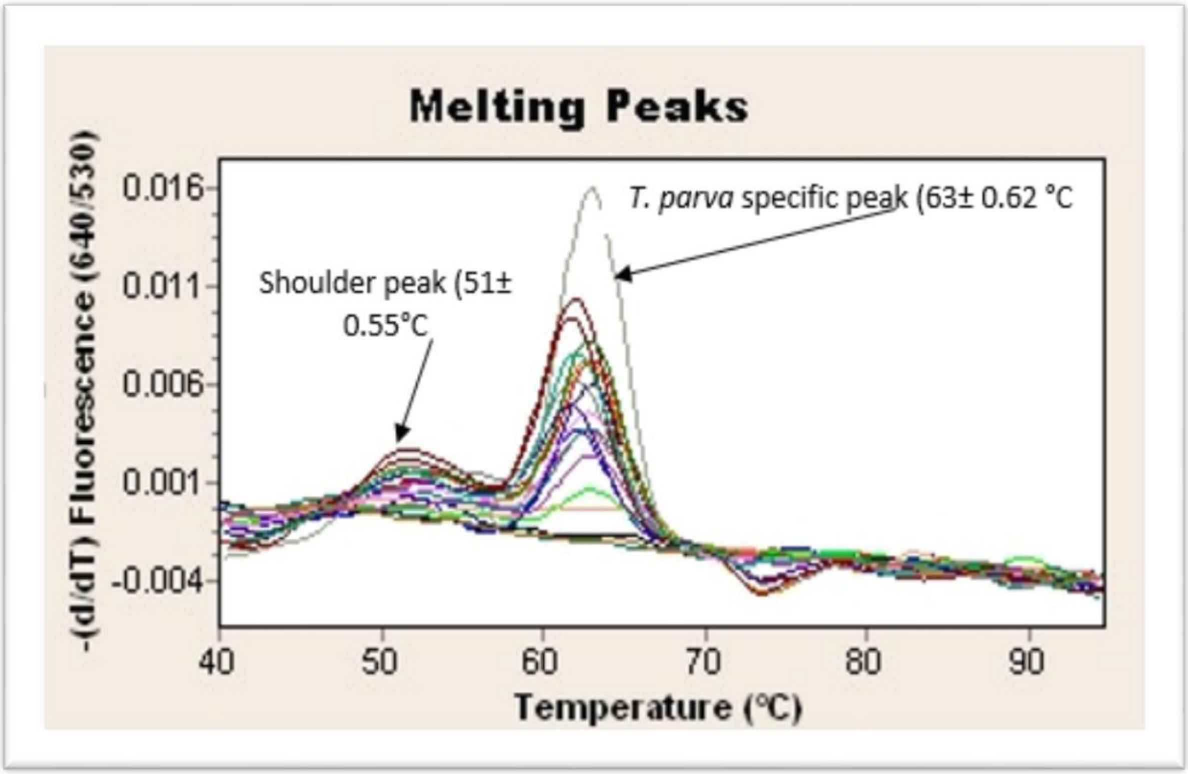


Figure 4.4: Graphical representation of real-time PCR melting peak analysis at 640 nm showing the specific detection *T. parva,* indicated by a melting peak at 63 ± 0.62°C.

4.3. The amplification of the full-length 16S and 18S rRNA gene

Six samples, which hybridized with the A/E genus-specific probes only without the signal on species-specific probes, were selected for the confirmation of the *Anaplasma* or *Ehrlichia* species. The PCR products obtained for the 16S rRNA gene were of the expected fragment size, 1 500bp (**Figure 4.5**). The amplicon of the near full-length 18S rRNA gene, was also of the expected fragment size, 1 700bp (**Figure 4.6**). No amplification was obtained from the water negative controls indicating that no contamination was introduced into the reactions during the preparation of the PCR reactions.

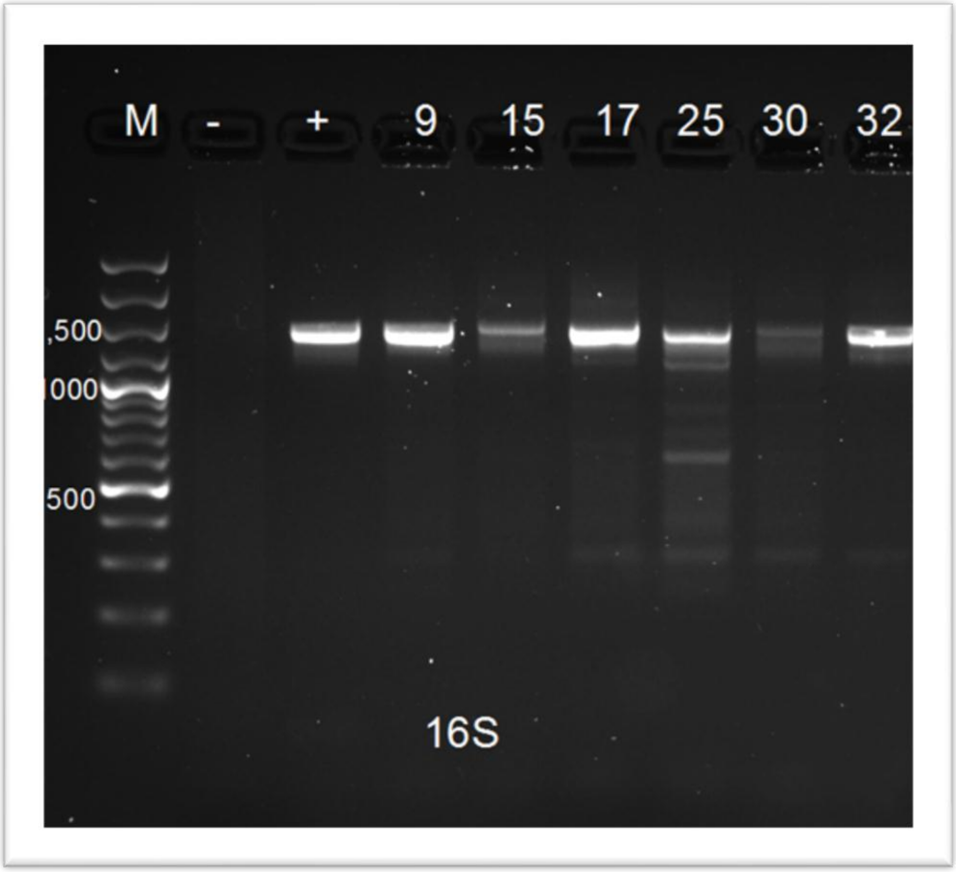


Figure 4.5: The 2% agarose gel image showing the PCR products obtained from the amplification of the full-length 16S rRNA gene (lanes labeled 9, 15, 17, 25, 30, and 32, according to the sample numbers). The size of the amplicons was determined using the 100bp molecular weight marker, shown in the lane marked with an ‘M’. Lanes 2 (-) and 3 (+) show amplification in negative and positive control reactions, respectively.

