

OCCURRENCE OF TICK-BORNE HAEMOPARASITES IN CATTLE IN THE MUNGWU DISTRICT, NORTHERN PROVINCE, ZAMBIA

by

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DEDICATION

To my late mother Mary Namfukwe Nakanyika for your love, encouragement and support during my childhood education.

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Occurrence of tick-borne haemoparasites in cattle in the Mungwi District, Northern Province, Zambia

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SUMMARY

The most important tick-borne diseases (TBDs) occurring in Zambia that affect domestic animals, particularly cattle and small ruminants, are theileriosis (East Coast fever and Corridor disease), anaplasmosis (gall sickness), babesiosis (red water), and heartwater (cowdriosis). Of these, theileriosis is the most important, causing significantly more deaths than the other tick-borne diseases combined. Despite their importance, little is known about the occurrence and prevalence of haemoparasites in cattle in the communal areas of Zambia. Clinical signs and post mortem lesions are pathognomonic of mixed tick-borne infections especially babesiosis, anaplasmosis and East Coast fever (ECF). The main objective of this study was, therefore, to screen selected communal herds of cattle for tick-borne haemoparasites and identify the tick vectors associated with the high cattle mortalities due to suspected TBDs in the local breeds of cattle grazing along the banks of the Chambeshi River in Mungwi, Zambia. East Coast fever is endemic to the district of Mungwi, Northern Province, Zambia and vector control using acaricides has proved to be very costly for the small scale farmers. Also, Mungwi experiences increased cattle mortalities between December to March and May to July. All age groups of cattle are affected.

A total of 299 cattle blood samples were collected from July to September 2010 from Kapamba (n=50), Chifulo (n=102), Chisanga (n=38), Kowa (n=95) and Mungwi central (n=14) in the Mungwi District, Northern Province, Zambia. Ticks were also collected from the sampled cattle from April to July 2011. DNA was extracted and the parasite hypervariable region of the small subunit rRNA gene was amplified and subjected to the reverse line blot (RLB) hybridization assay. The results of the RLB assay revealed the presence of tick-borne haemoparasites in 259 samples occurring either as single or mixed infections. The most prevalent species present were the benign *Theileria mutans* (54.5%)

and *T. velifera* (51.5%). *Anaplasma marginale* (25.7%), *Babesia bovis* (7.7%) and *B. bigemina* (3.3%) were also detected in the samples. Nine percent of the samples tested negative for the presence of haemoparasites. In a number of samples (4%) the PCR products failed to hybridize with any species-specific probes but hybridized only with the genus-specific probes which could suggest the presence of a novel species or variant of a species.

Of the four *Theileria* species known to occur in Zambia (*T. parva*, *T. mutans*, *T. velifera* and *T. taurotragi*), *T. parva* is the most economically important, causing Corridor disease in the Southern, Central, Lusaka and the Copper-belt provinces, while causing ECF in the Northern and Eastern provinces of Zambia. In our study, only one sample (from Kapamba) tested positive for the presence of *T. parva*. This was an unexpected finding; also because the tick vector, *Rhipicephalus appendiculatus*, was identified on animals from Kowa (14%), Chisanga (8.5%), Chifulo (6%) and Kapamba (1.4%). We can only speculate that the RLB hybridization assay may not have been able to detect the parasite in the animals sampled due to a too low parasitaemia. The samples should also be subjected to the *T. parva* specific real-time PCR assay to determine a more accurate *T. parva* prevalence in cattle in the Mungwi district, Northern Province.

In Zambia, *Babesia bovis* and *B. bigemina* are recognized as being of economic importance in cattle. In our study, *B. bovis* was present in 7.7% of the sampled animals and *B. bigemina* in 3.3% of the animals. We detected *B. bovis* in all of the five sampled areas with the highest detection in Mungwi central (14.3%) and Kowa (10.5%). As expected, the tick vector *Rhipicephalus (Boophilus) microplus* was identified from animals from all of these areas. *Babesia bigemina* was only reported from Kowa (10.5%). The most abundant ticks identified from the sampled animals from Kowa were *Rhipicephalus (Boophilus) decoloratus* (36.3%) and *Rhipicephalus (Boophilus) microplus* (8.8%). These tick vectors have been implicated in the transmission of *B. bigemina*. Our findings are in concordance with results obtained by other authors who have speculated that an increase in the detection of *B. bovis* may indicate that *B. bovis* is becoming endemic in this part of the country. This could be due to uncontrolled movement of cattle that frequently occurs within Zambia.

Heartwater (cowdriosis) is caused by *Ehrlichia ruminantium*, a rickettsial disease that affects domestic and wild ruminants. In Zambia, heartwater is mainly a disease of cattle, although outbreaks in sheep and goats have been reported and recorded. In our study, only one sample (from Kapamba) tested positive for the presence of *E. ruminantium* even though *Amblyomma variegatum* ticks were identified from 52.9% of the sampled animals from all study areas. The cattle sampled in our study

are not regularly dipped and no game has been spotted in cattle grazing areas. It is possible that these cattle may have attained a state of endemic stability to heartwater. It is also possible that the RLB hybridization assay may not have been sensitive enough to detect *E. ruminantium* infections if the parasitaemia was very low. Samples should also be subjected to the *E. ruminantium*-specific pCS20 real-time PCR assay to determine more accurately the *E. ruminantium* prevalence in cattle in the Mungwi district, Northern Province.

Anaplasma marginale (the causative agent of bovine anaplasmosis) has previously been shown to be present in all the provinces of Zambia and is the only *Anaplasma* species of importance to cattle in Zambia. In our study, 25.7% of the sampled cattle tested positive for *A. marginale*; it was detected in all areas except Chisanga. *Amblyomma variegatum* was identified from 52.9% of the sampled cattle, and *Rhipicephalus (Boophilus) microplus* from 12.1% of the cattle. *Rhipicephalus (Boophilus) microplus* has been incriminated as being a vector of *A. marginale*. Furthermore, three samples (from Kowa) tested positive for the presence of *Anaplasma centrale*. To our knowledge, no vaccination regime using *A. centrale* is being conducted in the Mungwi district of Zambia. The presence of *A. centrale* is, therefore, an interesting finding.

The results of our study suggest that the cause of cattle mortalities in Mungwi during the winter outbreaks is mainly due to *A. marginale*, *B. bovis* and *B. bigemina* infections. This was confirmed by the results of the RLB hybridization assay, clinical manifestation of the disease in the affected cattle (own observation) and the tick species identified on the animals. It appears that in Mungwi, babesiosis due to *B. bovis* mostly infects cattle above one year of age. Calves appear to be less affected by *B. bovis* infection.

There is need for further epidemiological surveys in Mungwi district, Northern Province, Zambia to get a better understanding of the epidemiology of these tick-borne haemoparasites affecting cattle. We conclude that integrated control policies should be developed to take account of multi-species pathogen communities that are commonly associated with clinical and sub-clinical TBD infections in Zambia.

TABLE OF CONTENTS

<i>Dedication</i>	2
<i>Acknowledgements</i>	3
<i>Summary</i>	4
<i>List of figures</i>	8
<i>List of tables</i>	9
<i>Abbreviations</i>	10
INTRODUCTION.....	11
LITERATURE REVIEW.....	14
1. Tick vectors	15
2. Life cycles.....	17
3. Disease syndromes.....	23
4. Control of tick-borne diseases caused by <i>Theileria</i> , <i>Babesia</i> , <i>Anaplasma</i> and <i>Ehrlichia</i> species...	25
5. Laboratory diagnosis.....	28
MATERIALS AND METHODS.....	31
1. Sample collection.....	31
2. Laboratory techniques	32
3. Tick counts and identification.....	37
RESULTS.....	38
1. RLB results.....	38
2. Tick identifications	45
DISCUSSION	47
CONCLUSIONS	57
REFERENCES	59

LIST OF FIGURES

LITERATURE REVIEW

Fig. 1: The life cycle of <i>Theileria parva</i>	18
Fig. 2: The life cycle of <i>Babesia bigemina</i> in cattle and the ixodid tick vector as currently understood.....	20
Fig. 3: Schematic of the development cycle of <i>A. marginale</i> in cattle and ticks	21
Fig. 4: Life cycle of <i>E. ruminantium</i> in cattle and the tick	23

MATERIALS AND METHODS

Fig. 1: Map showing the five localities of Mungwi, in Zambia, from which the blood samples were collected from cattle.....	31
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RESULTS

Fig. 1: RLB results of some of the bovine specimens screened. The species-specific oligonucleotide probes were applied in horizontal lanes and the PCR products in vertical lanes. The negative control was applied in lane 42.....	38
Fig. 2: Schematic representation of the occurrence of tick-borne haemoparasites in bovine blood samples from five localities in Mungwi, Zambia as determined by the RLB hybridization assay.....	41
Fig. 3: Mixed <i>Theileria</i> species infections in samples from the five areas of Mungwi as screened using the RLB hybridization assay.....	42
Fig. 4: Mixed <i>Babesia</i> species infections in samples from five areas of Mungwi as determined using the RLB hybridization assay.....	43
Fig. 5: <i>Anaplasma</i> species infections in samples from five areas of Mungwi as screened using the RLB hybridization assay.....	44
Fig. 6: Mixed <i>Ehrlichia</i> species infections in samples from five areas of Mungwi as screened using the RLB hybridization assay.....	45
Fig. 7: Tick species identified from the cattle sampled from five areas of Mungwi district, Zambia.....	46

LIST OF TABLES

MATERIALS AND METHODS

Table 1: Thermocycler program for <i>Theileria/Babesia</i> and <i>Ehrlichia/Anaplasma</i> touchdown PCR	33
Table 2: Genus- and species-specific probes and their sequences present on the in-house prepared membrane used for detecting pathogen DNA.....	34

RESULTS

Table 1: The occurrence of tick-borne haemoparasites in bovine blood samples from five localities in the Mungwi District, Zambia as determined by the RLB hybridization assay.....	40
Table 2: Occurrence of tick species on 299 cattle sampled from five areas of Mungwi district, Zambia ...	46

ABBREVIATIONS

AA	Adult tick
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence substrate
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
Ehr-F	<i>Ehrlichia / Anaplasma</i> forward primer
Ehr-R	<i>Ehrlichia / Anaplasma</i> reverse primer
ml	Milliliter
mm	Millimeter
NN	Nymph
PCR	Polymerase chain reaction
POD	Peroxidase-labeled
RLB	Reverse line blot
RLBF ₂	<i>Theileria / Babesia</i> forward primer
RLBR ₂	<i>Theileria / Babesia</i> reverse primer
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
s	Second(s)
SDS	Sodium dodecyl sulfate
sp.	Species
SSPE	Sodium Chloride/Sodium Phosphate/EDTA
TBDs	Tick-borne diseases
UDG	Uracil DNA glycosylase
%	Percent
°C	Degrees Celsius
μl	Microliter

INTRODUCTION

Tick-borne diseases (TBDs) are one of the most important constraints to livestock production in developing countries. The most important TBDs of cattle in sub-Saharan Africa are theileriosis caused by *Theileria parva*, babesiosis caused by *Babesia bovis* and *B. bigemina*, anaplasmosis caused by *Anaplasma marginale* and heartwater caused by *Ehrlichia ruminantium* (Makala et al., 2003). In Zambia, the most important TBDs that affect domestic animals, particularly cattle and small ruminants, are theileriosis (East Coast fever and Corridor disease), anaplasmosis (gall sickness); babesiosis (red water), and heartwater (cowdriosis). Of these, theileriosis is the most important; causing significantly more deaths than the other TBDs combined (Makala et al., 2003).