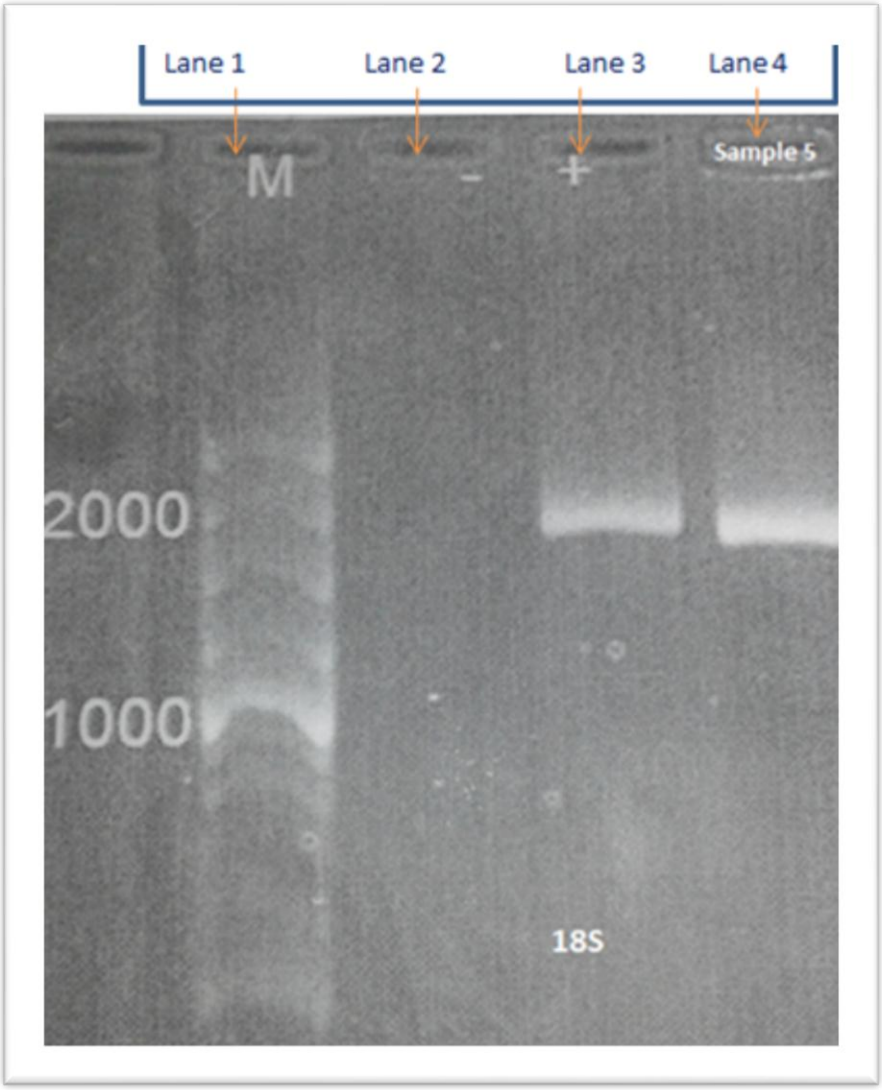


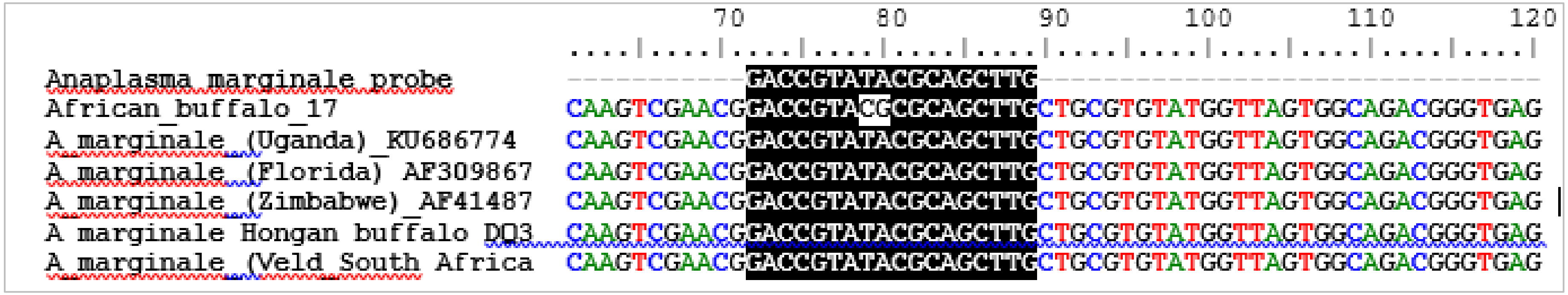
Figure 4.6: The 2% agarose gel image showing the PCR products obtained from the amplification of the full-length 18S rRNA gene from the RLB *Theileria* sp. (buffalo) positive sample (lane 4). The size of the amplicons was estimated using the 100bp molecular weight marker, shown in lane 1 marked with an ‘M’. A negative control was loaded in lane 2 and *T. parva* positive control in lane 3.

4.4. The 16S rRNA gene sequence and phylogenetic analysis

The homology analysis of the nine 16S rRNA gene sequences obtained in this study showed that one sequence had a 99% sequence identity to the published *A. marginale* 16S rRNA gene sequences obtained from the African buffalo from Florida (accession number; AF309867), Hongan (accession number: DQ341369), and Uganda (accession number: KU686792) as well as from African buffalo (accession number: KY287637), Eland (accession number: AF414872) and black wildebeest (accession number: AF414871) from South Africa. The other four sequences had 99% sequence identity to *A. centrale* isolates from Israel (accession number: CP001759), Uganda (accession number: KU686784) and isolates from *Rhipicephalus simus* from South Africa (accession number: AF414869) 16S rRNA gene sequences. The multiple sequence alignment of the V1 variable region of the 16S rRNA gene revealed two sequence variations in the RLB probe region of *A. marginale* and one in the region of the *A. centrale* probe (**Figures 4.7** and **4.8**, respectively)

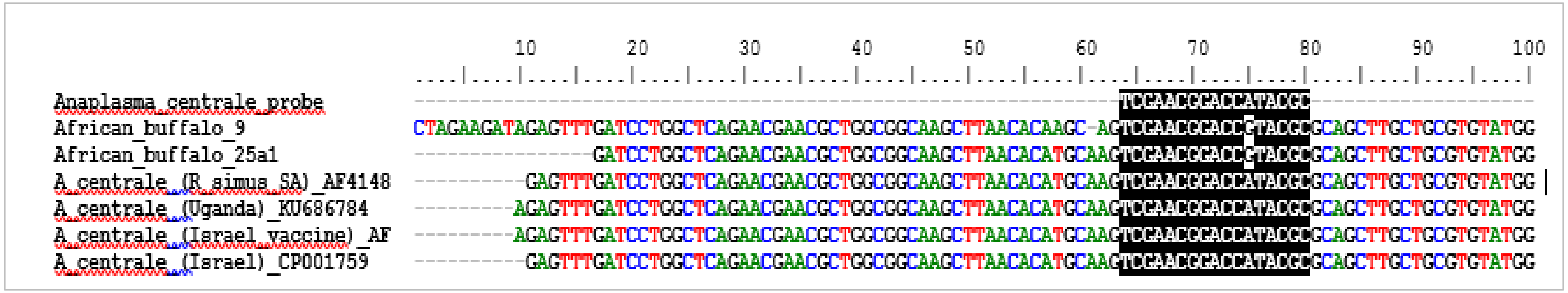
The pairwise analysis was performed to determine the differences between the sequences generated in this study and other published *A. marginale* and *A. centrale* 16S rRNA gene sequences. The number of nucleotides differences identified ranged from 1 to 10 base pairs for *A. marginale* and 1 to 5 for *A. centrale* (**Table 4.1**). The most nucleotide differences detected for *A. marginale* were between African buffalo 17 (Mozambique) from the current study and KU686789 (Uganda) sequences. While for *A. centrale* most nucleotide variations were detected between the sequence of African buffalo 32a2 and 25a1 (Mozambique) from the current study and published sequences AF414869 (from *R. simus*, South Africa), KU686784 (Uganda) and AF309869 (Israel vaccine).

Both neighbor-joining and maximum likelihood phylogenetic analyses were used to reveal the relationship among *A. marginale* and *A. centrale*. Since the bootstrap values and topologies obtained from the two analysis were similar, only the maximum-likelihood tree is presented (**Figure 4.9**). The *A. marginale* 16S rRNA sequences from this study grouped in one cluster with previously published sequences from cattle in Uganda, USA (Florida), Thailand, Hongan buffalo from China, wildebeest and African buffalo from South Africa. The *A. centrale* 16S rRNA sequences obtain in this study grouped with sequences from cattle from Uganda, as well as from African buffalo and *Rhipicephalus simus* from South Africa.



```
                                         70        80        90       100       110       120
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
Anaplasma_marginale_probe        -----------GACCGTATACGCAGCTTG-------------------------------
African_buffalo_17               CAAGTCGAACGGACCGTACGCGCAGCTTGCTGCGTGTATGGTTAGTGGCAGACGGGTGAG
A_marginale_(Uganda)_KU686774    CAAGTCGAACGGACCGTATACGCAGCTTGCTGCGTGTATGGTTAGTGGCAGACGGGTGAG
A_marginale_(Florida)_AF309867   CAAGTCGAACGGACCGTATACGCAGCTTGCTGCGTGTATGGTTAGTGGCAGACGGGTGAG
A_marginale_(Zimbabwe)_AF41487   CAAGTCGAACGGACCGTATACGCAGCTTGCTGCGTGTATGGTTAGTGGCAGACGGGTGAG
A_marginale_Hongan_buffalo_DQ3   CAAGTCGAACGGACCGTATACGCAGCTTGCTGCGTGTATGGTTAGTGGCAGACGGGTGAG
A_marginale_(Veld_South_Africa   CAAGTCGAACGGACCGTATACGCAGCTTGCTGCGTGTATGGTTAGTGGCAGACGGGTGAG
```

Figure 4.7: The nucleotide alignment of the partial 16S rRNA gene sequences of *Anaplasma marginale* showing nucleotide differences from one of the sequence obtained from the current study in the RLB probe region (between nucleotide 150bp) within the V1 hypervariable region.



```
                                           10        20        30        40        50        60        70        80        90       100
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Anaplasma_centrale_probe           ---------------------------------------------------------------TCGAACGGACCATACGC--------------------
African_buffalo_9                  CTAGAAGATAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAAGCTTAACACAAGC-AGTCGAACGGACCGTACGCGCAGCTTGCTGCGTGTATGG
African_buffalo_25a1               ----------------GATCCTGGCTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGACCGTACGCGCAGCTTGCTGCGTGTATGG
A_centrale_(R_simus_SA)_AF4148     ----------GAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGACCATACGCGCAGCTTGCTGCGTGTATGG
A_centrale_(Uganda)_KU686784       ---------AGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGACCATACGCGCAGCTTGCTGCGTGTATGG
A_centrale_(Israel_vaccine)_AF     ---------AGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGACCATACGCGCAGCTTGCTGCGTGTATGG
A_centrale_(Israel)_CP001759       ----------GAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGACCATACGCGCAGCTTGCTGCGTGTATGG
```

Figure 4.8: The nucleotide alignment of the partial 18S rRNA gene sequences of *Anaplasma centrale* showing nucleotide differences from two sequencces obtained from the current study in the RLB probe region (between nucleotides 170bp) within the V1 hypervariable region.

Table 4.1: The pairwise analysis of the near full-length 16S rRNA sequences of *A. marginale* and *A. centrale* showing the number of nucleotide differences (number of nucleotides) between sequences produced in this study and published sequences from different host species and geographic origin.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	**African buffalo 17 (*A. marginale*)**																				
2	**African buffalo 9 (*A. centrale*)**	9																			
3	**African buffalo 25a1 (*A. centrale*)**	5	2																		
4	**African buffalo 32a1 (*A. marginale*)**	2	6	7																	
5	**African buffalo 32a2 (*A. centrale*)**	10	4	5	7																
6	**African buffalo 25b1 (*A. centrale*)**	6	2	1	8	1															
7	**African buffalo 30b5 (*A. marginale*)**	6	6	7	4	7	8														
8	*A. marginale* (Florida) AF309867	6	9	9	0	10	8	4													
9	*A. marginale* (Zimbabwe) AF414878	8	9	9	2	10	8	4	2												
10	*A. marginale* (Veld_South_Africa) AF414873	7	8	8	1	9	7	3	1	1											
11	*A. marginale* (SA black wildebeest) AF414871	7	10	10	1	11	9	5	1	3	2										
12	*A. centrale* (*R. simus* SA) AF414869	8	3	1	7	5	1	7	10	10	9	11									
13	*A. centrale* (Uganda) KU686784	8	3	1	7	5	1	7	10	10	9	11	0								
14	*A. centrale* (Israel vaccine) AF309869	8	3	1	7	5	1	7	10	10	9	11	0	0							
15	*A. marginale* (Uganda) KU686789	10	11	8	1	9	7	3	4	4	3	5	12	12	12						
16	*A. centrale* (African buffalo) KC189842	7	2	1	8	5	1	8	8	8	7	9	1	1	1	9					
17	*A. centrale* (African buffalo) KC189841	8	3	2	9	6	2	9	9	9	8	10	2	2	2	10	3				
18	*A. centrale* (Israel) CP001759	8	3	1	7	5	1	7	10	10	9	11	0	0	0	12	1	2			
19	*A. marginale* (Florida) CP001079	7	10	10	1	11	9	5	1	3	2	2	11	11	11	5	9	10	11		
20	*A.marginale* (African buffalo South Africa) KY287637	1	7	6	1	7	6	5	1	3	2	2	6	6	6	2	7	8	6	2	

Except for sequences produced from buffalo from the current study, all the published sequences were obtained from cattle samples, unless otherwise indicated. The sequences obtained in this study are indicated in bold.