In Zambia, theileriosis poses a major constraint to the livestock industry with losses of about 10 000 cattle per annum (Nambota et al., 1994). While East Coast fever (ECF) occurs in the Eastern and Northern provinces of Zambia, Corridor disease is widespread in the Southern, Central and Lusaka provinces and has been reported in the Copper-belt province (Makala et al., 2003). The highest number of ECF cases occurs between January and March in the Northern and Eastern provinces, and the highest number of Corridor disease cases are recorded during the month of January in the Southern province (Samui, 1987). The disease is spreading at a very fast rate, beyond its original borders. The epidemiology is complicated by, among other factors, the wide distribution of the tick vector, *Rhipicephalus appendiculatus*, which is found all over the country (Nambota et al., 1994).

Bovine babesiosis is an economically important tick-borne disease of cattle in tropical and subtropical regions of the world (McCosker, 1981). The disease is primarily caused by two bovine intraerythrocytic protozoan parasites, *Babesia bovis* (Asiatic redwater) and *Babesia bigemina* (African redwater) which are mainly transmitted by the one-host ticks *Rhipicephalus (Boophilus) decoloratus* and *Rhipicephalus (Boophilus) microplus* (de Vos et al., 2004). In Zambia, *B. bovis* and *B. bigemina* are currently present in all the Zambian provinces (McCosker, 1981) and are recognized as being of economic importance in cattle and small ruminants (Luguru, 1985; Pegram et al., 1989; Pegram and Banda, 1990).

Heartwater, also known as cowdriosis, is caused by *Ehrlichia ruminantium* (previously known as *Cowdria ruminantium*), and is a rickettsial disease that affects domestic and wild ruminants in Zambia (Jongejan et al., 1988; Makala et al., 2003), the rest of sub-Saharan Africa and the Caribbean. In the agricultural areas of Zambia, *Amblyomma hebraeum* and especially *A. variegatum* are the main vectors of heartwater (Makala et al., 2003). In Zambia, heartwater is mainly a disease of cattle, although outbreaks in sheep and goats have been reported and recorded. Records of the Central Veterinary Research Institute (CVRI) for the period 1986–1997 revealed that the disease occurred throughout Zambia (Mangani, 1997; Makala et al., 2003). Heartwater is believed to be responsible for numerous deaths occurring throughout the year, but especially during the rainy season from March to September. The disease is mainly seen in areas where regularly dipped animals are in close proximity to indigenous kept cattle with no acaricidal treatment and also where game is frequently seen in cattle grazing areas. Heartwater also occurs in commercial farms when they have relaxed their normal tick control practices (Makala et al., 2003).

Bovine anaplasmosis (formerly known as gall sickness), caused by *Anaplasma marginale*, is an infectious but noncontagious disease that occurs in tropical and subtropical regions worldwide, including South and Central America, the United States, southern Europe, Africa, Asia and Australia (Aubry and Geale, 2011). *Anaplasma centrale*, a less pathogenic but closely related organism, is used as a live vaccine for cattle in Israel, South Africa, South America and Australia (de la Fuente et al., 2005). In general, tick vectors of *A. marginale* include *Rhipicephalus (Boophilus) spp.*, selected *Dermacentor spp.*, *Ixodes ricinus* and *Rhipicephalus spp.*, while *Amblyomma spp.* do not appear to transmit *A. marginale* (Kocan et al., 2004). *Anaplasma marginale* is present in all the provinces of Zambia (Jongejan et al., 1988); it is regarded as the only *Anaplasma* species of importance to cattle in Zambia (McCosker, 1981) and is transmitted by *A. variegatum*. There is, however, an overlapping distribution of *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus evertsi evertsi*, which also act as vectors (Friedhoff, 1997).

Despite their importance, little is known about the occurrence and prevalence of haemoparasites in cattle in the communal areas of Zambia (Marufu et al., 2010). Clinical signs and post mortem lesions are pathognomonic of mixed tick-borne infections especially babesiosis, anaplasmosis and ECF (own unpublished observations, 2010). The main objective of this study was, therefore, to screen selected communal herds of cattle for tick-borne haemoparasites and identify the tick vectors associated with the high cattle mortalities due to suspected TBDs in the local breeds of cattle grazing along the banks of the Chambeshi River in the Mungwi District, Northern Province, Zambia. The

district of Mungwi in Zambia is endemic to ECF, and vector control using acaricides has proved to be very costly for the farmers. Also, Mungwi experiences increased cattle mortalities between December to March and May to July. All age groups of cattle are affected.

Objective of the study:

The occurrence of tick-borne haemoprotozoan parasites (*Theileria*, *Babesia*, *Anaplasma*, and *Ehrlichia*) in the local breeds of cattle grazing along the banks of the Chambeshi River in the Mungwi District, Northern Province, Zambia was determined with the aim of monitoring and confirming that increased cattle mortalities experienced in the rainy season and early winter are due to tick-borne diseases. The specific objectives were:

1. To screen blood collected (on filter paper) from cattle from five areas in the Mungwi District (Kapamba, Chifulo, Chisanga, Kowa and Mungwi central) for the presence of *Theileria*, *Babesia*, *Ehrlichia* and *Anaplasma* species using the reverse line blot (RLB) hybridization assay.
 2. To collect ticks from the same animals in order to (i) identify the most prevalent tick species present, and (ii) to compare the tick prevalence with the occurrence of TBDs of the local breeds of cattle.
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LITERATURE REVIEW

Haemoparasites are parasites that live within their (animal) host's bloodstream. This dissertation will focus on four important tick-borne haemoparasites of cattle. Theileriosis, babesiosis, heartwater and anaplasmosis manifest haematologically as intracellular, haemoparasites (Ko et al., 2008). The piroplasms comprising the two genera, *Theileria* and *Babesia*, are protozoa that are highly pathogenic to cattle (Mehlhorn & Schein, 1984). Piroplasms are pear-shaped, rod, round or oval parasites with an intraerythrocytic stage (Mehlhorn & Schein, 1984). The causative agents of anaplasmosis and heartwater are rickettsial haemoparasites. The parasites are situated in the blood cells and are transmitted by ixodid tick species.

Among *Theileria* species, *Theileria parva* causes severe infections in ruminants, resulting in East Coast fever (ECF), Corridor disease and Zimbabwe theileriosis (also known as January disease). *Theileria annulata* is the cause of tropical theileriosis while *T. mutans* has been implicated as the cause of benign theileriosis. *Theileria parva* and *T. annulata* are the most pathogenic of the six species of this protozoan genus that infect cattle (Norval et al., 1992). East Coast fever can be relatively mild, causing low mortality, restricted to calves only, but full-scale epidemics affecting all age groups do occur (Berkvens, 1991). The disease is characterized by pyrexia, enlargement of superficial lymph nodes, severe pulmonary edema, wasting and terminates in death (Lawrence & Williamson, 2005). Corridor disease exhibits clinical signs of advanced ECF like emaciation, diarrhea and regression of lymph nodes. However, the course of clinical features in Corridor disease is usually shorter with death occurring within three to four days after onset of the first signs (Lawrence & Williamson, 2005). *Theileria annulata* causes tropical theileriosis but has not been reported in southern Africa (OIE, 2008).

Babesia parasites are the cause of bovine babesiosis or redwater. *Babesia* species known to infect cattle in southern Africa include *Babesia bovis* and *B. bigemina*. *Babesia bovis* is the more pathogenic form causing more severe disease. While *B. bovis* is the more virulent of the two, *B. bigemina* is important because of its widespread distribution in southern Africa (de Vos et al., 2004). Similar clinical signs develop during infection with both organisms. However, the course of the disease differs markedly. Infections are characterised by muscle tremor, reluctance to move,

haemoglobinuria and occasionally signs of cerebral derangement such as circling, head pressing, mania and convulsions. These signs are seen very early in the course of *B. bovis* infections and only develop in *B. bigemina* infections in the latter stages when parasitaemia is well advanced. Anaemia and jaundice develop steadily with *B. bovis*, but much more with *B. bigemina* (OIE, 2002).

Anaplasma marginale causes anaplasmosis in cattle. The disease is characterized by anaemia and icterus without haemoglobinemia and haemoglobinuria. These clinical signs result from the massive phagocytosis of infected erythrocytes by the bovine reticuloendothelial system (Potgieter & Stoltsz, 2004). Other clinical signs of anaplasmosis include fever, weight loss, abortion lethargy and death (Kocan et al., 2003). Cattle of all ages can be infected by *A. marginale*; however, the severity of the disease is age-dependent. Calves are less susceptible to clinical disease and disease is rare in calves under six months of age (Kocan et al., 2003).

In cattle, *Ehrlichia ruminantium* causes heartwater. Clinical disease may be classified as peracute, acute, subacute, mild or inapparent. The disease is characterized by terminal convulsions shortly before death. Mortality may reach 100% with the peracute disease. The acute disease is more common and is characterized by anorexia, rapid breathing, lethargy and empty chewing movements, twitching of the eyelids, circling, aggression and blindness. The animals often become recumbent shortly before death. The mild form of the disease is seen in indigenous breeds of cattle that have natural resistance. These animals will appear subclinical or exhibit mild symptoms (Radostits & Clive, 2007).