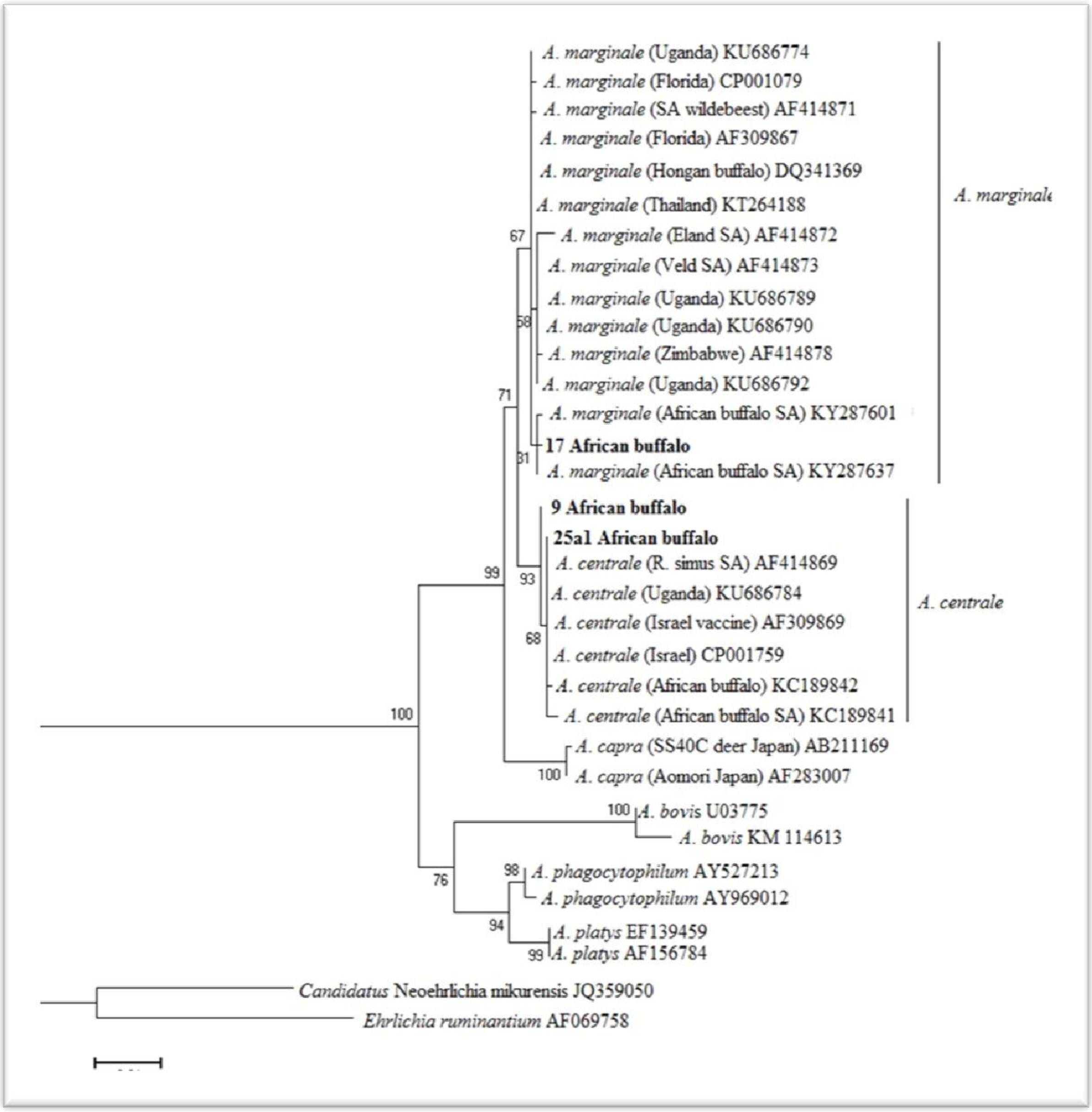


Figure 4.9: A maximum likelihood tree showing the phylogenetic relationship between the *A*. *marginale* and *A*. *centrale* 16S rRNA sequences, including other *Anaplasma* species. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. The sequences obtained in this study are shown in bold. The tree was rooted using the 16S rRNA gene sequences of *Candidatus Neoehrlichia mikurensis* and *Ehrlichia ruminantium*. The accession number of each sequence is indicated in the sequence name. Branch lengths are proportional to the estimated genetic distance (number of

nucleotide substitutions per site over a length of 1,113 bp of the 16S rRNA gene) between the taxa.

4.5. The 18S rRNA gene sequence and phylogenetic analysis

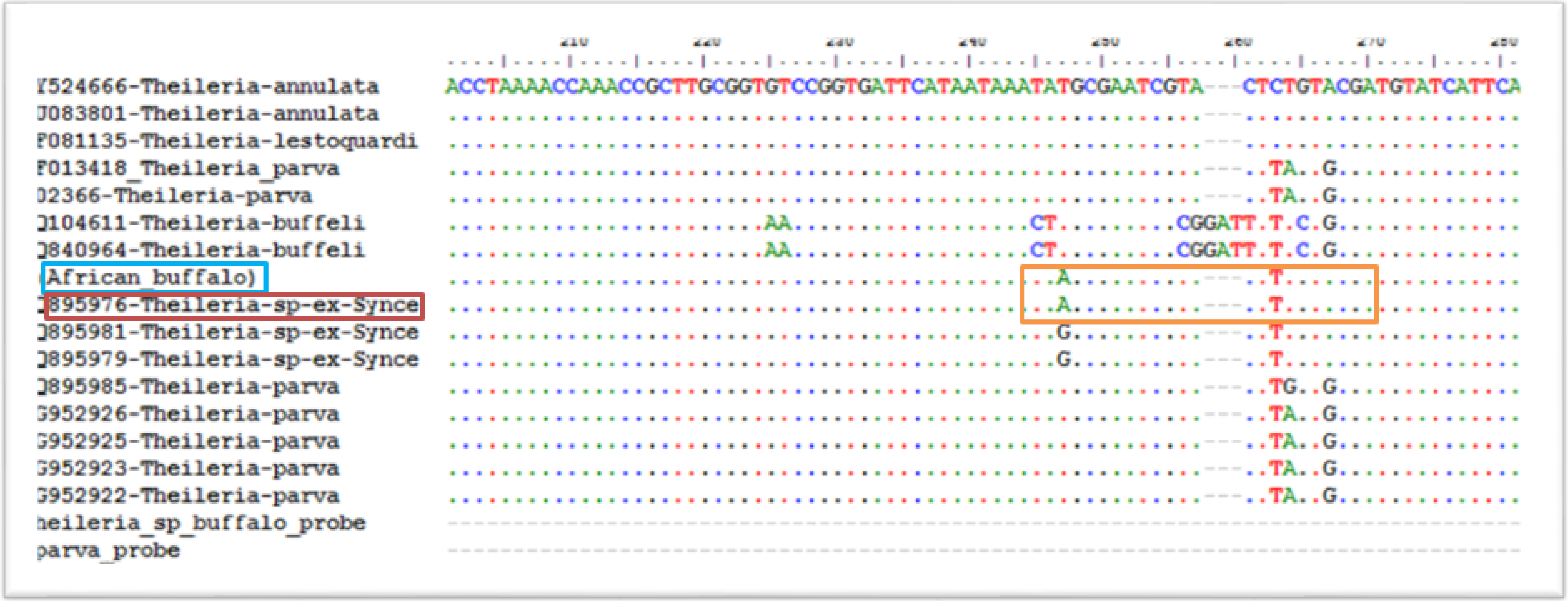
A BLASTn search analysis of the 18S rRNA gene sequence performed to confirm the detection of *Theileria* sp. (buffalo) by the RLB hybridization assay in one sample (sample 5) investigated in this study revealed high sequence identity to the *Theileria* sp. African buffalo 18S rRNA gene sequences from South Africa, particularly, the isolate with HQ895976 NCBI accession number. The pairwise analysis showed that the near full-length sequence of samples 5 was 100% identical to *Theileria* sp. African buffalo (HQ895981) sequence (**Table 4.2**). The multiple sequence alignment of the 18S rRNA *Theileria* sequences in the RLB oligonucleotides probe region, within the V4 variable region, revealed 100% sequence identity with *Theileria* sp. (buffalo) probe (**Figure 4.10**).