1. TICK VECTORS

By definition, ticks are parasites that are costly to their hosts as they divert resources for their growth, reproduction, and survival with no rewards for the hosts (Sorci & Garnier, 2008). Development in and transmission of haemoparasites by tick vectors are phenomena closely synchronized with the tick feeding cycle (Salih et al., 2007). In all known life cycles, initial infection of tick tissues occurs in midgut epithelial cells and transmission is effected when ticks feed after parasites have developed and multiplied in salivary glands (Salih et al., 2007).

Many factors affect the development and transmission of haemoparasites by ticks, including age of ticks, artificial temperature, climate and/or season, tick stage or sex, haemoparasite variation, concurrent infection of ticks with other pathogens, host cell susceptibility, transovarial transmission and effect of haemoparasites on tick biology. Tick-borne haemoparasites are dependent on ticks for biological transmission (Salih et al., 2007).

Infection of vertebrate hosts starts with the attachment and feeding of infected ticks. The ticks are unable to transmit parasites immediately after attachment of their mouthparts (Mehlhorn & Schein, 1984). This is mainly because their salivary glands become reduced after moulting. After attachment to a new host, salivary glands grow in a few days and only then do the piroplasms, which undergo multiplication inside the salivary gland cells, have a chance to develop (Mehlhorn & Schein, 1984). Thus infectious sporozoites of piroplasms are often not transmitted earlier than 3-5 days after first attachment of the ticks to their hosts (Mehlhorn & Schein, 1984).

Transmission is also said to be influenced by the density of competent hosts. Too low densities of competent hosts (i.e., hosts where transmission can occur) do not sustain the infection cycle, while too high densities of incompetent hosts may dilute the competent hosts so as to make infection persistence impossible (Rosà & Pugliese, 2007).

Important ticks reported in Zambia and the parasites they transmit:

Important ticks of cattle in Zambia include *Rhipicephalus appendiculatus*, *R. (Boophilus) microplus*, *R. (Boophilus) decoloratus* and *Amblyomma variegatum* (Pegram et al., 1986). *Rhipicephalus appendiculatus*, *R. (Boophilus) decoloratus* and *A. variegatum* are found throughout the country (Pegram et al., 1986; Berkvens et al., 1998). *Rhipicephalus (Boophilus) microplus* is only found in the Eastern and Northern Provinces (Berkvens et al., 1998), however, a study by Jongejan et al. (1988) suggested that this tick is spreading westwards into Central Zambia.

Theileria parva is transmitted by *Rhipicephalus appendiculatus*; it infests ears of cattle in the humid areas of southern and eastern Africa. Although *Rhipicephalus zambesiensis* and *R. duttoni* are also important vectors of *T. parva*, they have not been reported in Zambia. In eastern Zambia, peaks of ECF incidence occur in the rainy season (December to February) and the dry months (May to July), with nymph-to-adult tick transmission dominating the infection dynamics (Billiouw et al., 1999). The highest prevalence of *T. parva* was reported in the rainy season (October) (Salih et al., 2007).

Rhipicephalus (Boophilus) microplus is known to transmit *B. bovis*. *Babesia bigemina* can be transmitted by *R. (Boophilus) microplus*, *R. (Boophilus) decoloratus* and *R. evertsi evertsi*. Transmission by *R. evertsi evertsi* is transovarial and only nymphal stages infect the cattle (Norval & Horak, 2004).

Vectors of anaplasmosis include *R. evertsi evertsi*, *R. simus* and *Hyalomma* spp. In Zambia, *R. evertsi evertsi* and *R. simus* have been reported, while among the *Hyalomma* spp., only *H. marginatum rupifex* and *H. truncatum* have been reported (Walker et al., 2003). Anaplasmosis can be transmitted mechanically by various other biting arthropods e.g. horse flies and mosquitoes. However, biological transmission of *Anaplasma* by ticks is the most efficient means of spread for *A. marginale* because of replication and persistence capabilities within ticks (Kocan et al., 1992). Mechanical transmission by arthropods can occur in any location and is considered to be the major route of dissemination of *A. marginale* in some areas of Africa where tick vectors do not occur (Kocan et al., 2009) or where strains of *A. marginale* are not infective for, or transmissible by, ticks (Kocan, et al., 2004).

Heartwater is transmitted by ticks of the genus *Amblyomma* (Allsopp et al., 2004), with *A. variegatum* (also known as the tropical bont tick) and *A. hebraeum* (the bont tick), being the main transmitters in southern Africa. These are three-host ticks that undergo transstadial transmission (Radostits & Clive, 2007). However, *A. hebraeum* ticks have not been reported in Zambia.

2. LIFE CYCLES

The life cycles of these parasites are complex and involve prolonged exposure to host and vector defense mechanisms.

2.1 *Theileria parva* life cycle:

The tick acquires *T. parva* as it feeds on infected cattle at a stage of clinical disease or recovery when piroplasms are circulating in the blood (Fig. 1). Transmission between larva and adult does not occur if the intervening nymphal stage feeds on a non-susceptible host. Succeeding stages transmit the infection as they feed when the sporozoites in their salivary glands have matured (Lawrence et al., 2004). When ambient temperatures are moderate to low, maturation of sporozoites in adult ticks takes three to four days after feeding commences. However, maturation in nymphs is more rapid. Maturation of sporozoites may occur in unfed ticks at ambient temperatures of 37°C and above and infection can be transmitted as soon as the tick begins to feed (Lawrence et al., 2004).

Upon ingestion by *R. appendiculatus* ticks, the parasite undergoes differentiation first in the tick gut, leading to the formation of forms called kinetes. The kinetes migrate to the tick's salivary glands, where they differentiate into infective sporozoites. When the tick feeds on a new host, the sporozoites are injected into the blood along with tick saliva (Lawrence et al., 1994a). The sporozoites penetrate lymphocytes and develop into schizonts. A schizont is a mass of protoplasm contained within a membrane which is situated intracytoplasmically in the infected lymphoblast. Schizonts are found intracellularly in lymphoblasts in lymphoid tissues throughout the body and in the blood (Lawrence et al., 2004). The development of sporozoite to schizonts in the lymphocytes causes a lymphoproliferative disorder and lymphadenomegaly. Some schizonts undergo asexual division and develop into merozoites. Merozoites invade the host's erythrocytes and develop into piroplasms which are infective to ticks.

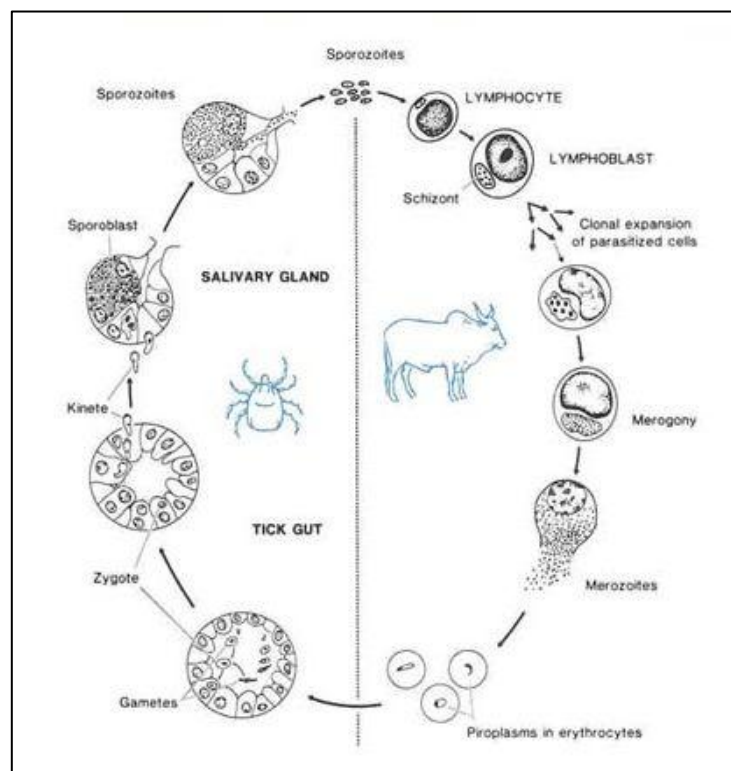


Fig. 1: The life cycle of *Theileria parva*. (Taken from: <http://www.ilri.org/InfoServ/Webpub/fulldocs/ilrad89/Theile.htm>)

2.2 Babesia life cycle:

All species of *Babesia* are naturally transmitted by the bite of infected ticks (almost all ixodids) and the main life cycle difference amounts to the presence of transovarial transmission in some species

(*Babesia* spp. sensu stricto) and not in others (*B. microti*-like) (Hunfeld et al., 2008). During the tick bite, sporozoites are injected into the host and directly infect red blood cells using an apical complex (Fig. 2). This distinguishes *Babesia* spp. from *Theileria* spp., where sporozoites do not readily infect red blood cells but initially penetrate a lymphocyte or macrophage in which development into schizonts takes place (Uilenberg, 2006). In the vertebrate host, *Babesia* sporozoites develop into piroplasms inside the infected erythrocyte resulting in two or sometimes four daughter cells that leave the host cell to infect other erythrocytes until the host dies or the immunity of the host clears the parasites. The spleen with its lympho-reticular filter function is essential in resisting primary infections of *Babesia* spp. by specifically removing infected cells from circulation, probably through a combination of spleen microcirculation and stimulated phagocytic cell activity (de Vos et al., 1987; Gray & Weiss, 2008).

In the adult *Rhipicephalus* (*Boophilus*) ticks, development of *Babesia* spp. occurs in the basophilic epithelial cells of the tick gut wall following ingestion of infected erythrocytes by the engorging female to produce ookinetes (syngamy). The ookinetes penetrate the tick gut epithelial cells and undergo merogony to produce merozoites. Merozoites undergo a secondary merogony and invade the developing ova. Oviposition will result in infected eggs (transovarial transmission) and infected larval stage of the tick. The merozoites will replicate in the gut of the infected larva by schizogony to produce sporokinetes. Sporokinetes invade the salivary gland of the tick where they undergo sporogony to sporozoites. These are infective to cattle (de Vos et al., 2004).