Both neighbor-joining and maximum likelihood phylogenetic analyses were used to reveal the relationship among *Theileria* spp. The bootstrap values obtain from maximum-likelihood and neighbor-joining trees were identical, thus only the maximum-likelihood tree is shown in (**Figure 4.11)**. Consistent to the BLASTn search analysis results, the sequence from samples 5 grouped with the published sequence for *Theileria* sp. African buffalo (HQ895976) from South Africa.

Table 4.2: The pairwise analysis of the near full-length 18S rRNA gene sequence produced from samples 5 (from the current study), and *Theileria parva* and *Theileria* sp. (buffalo) reference sequences from cattle and African buffalo

		1	2	3	4	5	6	7	8	9	10
1	*T. parva* L02366										
2	*T. parva* HQ895985	3									
3	*T. parva* Katete Zambia MG952926	0	3								
4	*T. parva* Kiambu5 MG952925	0	3	0							
5	*T. parva* Muguga2 MG952923	0	3	0	0						
6	*T. parva* Uganda MG952922	0	3	0	0	0					
7	*Theileria* sp. African buffalo HQ895976	14	12	14	14	14	14				
8	*Theileria* sp. African buffalo HQ895981	14	11	14	14	14	14	9			
9	*Theileria* sp. African buffalo HQ895979	14	11	14	14	14	14	9	2		
10	**5 (African buffalo)**	14	12	14	14	14	14	0	9	9	

The sequence from sample 5 obtained in this study is indicated in bold. The pairwise analysis was performed in a total of 1,477 nucleotide positions.



```
                                       210       220       230       240       250       260       270       280
                               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
524666-Theileria-annulata      ACCTAAAACCAAACCGCTTGCGGTGTCCGGTGATTCATAATAAATATGCGAATCGTA---CTCTGTACGATGTATCATTCA
083801-Theileria-annulata      .........................................................---.....................
081135-Theileria-lestoquardi   .........................................................---.....................
013418_Theileria_parva         .........................................................---..TA..G..............
02366-Theileria-parva          .........................................................---..TA..G..............
104611-Theileria-buffeli       ........................AA..................CT.........CGGATT.T.C.G..............
840964-Theileria-buffeli       ........................AA..................CT.........CGGATT.T.C.G..............
(African_buffalo)              ..............................................A..........---..T..................
895976-Theileria-sp-ex-Synce   ..............................................A..........---..T..................
895981-Theileria-sp-ex-Synce   ..............................................G..........---..T..................
895979-Theileria-sp-ex-Synce   ..............................................G..........---..T..................
895985-Theileria-parva         .........................................................---..TG..G..............
952926-Theileria-parva         .........................................................---..TA..G..............
952925-Theileria-parva         .........................................................---..TA..G..............
952923-Theileria-parva         .........................................................---..TA..G..............
952922-Theileria-parva         .........................................................---..TA..G..............
heileria_sp_buffalo_probe      ---------------------------------------------------------------------------------
parva_probe                    ---------------------------------------------------------------------------------
```

Figure 4.10: The nucleotide multiple sequence alignment of the RLB probe region, within the V4 hypervariable region of the 18S rRNA gene of *T. parva, Theileria* sp. (buffalo), *T. buffeli, T. annulata* and *T. lestoquardi*, against the query sequence from 170 base pair nucleotides. The sequence variations in the probe region are shown in orange block.

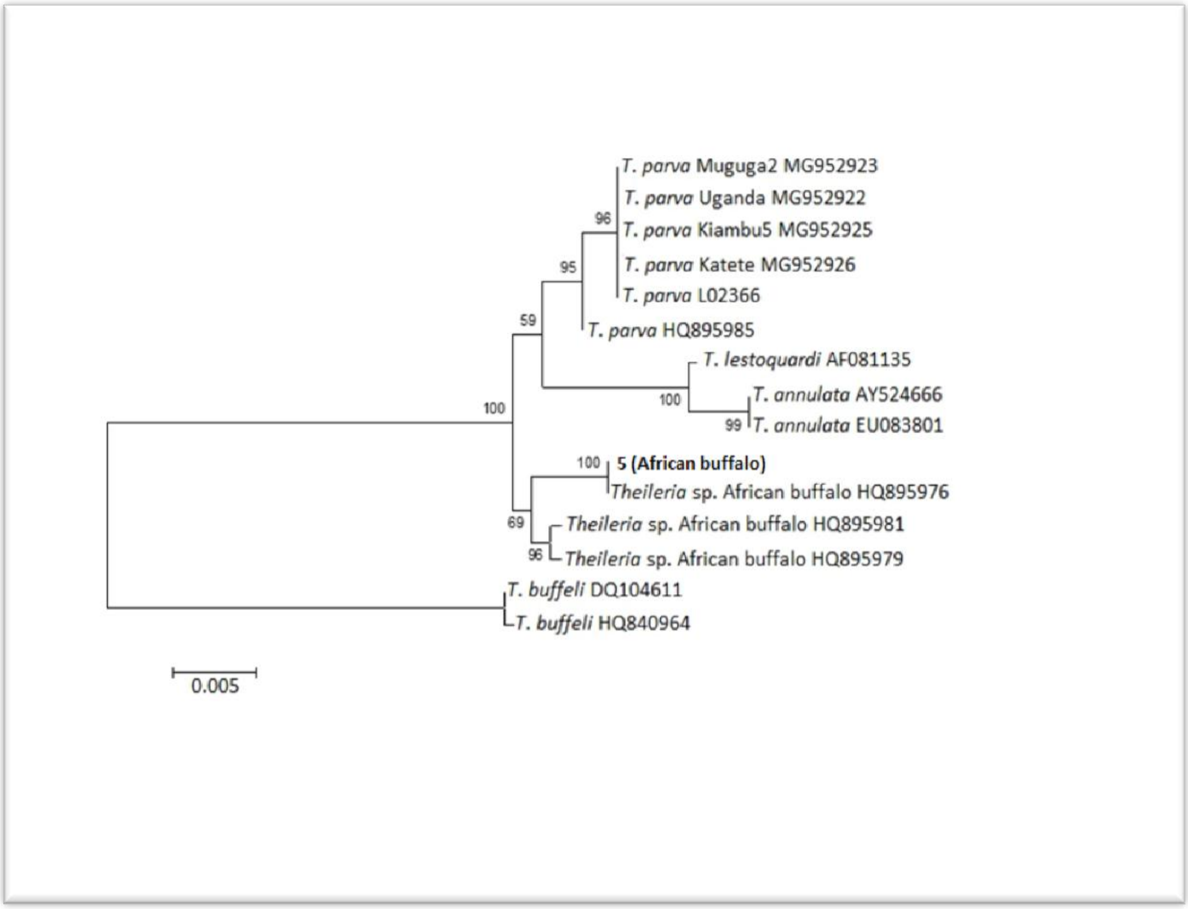


Figure 4.11: A maximum likelihood tree showing the phylogenetic relationship between sample 5 (African buffalo) (in bold) sequence, and published sequences from other *Theileria* species. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. The tree was rooted using the 18S rRNA gene sequences of *T. buffeli*. The accession number of each sequence is indicated next to the species name. Branch lengths are proportional to the estimated genetic distance (number of nucleotide substitutions per site over a length of 1,470 bp of the 18S rRNA gene) between the taxa.

CHAPTER 5

4. DISCUSSION

The buffalo population under investigation is from an area surrounded by a tsetse fly belt where trypanosome challenge is very high. For this reason the domestic animals and buffalos are kept separate. In addition, previous studies have shown that the species of ticks that parasitize buffaloes are similar to those that parasitize cattle and therefore they can transmit diseases, such as Corridor disease, between these animals (Miranpuri, 1988). In this study, the RLB hybridization assay results demonstrated that the investigated buffalos are infected with various haemoparasites including five *Theileria* and three *Anaplasma* spp. These species were detected either as single or mixed infections. This study is the first of its kind performed in buffalos in the area of Zambezi delta, thus the first report of pathogens occurring in this population.

The RLB analysis showed that *T. parva* (95%) was the most frequently occurring tick-borne haemoparasite species followed by *Theileria* sp. (sable) (85%), *A. marginale* (63%), *T. velifera*, (45%), *T.* mutans (43%), *A. centrale* (10%), *Theileria* sp. (buffalo) (3%) and *Anaplasma* sp. (Omatjenne) (3%). This assay has previously been used by various authors to detect parasites in infected host and vectors (Gubbels *et al*., 1999, Nijhof *et al*., 2003, Oura *et al*., 2011), which showed that buffaloes are usually infected with multiple *Theileria* spp. Consistent with this, the buffalo samples investigated were infected with more than one *Theileria* spp. among which were *T. parva, Theileria* sp. (buffalo), *T. mutans, T. velifera* and *T. buffeli* (Allsopp, *et al*., 1993; Gubbels, *et al*., 1999; Oura, *et al*., 2004; Pienaar, *et al*., 2011; Chaisi, *et al*., 2011). Furthermore, these findings were not unexpected as it is well-known that the African buffalo is a natural reservoir host of both pathogenic and non-pathogenic *Theileria* species (Young*, et al*., 1992; Uilenberg, 1995). It is also notable that these parasites always appear together, suggesting that they are common and widely distributed. Moreover, they infect both cattle and buffaloes. In our study, a total of 17 different combinations of mixed infections were observed. Chaisi *et al*

2011 reported 18 different combinations of mixed infections by these parasites in the African buffalo in Kruger National Park, Hluhluwe-iMfolozi Park, Greater Limpopo Transfrontier Park and from a game ranch near Hoedspruit. Mixed infections of *Theileria* spp, have been reported in field samples from different countries including eastern Africa and southern Africa, such as Rwanda (Bazarusanga *et al*., 2007), Uganda (Oura *et al*., 2010) and South Africa (Chaisi *et al.*, 2011; Pienaar *et al.*, 2011). In these studies, cattle and buffaloes were infected with the same *Theileria* parasites spp. detected in the current study, including *T. parva, T. mutans, T. velifera,* and *Theileria* sp. (buffalo).

The high prevalence of *T. parva* by qPCR is in agreement with findings of previous studies conducted on African buffalo in South Africa (Chaisi *et al*., 2011; Pienaar *et al*., 2011). This suggests that buffaloes in the investigated population are exposed to the constant presence of the tick vector that transmits *T. parva*, which might result in the persistent transmission of this haemoparasite between the buffalos in this location. Importantly, the high occurrence of *T. parva* indicates a high risk of spreading Corridor disease, should there be contact of infected buffalo with cattle in the area. Bhoora *et* al., 2010; Oura *et al*., 2005; Sibeko *et al*., 2008 also demonstrated that real-time PCR is more sensitive than the RLB hybridization assay and that *Theileria* and *Babesia* spp. infections can be detected by real-time PCR in samples that test negative by the RLB hybridization assay. All *Theileria* spp. detected in this study were also identified by Chaisi *et al*., (2011) and Pienaar *et al*., (2011) in their investigations. This suggest that these parasites and their vectors are widely distributed, explaining why the buffalos from Mozambique and South Africa were infected with similar *Theileria* species*. Theileria* sp. (sable) was the second most occurring *Theileria* spp. detected in this study. This parasite has been reported in buffalo, Nyala and Reedbuck in South Africa, African short-horn cattle in Tanzania, blesbok and wildebeest in Swaziland (Nijhof, *et al*., 2005; Debeila *et al*., 2013). The parasite has also been isolated in Sable (*Hippotragus niger*) and Roan (*Hippotragus equinus*) antelopes in South Africa. The main vectors transmitting *Theileria* sp. (sable) include *Rhipicephalus zambeziensis, R. evertsi evertsi* and *R. appendiculatus* and their distribution have been reported in Mozambique with no specific information regarding their geographic distribution in the country (Dias 1993). *Rhipicephalus appendiculatus* is the vector for *Theileria parva* which explains the reason why *Theileria* sp. (sable) and *T. parva* distributions overlap. Similar results were obtained by Debeila *et al* (2013) in that 61% of the samples tested positive for the presence of *Theileria* sp. (sable) in the Kruger National Park, using the RLB assay.

Theileria mutans and *T. velifera* were frequently detected in the current study. *Theileria mutans* has been considered a common parasite of cattle in many parts of the world (Neitz, 1957), but also harboured by African buffalo. Through field and experimental evidence it has been demonstrated that *T. mutans* strains from buffalo are more pathogenic to cattle than those derived from cattle (Theiler 1906). *Theileria velifera* had also been reported by a number of authors who detected the parasite from analysis of buffalo samples from central, east and southern Africa, including Rwanda, Uganda, Botswana, Zimbabwe, South Africa (Bazarusanga *et al*., 2007; Chaisi *et al*., 2011; Pienaar *et al*., 2011; Oura *et al*., 2005 Eygelaar *et al*., 2016). *Theileria velifera* is considered non-pathogenic and has no economic importance. The parasite has been reported from cattle and buffalo in South Africa (Berger 1979 and Debeila *et al*., 2013).

Theileria sp. (buffalo) was the least detected *Theileria* sp. and this species is known to be widely distributed in the eastern and southern Africa, including Kenya and East Africa (Conrad *et al*., 1987; Allsopp *et al*., 1993), Uganda (Oura *et al*., 2011) as well as in South Africa (Chaisi *et al*., 2011; Piennar *et al*., 2011; Eygelaar *et al*., 2016). It has been previously detected in cattle at high frequencies in buffalo (Pienaar *et al*., 2014; Mans *et al*., 2011). Additionally, *Theileria* spp.*, Anaplasma* spp.*, A. marginale, A. centrale and Anaplasma* sp. (Ommatjienne), were also detected in this study. Anaplasmosis is an important tick-borne disease of domestic and wild ruminant in tropical and subtropical regions of the world. To date, *A. marginale, A. centrale* and *Anaplasma* sp. (ommatjenne) is well documented in cattle but among the three *A. marginale* is an important cause of the disease in cattle while *A. centrale* and *A.* sp. (ommatjienne) do not cause clinical diseases, little is known about the disease and its impact in buffalo (Debeila, 2012; Fyumagwa *et al*., 2009). In the current study, the most common of the three *Anaplasma* spp. detected was *A. marginale* (63%) and this finding is comparable to that of Machado *et al*., (2016) where by *A. marginale* was identified in 72.2% of buffalo from Mozambique. This suggests that this parasite and its vector are well distributed in Mozambique. This species has also been detected at high frequencies in other studies. In buffalo samples from the National Parks in Uganda *A. marginale* was detected in 68% of the samples and *A. centrale* in 56% (Oura *et al*., 2011). Conversely, Eyglaar *et al* 2015 detected the same pathogen in only 20% of investigated buffalo samples from Botswana and Zimbabwe and 30% of *A. centrale* in the same samples. *Anaplasma marginale* has also been reported in water buffalos in Brazil and these animals are incriminated as important reservoirs of infection (Silva *et al*., 2015; Kocan *et al*., 2003).