In 2004, de Vos et al. reported evidence of vertical transmission of *Babesia* in *Rhipicephalus* (*Boophilus*) ticks, with infection passing from one tick generation to the next in the absence of re-infection from the vertebrate host. Inside the tick, *Babesia* zygotes multiply as 'vermicules,' which invade many organs of the tick including the ovaries; *Babesia* species are readily passed to the next generation of ticks in the egg. These parasites can sometimes be passed transovarially through several generations, although this varies with the species of *Babesia* and the species of tick (OIE, 2008). Transovarial transmission occurs with further development taking place in the larval stage. In *B. bovis*, the final cycle of sporogony takes place in the salivary glands of feeding larvae leading to formation of small sporozoites. For *B. bigemina*, some development occurs in the feeding larva but schizogony in the salivary glands only occurs in the nymphal and adult ticks (de Vos et al., 2004).

Development of *Babesia* spp. in cattle occurs only in the erythrocytes. Sporozoites penetrate erythrocyte cell membranes using an apical complex. When inside the erythrocyte, it transforms into a trophozoite from which two merozoites develop by a process of merogony (de Vos et al., 2004).

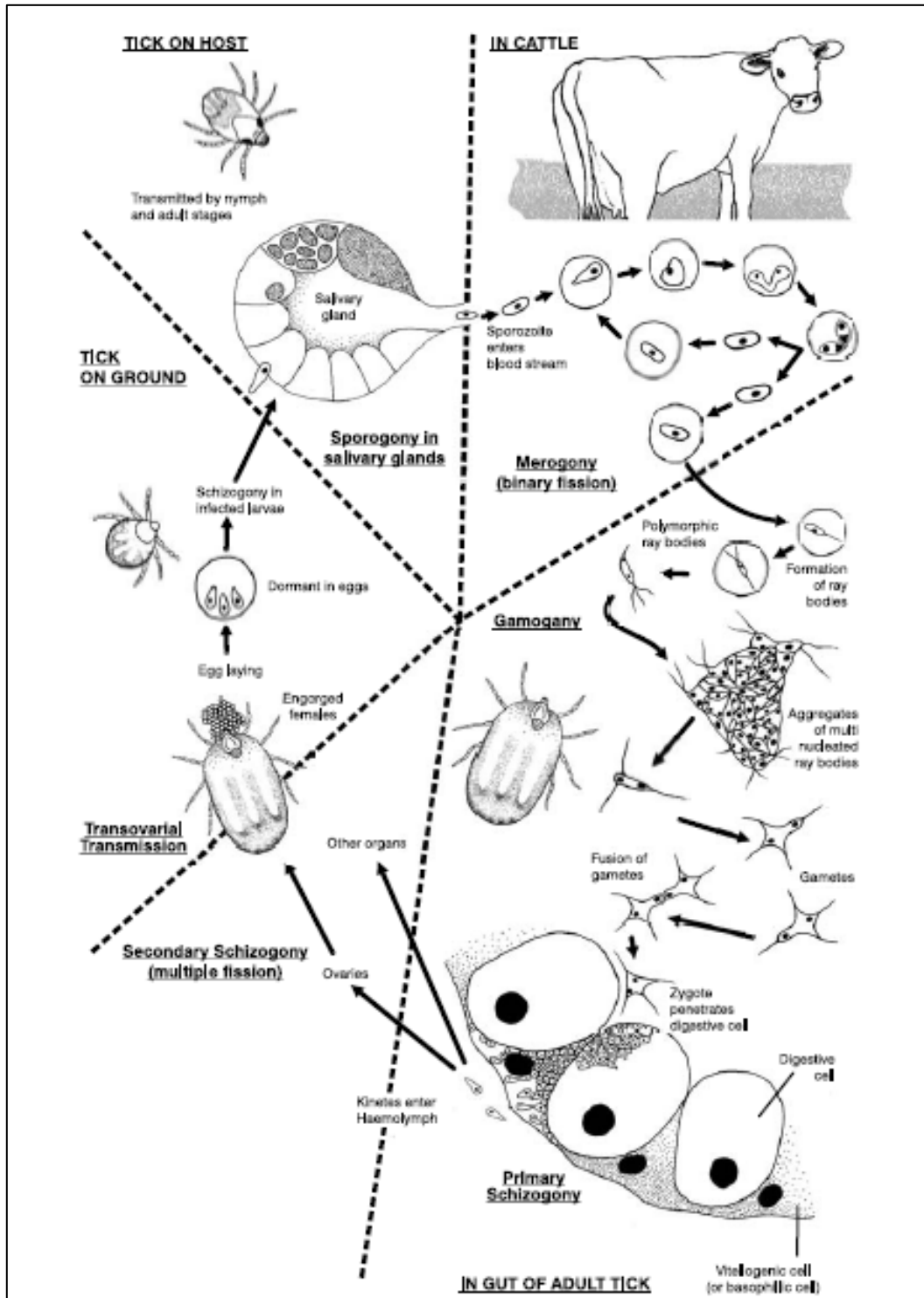


Fig. 2: The life cycle of *Babesia bigemina* in cattle and the ixodid tick vector as currently understood (taken from: Bock et al., 2004; adapted from Mehlhorn & Shein, 1984; Mackenstedt et al., 1995; Gough et al., 1998).

2.3 Life cycle of *Anaplasma species*:

Anaplasma species can be transmitted biologically, mechanically and via the transplacental route. *Anaplasma marginale* invades erythrocytes and undergoes cycles of replication, removal of infected erythrocytes by the reticuloendothelial system and subsequent reinvasion of erythrocytes within the ruminant. During the initial infection, there is a geometrical increase phase where the number of infected erythrocytes doubles about every 24 hours (Richey & Palmer, 1990).

The developmental cycle of *A. marginale* in ticks is complex (Fig. 3) (Kocan et al., 2003). Infected erythrocytes taken into ticks with the blood meal provide the source of *A. marginale* infection for tick gut cells, after which many other tick tissues become infected, including the salivary glands. Rickettsiae are transmitted from the tick salivary glands to vertebrates during feeding. *Anaplasma marginale* develops within membrane-bound vacuoles or colonies at each site of infection in ticks. The first form seen within the colony is the reticulated (vegetative) form, which divides by binary fission to form large colonies that may contain hundreds of organisms. The reticulated form then changes into the dense form, the infective form which can survive outside the host cells. Cattle become infected when the dense form is transmitted during tick feeding via the salivary glands (Kocan et al., 2003).

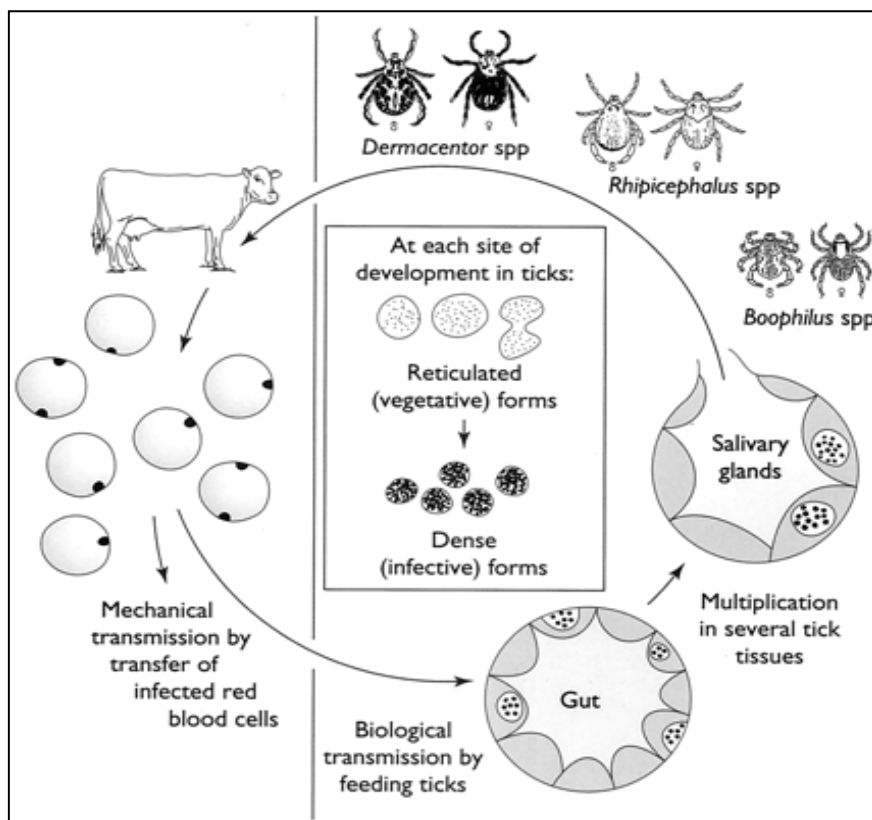


Fig. 3: Schematic of the development cycle of *A. marginale* in cattle and ticks (Taken from: Kocan et al., 2003).

2.4 Life cycle of Ehrlichia species:

Ehrlichia ruminantium is the cause of heartwater. It is transmitted transtadially by three host ticks of the genus *Amblyomma*. It is an obligate intracellular rickettsial organism that parasitizes macrophages (Stephen & Feldman, 2000). In the tick, initial replication occurs in the epithelium of the intestine of the tick after ingestion of an infected blood meal and the salivary glands eventually become parasitized (Fig. 4). Transmission of the parasite to the vertebrate host probably occurs through the saliva of the tick while feeding (Allsopp et al., 2004). In 1987, Bezuidenhout demonstrated *E. ruminantium* in the salivary glands of *Amblyomma* ticks and suggested that transmission occurs via the salivary glands. *Ehrlichia ruminantium* can also be transmitted vertically from cows to their calves (Deem et al., 1996).

The *E. ruminantium* growth cycle in ruminant host cells closely resembles that of the biphasic *Chlamydia* cycle as was shown by studying bovine endothelial cell cultures (Jongejan et al., 1991). It was shown that elementary bodies (EBs), the electron-dense, metabolically inactive form of the organism will infect a new host cell and once inside, will transform to active, non-infective reticulate bodies. This reproductive stage multiplies by binary fission or possibly endosporulation and reverts to the EB stage after which the pathogen is released from the host cell (Prozesky & Du Plessis, 1987; Jongejan et al., 1991; Allsopp et al., 2004; Marcelino et al., 2005). In this form, the EBs are infective stages and can infect new endothelial cells and feeding ticks.