Similarly, *A. centrale* finds its host in domestic and wild animals. This tick-transmitted *Rickettsia* has been detected in Buffalo, Black Wildebeest, Blue Wildebeest, Eland, Waterbuck, Impala and cattle (Khumalo *et al*., 2016; Oura *et al*., 2004). Although, *A. centrale* (10%) and *Anaplasma* sp. (Ommatjenne) (3%) were detected in low percentages in our study, suggesting that there is low circulation of this species in this area. The RLB analysis revealed that none of the analysed buffalo samples were carriers of *Babesia* and *Ehrlichia* species. However, *Babesia bovis* was reported by Penzhorn (2006) to be present in low percentage in buffalo from Okavango delta. This suggests that buffalos are not natural reservoir hosts of *Babesia* species. Conversely, *Ehrlichia ruminantium*, has been reported to infect domestic and wild ruminants, however, this parasite in most wildlife species appear not be associated with clinical disease (Peter, *et al*., 2002). Although this parasite has been reported in buffalo, it does not seem that buffalo is an important reservoir host; however, this subject remains open for further investigation.

The results of the present study showed that buffalos are exposed to high levels of challenge by the tick vectors. This observation is corroborated by the finding of similar levels of *T. parva* infection in animals from different age groups. *Theileria parva* infections were confirmed by qPCR analysis. There were slight discrepancies observed between results obtained using the qPCR and RLB hybridization assays in the detection of *T. parva*, with the former detecting the parasite in all 40 buffalo samples analysed while the latter detected *T. parva* in 95% of the samples tested. The sensitivity of the RLB hybridization assay is usually compromised when analysing field samples with mixed infections, which is the case in this study with the detection of multiple *Theileria* spp. in a single sample. Especially when the parasitemia of the pathogen of interest is very low. This scenario results in primer annealing being biased towards the more abundant DNA thus the diminished sensitivity for the detection of the target pathogen (Eygelaar *et al*., 2015). Another possible explanation for low sensitivity by the RLB hybridization assay is the poor hybridization probes which lead to under detection of some pathogens.

DNA samples that only hybridized with the genus-specific probe on the RLB were further subjected to sequence analysis in an attempt to identify the novel pathogens. The 16S and 18S rRNA genes have been useful in the characterization of tick-borne haemoparasites (Byaruhanga *et al*., 2016). The sequence analysis of the full-length 16S rRNA gene of three samples that only hybridized with the *Anaplasma/Ehrlichia* genus-specific probes, and had no signal on the species-specific probe, indicated that one of these was similar to the published *A. marginale* 16S rRNA gene sequences (KY287637; KY287601), two of which were obtained from buffalo samples from China and South Africa respectively. While, the other two 16S rRNA gene sequences were similar to those published for *A. centrale,* which were obtained from cattle from Israel and *Rhipicephalus simus* ticks from South Africa. The sequence similarities observed were confirmed by phylogenetic analyses and the new sequences clustered perfectly with the respective *A. centrale* and *A. marginale* sequence clades. Interesting finding was the presence of one full-length *Anaplasma margianle* sequence that was 100% identical to that of the published uncultured *Anaplasma* sp. clone from African buffalo from South Africa in the Monkala National Park and also 99% identical to *A. marginale* from buffalo found in China. Two full-length *A. centrale* sequences were identical to *Anaplasma centrale* from *Rhipicelulus simus* from South Africa, and 100% identical to sequences from cattle from Uganda.

The highest number of nucleotide variations identified for *A. centrale* was six, which was between sequences from African buffalo and sequences from Hongan buffalo; while for *A. marginale* it was 10, between sequences from African buffalo from South Africa and China, over a 1,113 bp region. The nucleotides variation observed might be the reason why these species were not detected by the RLB hybridization probe. The initial analysis of the *Theileria* 18S rRNA sequence from a sample that produced a signal only on the *Theileria/Babesia* genus-specific probes but no other signal from species-specific probes, using blast showed high similarity with a *T. parva* sequence. However, subsequent to further analysis by alignment with additional *T. parva* and *Theileria* sp. (buffalo) 18S rRNA sequences, obtained from the Genbank. It was found that our sequence was more similar to *Theileria* sp. (buffalo) with consideration of the region where the RLB probes for specific detection of *T. parva* and *Theileria* sp. (buffalo) were designed. Nine nucleotide base difference were detected, when *T. parva* sequence (the query) was compared to published *Theileria* sp. (buffalo). *T. parva* was generally identical to sequences (99-100%) originating from parasites isolated from South Africa and this suggest that the tick is widely distributed worldwide and that tick vector from buffalo in Mozambique is the same as the one found in South Africa.

From the analysis of the probe region on the 18S rRNA gene sequences, *T. parva* and *Theileria* sp. (buffalo) probes were aligned with the *T. parva* and *Theileria* sp. (buffalo) from the Genbank to detect if these sequences would bind to the probe region, interesting finding was that *T. parva* did not bind with the *T. parva* probe but instead *Theileria* sp. (buffalo) was the one that binded with *T. parva* probe and this could be the reason why this species was negative on RLB. Therefore, further studies should consider sequencing more *T. parva* and *Theileria* sp. (buffalo) 18S rRNA genes for design of probes that will detect sequence variants of these tick-borne haemoparasites.

CHAPTER 6

5. CONCLUSION

This study is the first to report on the occurrence and diversity of tick-borne haemoparasites circulating in an isolated buffalo herd in the area of Zambezi delta in Mozambique. The haemoparasites spp. detected were *T. parva, T. mutans, T. velifera, Theileria* sp. (sable), *Theileria* sp. (buffalo)*, A. marginale, A. centiale, Anaplasma* sp. (*Omatjinne*), which are common tick-borne parasite infections in buffalo, especially in southern Africa. Considering the livestock-buffalo contact in this area, of very low probability, it can be suggested that buffalo are natural hosts of the detected parasites. Among the detected pathogens were parasite species pathogenic to cattle; notable is the high occurrence of *T. parva* and *A. marginale.* This suggests that buffalos in the current study are likely to be infected due to the constant presence of the tick vectors that transmits these parasites, which might result in the persistent transmission of this haemoparasite between buffalos in this area. The study also elucidates the phylogenetic relationships of *A. marginale* and *A. centrale* as well as *Theileria* sp. (buffalo) and *T. parva* based on the sequences of molecular markers 16S rRNA and 18S rRNA*,* which revealed sequence variations in parasites occurring in this herd of buffalos.

The data generated in this study can be useful as baseline information for relocation operations, to evaluate the risk of introducing haemoparasites in areas with vulnerable hosts, especially where conditions are suitable for the establishment of the relevant tick vectors. Consequently, this data can contribute to the development and implementation of control strategies to mitigate the spillover of tick-borne diseases from wildlife to other wildlife or even to local livestock populations.

Cattle should also be investigated of tick-borne haemoparasites in this area as buffalo are known to be carriers of many infectious diseases of cattle in South Africa. Further studies can focus on molecular and immunological characterisation of *T. parva* and *A. marginale* strains from these buffalo. This will be useful in the search for disease control candidates that will provide broad protection against infection by these tick-borne parasites.