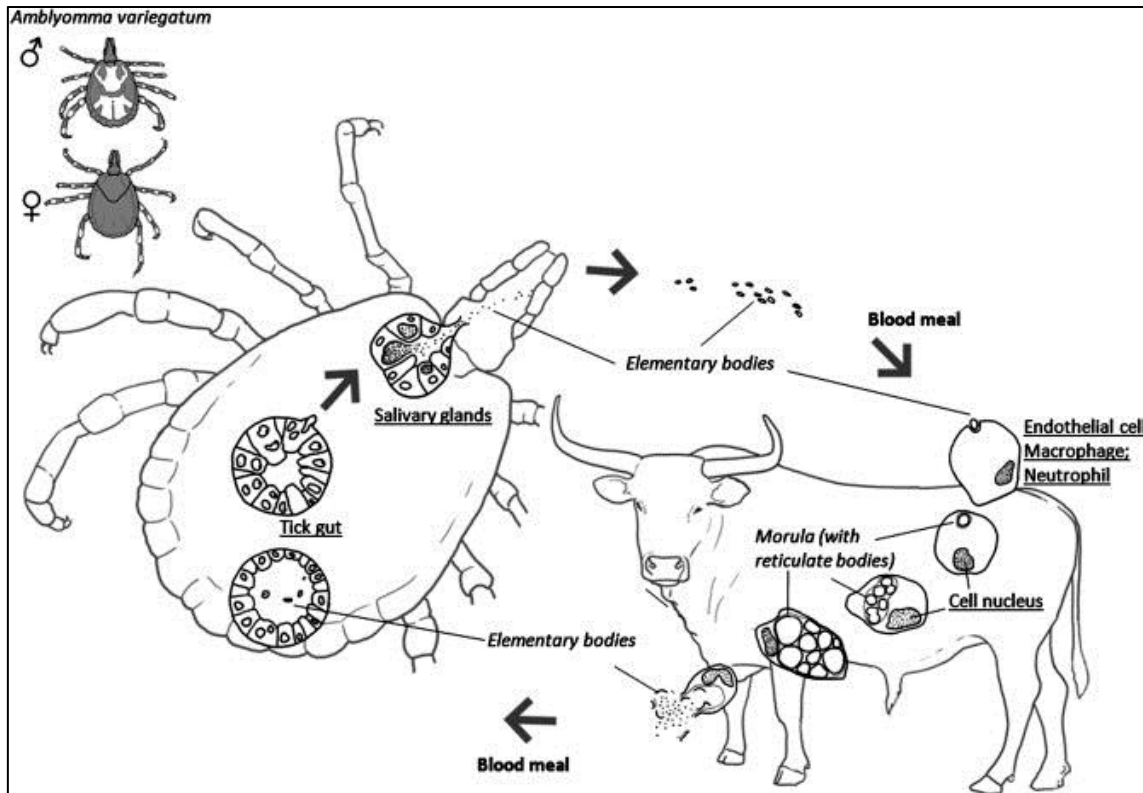


Fig. 4: Life cycle of *E. ruminantium* in cattle and the tick (Taken from Marcelino et al., 2012).

3. DISEASE SYNDROMES

3.1 Theileriosis:

Theileria parva, a tick-transmitted apicomplexan parasite, is the causative agent of ECF, Corridor disease and January disease in cattle (Uilenberg et al., 1982; Perry et al., 1991). East Coast fever, caused by cattle-associated *T. parva* parasites, is characterized by fever, anorexia, pyrexia, enlargement of superficial lymph nodes, severe pulmonary edema, wasting and death (Lawrence et al., 1994a). The animal is depressed and lethargic. There are increased pulse and respiratory rates. Lacrimation accompanies edema of the eyelids and constipation is common. The precrural and prescapular lymph nodes are prominently enlarged. Corneal opacity and petechiae of the mucous membranes of the vulva and beneath the tongue have been reported. In the terminal stages of ECF, dyspnea develops with an increased respiration, a watery cough and a frothy discharge from the nostrils (Lawrence et al., 1994a).

Corridor disease is caused by buffalo-associated *T. parva* parasites and exhibits similar clinical features as ECF except the course of the disease is short and death occurs three to four days after

onset of clinical signs. Severe pulmonary edema precedes death (Lawrence et al., 2004). Corridor disease is characterized by low schizont parasitosis and piroplasm parasitaemia. The schizonts are very scanty and most cattle die before significant piroplasm parasitaemia develops. The erythrocytic piroplasms are too scanty to even infect new ticks (Uilenberg, 1999). Using light microscopy, it is impossible to differentiate *T. parva* schizonts and piroplasms from most other *Theileria* spp. which may occur simultaneously within the sample (Norval et al., 1992).

Outbreaks of January disease or Zimbabwe theileriosis follow a seasonal pattern with peak adult tick vector activity between January and March. Chronic signs such as emaciation and diarrhoea are rarely seen in January disease due to the short disease course before death. Froth may be seen from the nostril prior to death due to pulmonary edema (Lawrence & Williamson, 2005; Norval et al., 1985). Serological evidence and experimental infection suggests that the disease is commonly subclinical or mild (Lawrence et al., 1994c).

3.2 Babesiosis:

Babesiosis in cattle is caused by two *Babesia* spp. namely *Babesia bovis* and *B. bigemina*. Infection by *Babesia* spp. may cause pregnant cattle to abort and bulls to show reduced fertility lasting six to eight weeks. Cerebral babesiosis is manifested by a variety of signs of central nervous system involvement (Bock et al., 2004). *Babesia bovis* causes depression, weakness, reluctance to move and nervous signs. Head pressing, hyperaesthesia, circling, nystagmus, aggression, paralysis and convulsions are due to nervous involvement (De vos & Potgieter, 1994). Haemoglobinuria is common and hence the name redwater. Anaemia and icterus are common in protracted cases.

In *B. bigemina*, infection is not as severe as in *B. bovis*. However, the disease can develop suddenly with anaemia, icterus and death. Haemoglobinuria occurs early and is more consistent than in *B. bovis* infection. Nervous involvement is absent in *B. bigemina* infection (de Vos et al., 2004). Babesiosis is characterised by anorexia, fever, anemia, and icterus (Ko et al., 2008).

3.3 Heartwater:

Heartwater, caused by *Ehrlichia ruminantium*, is characterised by a sudden high fever, loss of appetite and respiratory involvement. Infected animals exhibit nervous signs such as excessive chewing movements, incoordination, head tilting, rigid posture and a high-stepping gait. Some cattle develop convulsions and death follows. However, in some cases nervous signs may not be observed prior to death (Allsopp et al., 2004).

3.4 Anaplasmosis:

In cattle, *Anaplasma marginale* infections cause anaplasmosis. The severity of the disease depends on age. Older bovines are severely affected while the disease is subclinical in calves less than one year. Anemia is common. The disease occurs frequently in purebred and high producing cows (Potgieter & Stoltsz, 2004). Acute anaplasmosis causes pallor of mucous membranes, inappetance, decreased milk production and general nervousness. As the disease progresses, there is constipation, ruminal stasis, weight loss, dehydration, icterus and labored respiration. Initially, diarrhea may be present and the faeces are usually bile-stained. Abortions occur in severely affected cattle. Chronic anaplasmosis is manifested by slow recovery after the acute disease. A recovered animal may take several months to regain its previous condition (Potgieter & Stoltsz, 2004).

4. CONTROL OF TICK-BORNE DISEASES CAUSED BY *THEILERIA*, *BABESIA*, *ANAPLASMA* AND *EHRlichia* SPECIES

Potential control methods for haemoparasites that target parasites as they are developing in their respective tick hosts include drugs (against ticks and parasites) and vaccines (against ticks and parasites). Successful application of control strategies will be dependent upon thorough understanding of parasite developmental cycles, biology of the tick vectors and the immune response of cattle to ticks and to haemoparasites (Salih et al., 2007).

The current strategy of relying on tick control and therapeutic drugs as a way of controlling TBDs is becoming increasingly difficult for Zambia (Nambota et al., 1994). This is because both curative drugs and acaricides are very costly. Immunization against theileriosis using the infection and treatment method as a way of controlling the disease is becoming increasingly accepted, provided local *Theileria* stocks are used (Nambota et al., 1994).

4.1 Chemical control of ticks:

Acaricides are essential in the short-term but do not offer a permanent solution to tick control (Frisch, 1999). The reliability of acaricides declines when resistance to their use builds up in tick populations. This is common with *Rhipicephalus (Boophilus)* spp. and less so for *Rhipicephalus* spp. (Jongejan et al., 1988). The establishment of acaricide resistance, the increasing cost of chemical control and the data on production loss as an integral part of tick infestation are factors which led to the incorporation of ecological principles into tick control (Spickett, 1994). Today tick control rests overwhelmingly on the twin approaches of vaccination and application of chemical acaricides to

achieve a state of endemic stability (Willadsen, 2006). The effectiveness of this approach is greatest for breeds of high tick resistance. Total resistance is achievable with high host resistance and provides a permanent solution to tick control (Frisch, 1999). The availability and expedient use of acaricides for the control of ticks, together with the development and application of vaccination procedures against TBDs is thus a matter of priority (Spickett, 1994).

Total elimination of ticks is undesirable as it removes the challenge to TBDs which is required to establish endemic stability to these diseases. This is a biologically more robust state than maintaining populations of tick-free but disease-naïve animals, which can suffer severe mortalities if tick control breaks down (Ndou et al., 2010). Attempts to achieve a state of endemic stability for tick-borne pathogens may be unreasonable on smallholder farms. However, reductions in the frequency of use of acaricides may be possible following prospective studies of effects on mortality and morbidity due to tick-borne pathogens (Swai et al., 2005).

4.2 Immunization of cattle:

In order for a vaccine to be accepted for field use, it must be safe, effective against the targeted disease, and pure and free of contaminants or other pathogens. Routine chemo-immunization should be carried out in the tropics as a method of immunizing susceptible cattle against haemoparasitic infections before exposure to natural infection in the field. The method is particularly suitable for introduction of susceptible cattle into an enzootic area alongside an adequate tick control programme (Aliu, 1980). The use of host immunity is important for the establishment of economically suitable strategies of tick control (Spickett, 1994). However, considerable diversity is observed in field populations of each parasite and protection is only assured against homologous challenge (McKeever, 2009). The acquisition of molecular information is likely to have immediate impact on the identification of potential antigens for improved vaccines and novel targets for acaricide action (Willadsen, 2006).

Immunization significantly reduces the mortalities due to ECF (Berkvens et al., 1988). In considering the life cycle of *T. parva* in its mammalian host, there are two potential levels at which immunity against infection could be induced, namely the sporozoite and the macroschizont-infected cell. Ideally the prevention of infection at the stage when the host first encounters the parasite would be most desirable (Morrison, 1984). The sporozoites stabilate vaccine for the control of theileriosis by infection and treatment method (ITM) is being applied in ECF endemic areas with success (Nambota et al., 1994). Assistance to the Veterinary Services in Zambia (ASVEZA) was a Belgian sponsored

project that focused on control of ECF in Southern and Eastern Provinces from 1982 to 2002 under the supervision of the Institute of Tropical Medicine, Antwerp. The project employed the ITM method to vaccinate calves against ECF and managed to reduce calf mortality from more than 25% to less than 2.5% (Geerts, 2011). The ITM method of immunization against ECF involves inoculating a *Theileria* isolate or isolates which provide protection against the challenge within a particular location without the introduction of new strains, followed by the application of a drug capable of controlling the immunizing infection without the development of clinical disease. However, immunization by means of the ITM method creates a reservoir in both cattle and vector, *Rhipicephalus appendiculatus* (Bishop et al., 1992; Koch et al., 1992). The method is based upon the original observation of Neitz (1953) who infected cattle by the application of *T. parva*-infected *R. appendiculatus* ticks and treated them with chlortetracycline at 10 mg/kg from the day after tick application until the macroschizont parasitosis began to decrease. Since then many tetracyclines have been shown to control *T. parva* infections when used in this way (Dolan, 1985). In the southern province of Zambia, the Chitongo strain was used, while in eastern Zambia, the Katete strain was used to vaccinate calves against ECF. The *T. parva* stabilate is being produced at the Centre for Tick and Tick-borne Diseases (CTTBD) in Lilongwe, Malawi. Buparvaquone (Butalex[®], Coopers Animal health limited) and parvaquone (Parvexon[®], Bimeda[®]) are the drugs of choice for the treatment of ECF in Zambia.

Vaccines for babesiosis, anaplasmosis (*A. centrale*) and heartwater are not manufactured in Zambia and are not readily available on the market. Previously, some commercial livestock farmers would import these vaccines to vaccinate their cattle but not for sale. The government through the veterinary council of Zambia is now closely monitoring, supervising or restricting the importation of the vaccines so as to prevent the possibility of introducing new strains of tick-borne pathogens into the country.

4.3 Chemotherapy:

East Coast fever: Tetracyclines have been used successfully in immunization against ECF by the infection and treatment technique. Parvaquone is used at 10 mg/kg repeated after 48 hours to treat theileriosis. The drug is effective against piroplasms and schizonts but does not achieve parasitological cure and recovered animals remain intermittent carriers (Lawrence et al., 2004). For chemotherapy to be effective, the disease must be diagnosed early enough so that treatment can be given at the start of clinical disease. This is, however, difficult in *T. parva* infections because in most

cases the disease becomes clinically apparent only when it has reached an advanced stage (Nambota et al., 1994).

Babesiosis: Imidocarb (3mg/kg) and diminazene (3.5 mg/kg) are the only babesiacides used prophylactically for the short-term control and prevention of babesiosis. However, use of imidocarb may interfere with the development of immunity after vaccinations as the residual effect of the drug may eliminate or suppress infection. Thus the interval between use of imidocarb and vaccination should be at least eight weeks if immunity to *B. bovis* is required and 16 weeks for *B. bigemina* (de Vos et al., 2004).

Heartwater: Short-acting formulations of oxytetracycline have been used at a dosage rate of 10 to 20 mg/kg body weight to treat heartwater (Allsopp et al., 2004). The treatment is repeated every 24 hours. It is effective during the early, febrile stages of this disease. Antibiotic treatment alone is not always successful in later stages. Animals often die before treatment can be instituted (OIE, 2009).

Anaplasmosis: Tetracyclines such as chlortetracycline, tetracycline, oxytetracycline and doxycycline are commonly used for the treatment of acute and chronic anaplasmosis. Single injection of long-acting formulations at the rate of 20 mg/kg is very effective. At the recommended doses, tetracyclines do not sterilize *Anaplasma* infections in cattle but effectively inhibit the multiplication of *Anaplasma* in erythrocytes (Potgieter & Stolstz, 2004).

The most difficult problem remains to translate laboratory research into the extremely diverse parasite control requirements of farming systems in a way that is practically useful (Willadsen, 2006). Any one of these methods may not be adequate to control the problem on its own but when several of the methods are combined an economic and robust integrated control is likely to result.

5. LABORATORY DIAGNOSIS

Direct and indirect methods have been developed for the diagnosis of tick-borne haemoparasites of cattle. The direct methods involve identifying the parasite in Giemsa-stained blood smears or lymph node biopsy samples. Indirect methods are more accurate and have been used to screen and detect TBDs in cattle. These include immunofluorescent antibody test (IFAT), enzyme linked immunosorbent assay (ELISA) and nucleic acid-based tests (Marufu et al., 2010).

5.1 Conventional parasitological techniques:

Examination of blood and organ smears can confirm ECF by demonstration of schizonts in lymphoblasts and piroplasms in erythrocytes. Detection of *Babesia* species using thick blood smears is quite sensitive. Microscopic pathology has also been employed in the detection of *Babesia* spp. *Babesia bovis* is seen to parasitize red blood cells in the capillaries of the brain (de Vos et al., 2004).

Anaplasma can be confirmed by light microscopic demonstration of intraerythrocytic parasites on Giemsa-stained blood films. It has been reliable when used in the absence of an anemia crisis (Potgieter & Stoltsz, 2004).

Ehrlichia ruminantium can be detected by microscopic examination of Giemsa-stained brain smears. Demonstration of *E. ruminantium* colonies within the capillary endothelial cells of the brain is confirmative (Zwart, 1985).

5.2 Serological techniques:

The serological test of choice for diagnosis of ECF is a *T. parva*-specific antibody detection ELISA. This is based on the use of a recombinant polymorphic immunodominant molecule (PIM) (Lawrence et al., 2004).

Enzyme-linked immunosorbent assays are also used for detection of *B. bovis*. However, there is still no validated ELISA for *B. bigemina* (de Vos et al., 2004).

Using the laboratory-standardized “sandwich” ELISA (SELISA) with a sensitivity of 94.4%, the seroprevalence of babesiosis can be studied in cattle from endemic areas of the disease. In comparison to IFAT, SELISA detects higher numbers of serum samples positive for bovine babesiosis (Ravindran et al., 2007).

The IFAT is also used to detect antibodies to *Babesia* parasites in cattle. It is not only reproducible but also has high sensitivity and specificity (Krause et al., 1994).

A complement ELISA (cELISA) based on serum antibody inhibition of MAb AnaF16C1 binding to rMSP5 has been developed for sero diagnosis of *Anaplasma marginale* with a specificity of 100% (Knowles et al., 1996). The MAP1-B ELISA technique, based on an immunogenic region of the MAP1

protein (MAP1-B fragment) conserved between stocks of *E. ruminantium*, has been used to detect *E. ruminantium* in cattle (Semu et al., 2001).

5.3 Molecular biological techniques:

The commonly used molecular methods in diagnosis of tick-transmitted haematic protozoa are the following: (i) reverse line blot (RLB) hybridization; (ii) quantitative real-time polymerase chain reaction (qPCR); (iii) isothermal amplification methods: loop-mediated amplification (LAMP) and self-sustaining sequence replication (3SR, also called "Nudeic Acid Sequence Based Amplification", NASBA, or Transcription Mediated Amplification, TMA) (Criado-Fornelio, 2007).

In general, none of these methods could be considered better than another. Their use in diagnostic applications greatly depends on the laboratory size. Large-scale laboratories prefer methods amenable to automation, like RLB, PCR-ELISA or qPCR (Criado-Fornelio, 2007).

An RLB assay has been developed for the simultaneous identification and differentiation of *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* spp. (Gubbels et al., 1999; Bekker et al., 2002). The sensitivity of the RLB assay was determined at 0.000001% parasitaemia, enabling detection of the carrier state of most parasites. The assay is also able to correctly identify mixed DNAs from different parasites (Gubbels et al., 1999). The RLB is recommended for use in integrated epidemiological monitoring of TBDs, since RLB can also be used for screening ticks and can easily be expanded to include additional haemoparasite species (Gubbels et al., 1999). Using RLB, it is possible to detect and differentiate several different piroplasms simultaneously (Salih et al., 2007).

MATERIALS AND METHODS

1. SAMPLE COLLECTION

Bovine blood samples were collected on Whatman® filter paper (Merck) between July and September of 2010. The samples were collected from the ear vein of the cattle. The animals were restrained in a crush pen during routine deworming and treatment. The blood samples (n = 299) were collected from five areas namely Kapamba (n = 50), Chifulo (n = 102), Chisanga (n = 38), Kowa (n = 95) and Mungwi central (n = 14) (Fig. 1). These areas are all in Mungwi district of Northern Province, Zambia. Kapamba, Chifulo, Chisanga and Kowa are located along the Chambeshi flood plains while Mungwi central is on the upland. Information on the owner, age, sex and color of each animal sampled was captured. Cattle of all age groups were sampled. The sampled cattle included 84 males and 215 females.