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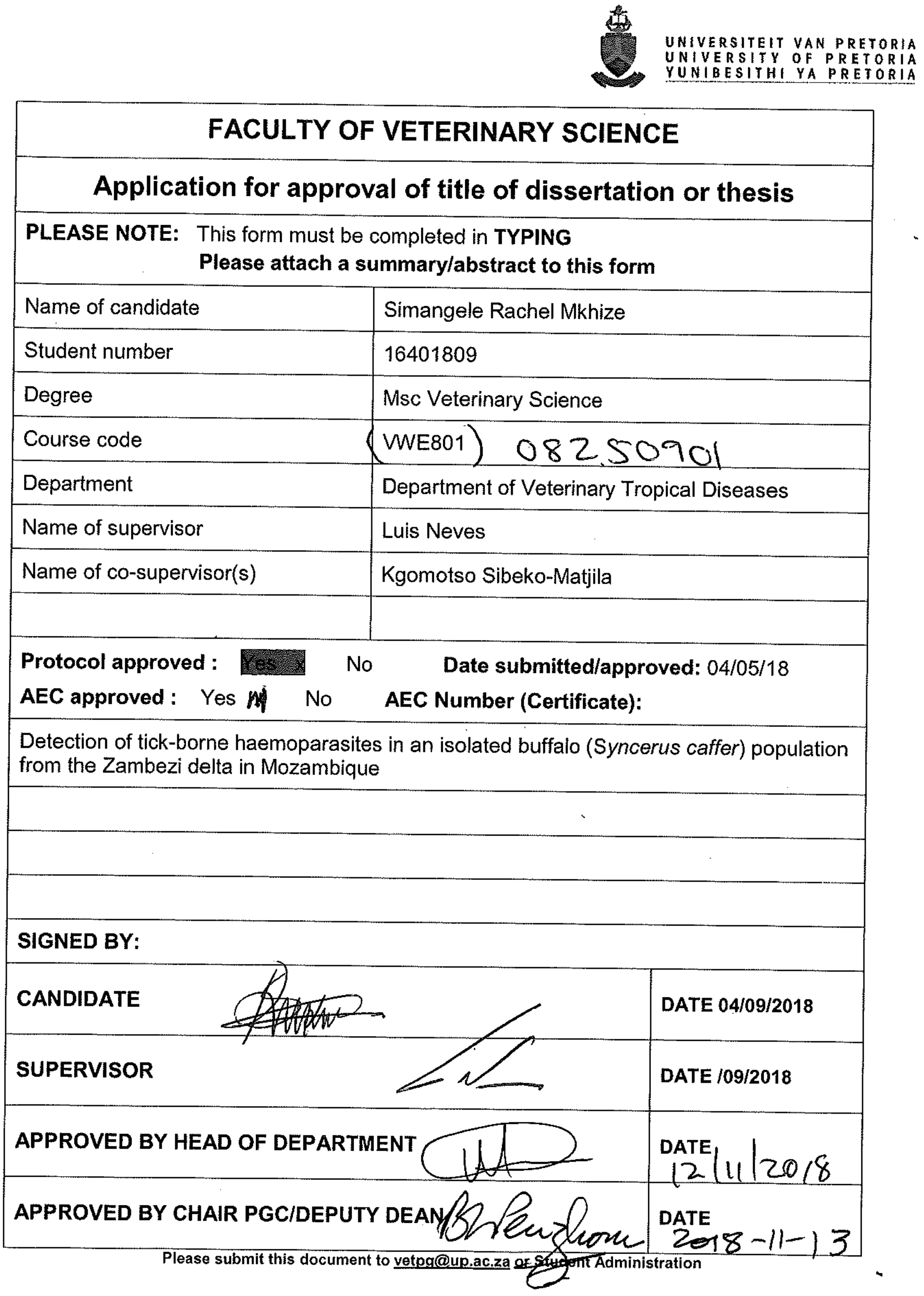
8. APPENDIX



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FACULTY OF VETERINARY SCIENCE

Application for approval of title of dissertation or thesis

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Name of candidate	Simangele Rachel Mkhize
Student number	16401809
Degree	Msc Veterinary Science
Course code	(VWE801) 08250901
Department	Department of Veterinary Tropical Diseases
Name of supervisor	Luis Neves
Name of co-supervisor(s)	Kgomotso Sibeko-Matjila

Protocol approved : Yes x No **Date submitted/approved:** 04/05/18
AEC approved : Yes N No **AEC Number (Certificate):**

Detection of tick-borne haemoparasites in an isolated buffalo (*Syncerus caffer*) population from the Zambezi delta in Mozambique

SIGNED BY:

CANDIDATE	**DATE 04/09/2018**
SUPERVISOR	**DATE /09/2018**
APPROVED BY HEAD OF DEPARTMENT	**DATE** 12/11/2018
APPROVED BY CHAIR PGC/DEPUTY DEAN	**DATE** 2018-11-13

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Research Ethics Committee

PROJECT TITLE	Detection of tick-borne haemoparasites in an isolated buffalo (*Syncerus caffer)* population from the Zambezi delta in Mozambique.
PROJECT NUMBER	RECIOO-18
RESEARCHER/PRINCIPAL INVESTIGATOR	Simangele Mkhize
DISSERTATION/THESIS SUBMITTED FOR	MSc

SUPERVISOR	Luis Neves

APPROVED	Date 12 December 2018
CHAIRMAN: UP Research Ethics Committee	Signature

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forestry & fisheries

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Reference: 12/11/1/1/9

Professor Luis Neves
Faculty of Veterinary Science
Department of Tropical Diseases

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

Dear Professor Luis Neves

Your fax / memo / letter/ Email dated 14 April 2016, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

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

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. A veterinary import permit must be obtained prior to the importation of the DNA extracted from blood collected from Buffalo in Mozambique.
4. DNA extracted from Buffalo blood must be flown into OR Tambo airport, South Africa where the state veterinarian must issue a Red Cross Permit for DNA to be moved to ARC-OVI Transboundary Animal Diseases (TAD).
5. DNA extracted from Buffalo blood must be transported from port of entry to TAD facility under Red Cross Permit.
6. The extracted DNA samples from buffalo blood will be heat treated in accordance with the specifcations of TAD to render the DNA non-infectious.

7. DNA extracted from Buffalo blood must be transported and packaged in compliance with the Regulations of the National Road Traffic Act, 1996 (Act No 93 of 1996) and/or IATA requirements

Title of research/study: Identification and characterisation of tick-bourne heamoparasites in an isolated buffalo population in the south wetlands of Zambezi delta
Researcher (s): Professor Luis Neves

Institution: Department of Tropical Diseases

Your Ref./ Project Number:
Our ref Number: 12/11/1/1/9

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date:
2016 -06- 03

agriculture,
forestry & fisheries

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Reference: 12/11/1/1/9

Professor Luis Neves
Faculty of Veterinary Science
Department of Tropical Diseases

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "IDENTIFICATION AND CHARACTERISATION OF TICK-BOURNE HEAMOPARASITES IN AN ISOLATED BUFFALO POPULATION IN THE SOUTH WETLANDS OF ZAMBEZI DELTA"

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

i) DNA extracted from blood collected from Buffalo must be stored at the access controlled BSL 2 facility at the Faculty of Veterinary Sciences, Department of Tropical diseases;

ii) Stored DNA samples may not be outsourced without prior written approval from DAFF

iii) Stored DNA samples may not be used for further research without prior written approval from DAFF.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2016 -06- 03