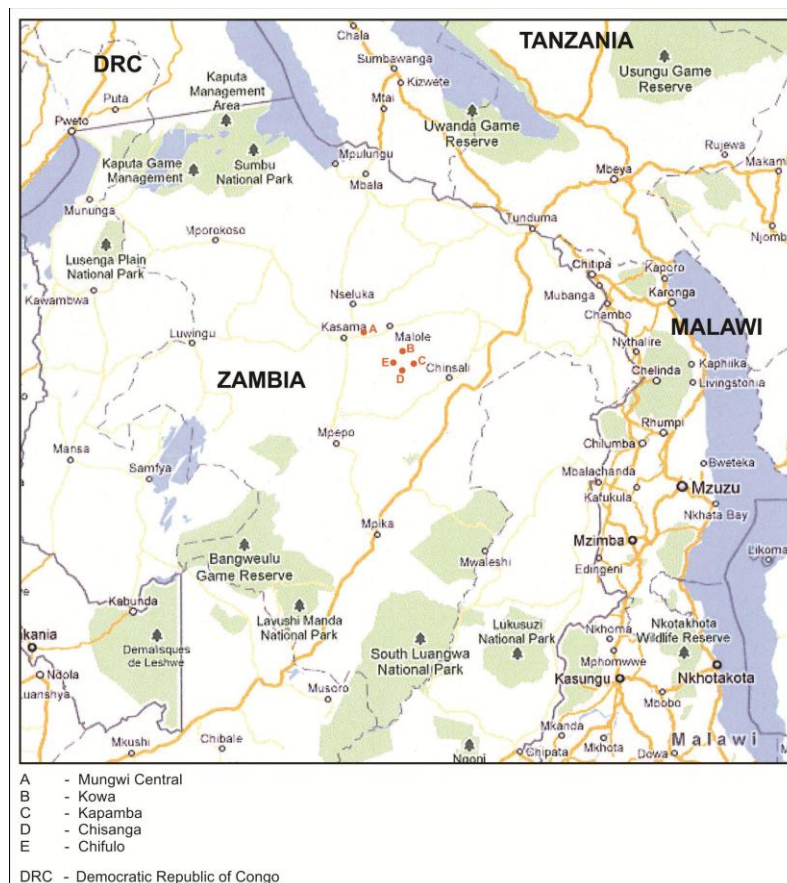


Fig. 1: Map showing the five localities of Mungwi, in Zambia, from which the blood samples were collected from cattle.

2. LABORATORY TECHNIQUES

The 299 bovine blood samples on filter paper collected in Mungwi were analysed in the Molecular Biology laboratory of the Department of Veterinary Tropical Diseases in the Faculty of Veterinary Science at University of Pretoria Onderstepoort campus as described below.

2.1 DNA extraction

DNA was extracted from dried blood spots using the QIAamp[®] DNA mini kit (QIAGEN, Southern Cross Biotechnologies). The manufacturer's extraction protocol was followed. Three circles (three millimeter diameter) were punched out from a filter paper on which bovine blood was dried using a single-hole paper puncher. The three punched-out circles from a dried blood spot were placed into a 1.5 ml microcentrifuge tube and 180 µl of buffer ATL was added. This was incubated at 85°C for 10 minutes after which the microcentrifuge tube was briefly centrifuged to remove drops from inside the lid. Twenty µl of proteinase K stock solution (20 mg/ml) was added to the solution, mixed by vortexing and incubated at 50°C for one hour. The sample was briefly centrifuged and 200 µl of buffer AL was added and then incubated at 70°C for 10 minutes. After brief centrifugation, 200 µl of ethanol (96-100%) was added to the sample and mixed thoroughly. The sample was applied to the QIAamp Mini spin column and centrifuged at 8000 rpm for one minute. The spin column was then placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded. Each spin column was tightly closed to avoid aerosol formation during centrifugation. The spin column was carefully opened and 500 µl of buffer AW1 was added without wetting the rim. The spin column was then placed in a clean 2 ml collection tube, and centrifuged at 8000 rpm for one minute; the tube containing the filtrate was discarded. The spin column was carefully opened and 500 µl of buffer AW2 was added without wetting the rim and centrifuged at 14000 rpm for three minutes. The spin column was then placed into a new 2 ml collection tube and the old collection tube containing filtrate was discarded. The column was spun at 14000 rpm for one minute to eliminate any AW2 that may have been carried over. The spin column was then placed into a new 1.5 ml microcentrifuge tube and the collection tube containing filtrate was discarded. The spin column was carefully opened and eluted in 150 µl of buffer AE. The solution was incubated at room temperature for one minute and then centrifuged at 8000 rpm for one minute. The extracted DNA was stored at 4°C.

2.2 Polymerase chain reaction (PCR)

Theileria and *Babesia* genus-specific forward primer, RLB F2 [5'-GAC ACA GGG AGG TAG TGA CAA G-3'] and biotin labelled reverse primer, RLB R2 [5'-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3'] were used to amplify the V4 hypervariable region of the parasite 18S rRNA gene as previously described

(Nijhof et al., 2005). For the simultaneous detection of *Ehrlichia* and *Anaplasma* spp. the V1 hypervariable region of the 16S rRNA gene was amplified using the *Ehrlichia* and *Anaplasma* genus-specific forward primer Ehr-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') (Schouls et al., 1999) and biotin labelled reverse primer Ehr-R (5'-Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3') (Bekker et al., 2002). The PCR reaction mixture consisted of 12.5 µl of Platinum® Quantitative PCR SuperMix-UDG (containing 60 U/ml Platinum Taq DNA polymerase, 40 mM Tris-HCl (pH 8.4), 100 mM KCl, 6 mM MgCl₂, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 400 µM dUTP and 40 U/ml UDG) (Celtic molecular diagnostics, South Africa), and 0.25 µl of each of the forward and reverse primers at a final concentration of 8 pmol/µl each, 2.5 µl of DNA template and 9.5 µl of water to a total volume of 25 µl. Positive (*A. centrale* Onderstepoort Biological Products vaccine strain) and negative controls (master mix without DNA template) were included to monitor false positive and false negative results. A touchdown PCR thermocycler program (Table 1) was applied for amplification.

Table 1: Thermocycler program for *Theileria* / *Babesia* and *Ehrlichia* / *Anaplasma* touchdown PCR.

1 cycle	3 min	37°C	Activate UDG
1 cycle	10 min	94°C	Inactivate UDG & activate <i>Taq</i>
	20 sec	94°C	Denature double stranded DNA template
2 cycles	30 sec	67°C	Anneal primers
	30 sec	72°C	Extension of PCR products by <i>Taq</i> polymerase
	20 sec	94°C	
2 cycles	30 sec	65°C	
	30 sec	72°C	
	20 sec	94°C	
2 cycles	30 sec	63°C	
	30 sec	72°C	
	20 sec	94°C	
2 cycles	30 sec	61°C	
	30 sec	72°C	
2 cycles	20 sec	94°C	

	30 sec	59°C	
	30 sec	72°C	
40 cycles	20 sec	94°C	
	30 sec	57°C	
	30 sec	72°C	
1 cycle	7 min	72°C	Final extension
		4°C	Keep at 4°C

2.3 Preparation of the RLB membrane:

A Biotinylated membrane prepared in-house was used. Genus- and species-specific probes (containing a N-terminal N-(trifluoroacetamidehexyl-cyanoethyl,N,N-diisopropyl phosphoramidite [TFA]) – C6 amino linker) included on the membrane are listed in Table 2. Preparation of the in-house membrane was done by diluting 8 µl (0.25 pmol/µl) of each species-specific oligonucleotide in 142 µl of 0.5 M NaHCO₃ at pH 8.4 to a final concentration of 2 pmol /150 µl (0.13 pmol/µl). The final volume of 150 µl of diluted probe was loaded onto the membrane by use of the miniblottedter. The membrane was marked and incubated for 10 minutes in 10 ml of freshly prepared 16% EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide). The membrane was then incubated at room temperature for 2 minutes after which the membrane was inactivated with 100 mM of NaOH for 8 minutes at room temperature with gentle shaking. The membrane was then rinsed with 100 ml 2 X SSPE / 0.1% SDS at 60°C for 5 minutes. The membrane was ready to be used for the RLB hybridization assay.

Table 2: Genus- and species-specific probes and their sequences present on the in-house prepared membrane used for detecting pathogen DNA. Symbols used to indicate degenerate positions:

R=A/G, W=A/T.

Oligonucleotide probe identification	Probe Sequence (5'--> 3')
<i>Anaplasma bovis</i>	GTA GCT TGC TAT GRG AAC A
<i>Anaplasma centrale</i>	TCG AAC GGA CCA TAC GC
<i>Anaplasma marginale</i>	GAC CGT ATA CGC AGC TTG
<i>Anaplasma (Ehrlichia) sp. Omatjenne</i>	CGG ATT TTT ATC ATA GCT TGC

<i>Anaplasma phagocytophilum</i>	TTG CTA TAA AGA ATA ATT AGT GG
<i>Babesia</i> genus-specific probe 1 (<i>Babesia catch-all 1</i>)	ATT AGA GTG TTT CAA GCA GAC
<i>Babesia</i> genus-specific probe 2 (<i>Babesia catch-all 2</i>)	ACT AGA GTG TTT CAA ACA GGC
<i>Babesia bicornis</i>	TTG GTA AAT CGC CTT GGT C
<i>Babesia bigemina</i>	CGT TTT TTC CCT TTT GTT GG
<i>Babesia bovis</i>	CAG GTT TCG CCT GTA TAA TTG AG
<i>Babesia caballi</i>	GTG TTT ATC GCA GAC TTT TGT
<i>Babesia canis</i>	TGC GTT GAC CGT TTG AC
<i>Babesia divergens</i>	ACT RAT GTC GAG ATT GCA C
<i>Babesia felis</i>	TTA TGC GTT TTC CGA CTG GC
<i>Babesia gibsoni</i>	CAT CCC TCT GGT TAA TTT G
<i>Babesia leo</i>	ATC TTG TTG CTT GCA GCT T
<i>Babesia major</i>	TCC GAC TTT GGT TGG TGT
<i>Babesia microti</i>	GRC TTG GCA TCW TCT GGA
<i>Babesia occultans</i>	CCT CTT TTG GCC CAT CTC GTC
<i>Babesia rossi</i>	CGG TTT GTT GCC TTT GTG
<i>Babesia</i> sp. (sable)	GCT GCA TTG CCT TTT CTC C
<i>Babesia vogeli</i>	AGC GTG TTC GAG TTT GCC
<i>Ehrlichia/Anaplasma</i> genus-specific probe	GGG GGA AAG ATT TAT CGC TA
<i>Ehrlichia canis</i>	TCT GGC TAT AGG AAA TTG TTA
<i>Ehrlichia chaffeensis</i>	ACC TTT TGG TTA TAA ATA ATT GTT
<i>Ehrlichia ruminantium</i>	AGT ATC TGT TAG TGG CAG
<i>Theileria/Babesia</i> genus-specific probe	TAA TGG TTA ATA GGA RCR GTT G
<i>Theileria</i> genus-specific probe	ATT AGA GTG CTC AAA GCA GGC
<i>Theileria annae</i>	CCG AAC GTA ATT TTA TTG ATT TG
<i>Theileria annulata</i>	CCT CTG GGG TCT GTG CA
<i>Theileria bicornis</i>	GCG TTG TGG CTT TTT TCT G
<i>Theileria buffeli</i>	GGC TTA TTT CGG WTT GAT TTT
<i>Theileria</i> sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
<i>Theileria equi</i>	TTC GTT GAC TGC GYT TGG
<i>Theileria</i> sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
<i>Theileria lestoquardi</i>	CTT GTG TCC CTC CGG G
<i>Theileria mutans</i>	CTT GCG TCT CCG AAT GTT
<i>Theileria ovis</i>	TTG CTT TTG CTC CTT TAC GAG
<i>Theileria parva</i>	GGA CGG AGT TCG CTT TG

<i>Theileria</i> sp. (sable)	GCT GCA TTG CCT TTT CTC C
<i>Theileria separata</i>	GGT CGT GGT TTT CCT CGT
<i>Theileria taurotragi</i>	TCT TGG CAC GTG GCT TTT
<i>Theileria velifera</i>	CCT ATT CTC CTT TAC GAG T

2.4 Reverse line blot (RLB) hybridization assay

The in-house prepared membrane was incubated for five minutes in 10 ml 2 x SSPE / 0.1% at room temperature. Twenty μ l of PCR product (10 μ l of PCR product obtained with the *Theileria* / *Babesia* primers and 10 μ l of PCR product obtained with the *Ehrlichia* / *Anaplasma* primers) was diluted in 130 μ l 2 x SSPE / 0.1% SDS. The diluted PCR products were denatured at 99.9°C for 10 minutes in a thermocycler and then cooled on ice immediately. The membrane was placed in the miniblotter with slots perpendicular to the line pattern of applied probes. The ink-lanes were placed directly under the opening of the slots. Residual fluid was removed by aspiration (vacuum). The slots were filled with 142 μ l of diluted PCR product according to the sample list. Air bubbles were avoided in the slots. Empty slots were filled with 2 X SSPE/0.1% SDS, to avoid cross flow. Hybridization was achieved by placing the miniblotter in an incubator at temperature 42°C for 60 minutes on a horizontal surface. The samples were removed by aspiration and the membrane was removed from the blotter. The membrane was washed twice in preheated 2 X SSPE/0.5% SDS for 10 minutes at 50°C in the incubator with shaking platform under gentle shaking. The 2 X SSPE/0.5% SDS was discarded in the sink after each wash.

The membrane was then incubated with pre-heated 10 ml 2 X SSPE/0.5% SDS + 12.5 μ l peroxidase labelled streptavidin-POD (Roche diagnostics, South Africa) conjugate (1.25 U) for 30 minutes at 42°C under gentle shaking. The solution was discarded in the sink. The membrane was washed twice in preheated 2 X SSPE/0.5% SDS for 10 minutes in a 42°C incubator under gentle shaking. The 2X SSPE/0.5% SDS was discarded in the sink after each wash. The membrane was then washed twice with 2X SSPE for 5 minutes at room temperature under gentle shaking. The 2X SSPE was discarded in the sink after each wash. The membrane was kept wet with 5 ml ECL1 and 5 ml ECL2 (Separation Scientific, South Africa). Mixing was done by shaking the container gently and keeping the membrane wet with ECL for one minute at room temperature. The membrane was placed between the two clean overhead sheets. Air bubbles were removed by pressing out extra fluids to the side of the membrane. This assembly was placed into an exposure cassette and all the corners of the overhead sheets were secured in the cassette with tape, and an X-ray film (Separation Scientific,

South Africa) was applied in a darkroom. The X-ray film was exposed for 20 minutes and then developed to detect the hybridized PCR products which were visible by chemiluminescence. The film was placed on a grid for each sample lane to correlate with the oligonucleotide probes.

2.5 Stripping of oligonucleotide/probe membrane

The membrane was stripped immediately after use. The procedure involved two washes with 1% SDS, preheated to 80°C for 30 minutes under gentle shaking and one wash with 20 mM EDTA at room temperature for 15 minutes under gentle shaking. After stripping, the membrane was stored in 20 mM EDTA, pH 8 at a temperature of 4°C in a plastic container.

3. TICK COUNTS AND IDENTIFICATION

Half body tick counts were conducted on all the 299 cattle. Ticks were identified to genus and species level using a stereomicroscope and dichotomous identification keys of ticks as illustrated by Walker et al. (2003). The identified ticks were compared to species descriptions and distribution (Walker et al., 2003). The ticks counts and identifications were done once on each animal between April and July 2011.

3. RESULTS

1. RLB RESULTS

The reverse line blot (RLB) hybridization assay was used to simultaneously screen for the presence of *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species in a total of 299 blood samples collected from cattle in five areas of the Mungwi district of Zambia. An example of a blot is illustrated in Fig. 1.

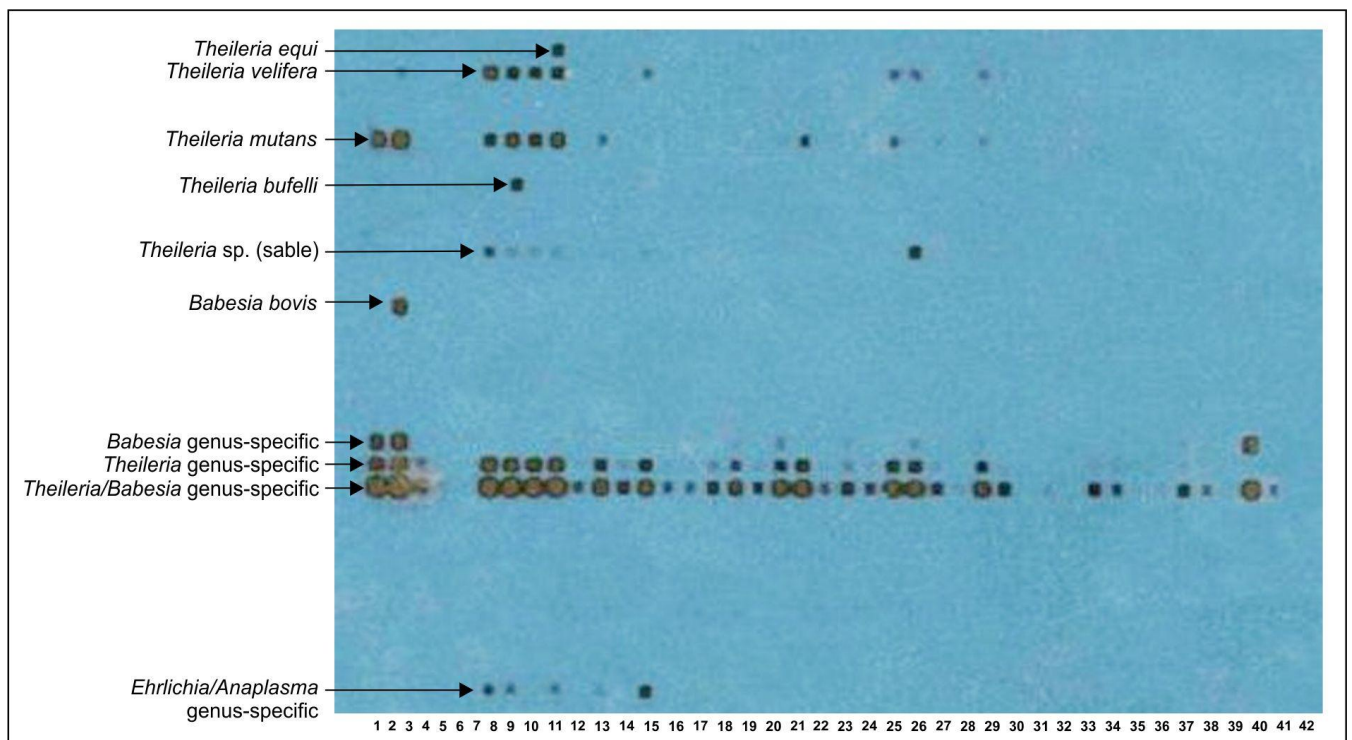


Fig. 1: RLB results of some of the bovine specimens screened. The species-specific oligonucleotide probes were applied in horizontal lanes and the PCR products in vertical lanes. The negative control was applied in lane 42.

Results indicated that 11% (n = 33) of the sampled animals tested positive for single infections of haemoparasites. The most prevalent haemoparasites present as single infections were *Theileria mutans* (4.7%) and *T. velifera* (4%). Three samples tested positive for *Ehrlichia canis* while two tested positive for *Theileria sp. (sable)*. One sample was positive for *Babesia bovis* while another was

positive for *Theileria equi*. The majority of the single infections were from Chifulo (6%) followed by Chisanga (2%) (Table 1; Fig. 2).

Samples that tested positive for mixed infections comprised 75.6% (n = 226) of the sampled animals. The samples with mixed infections exhibited a combination of one or two *Theileria* species and another species (85%) and occurred in all of the five areas sampled. Fifteen percent of the mixed infections exhibited a combination of *Babesia* species and *Anaplasma marginale* and were from Chifulo (Table 1; Fig. 2).

A total of 28 of the samples screened (9.4%) were negative for haemoparasites and/or below the detection limit of the test. These were from Chifulo, Chisanga and Kowa. Kapamba and Mungwi central did not record any negative samples. In 12 samples (4%) the PCR products failed to hybridize with any species-specific probes but hybridized only with the either the *Ehrlichia / Anaplasma* (n = 6) or the *Theileria / Babesia* (n = 6) genus-specific probes (Table 1; Fig. 2).

Table 1: The occurrence of tick-borne haemoparasites in bovine blood samples from five localities in the Mungwi District, Zambia as determined by the RLB hybridization assay.

	Kapamba (n=50)	Chifulo (n=102)	Chisanga (n=38)	Kowa (n=95)	Mungwi central (n=14)	TOTAL (n=299)
SINGLE INFECTIONS						
<i>Theileria equi</i>	0	1 (0.98%)	0	0	0	1 (0.33%)
<i>Theileria mutans</i>	1 (2%)	8 (7.8%)	2 (5.3%)	0	3(21.4%)	14 (4.7%)
<i>Theileria sp. (sable)</i>	0	0	2 (5.3%)	0	0	2 (0.67%)
<i>Theileria velifera</i>	3 (6%)	7 (6.9%)	0	2 (2.1%)	0	12 (4%)
<i>Babesia bovis</i>	0	0	1 (2.6%)	0	0	1 (0.33%)
<i>Ehrlichia canis</i>	0	2 (1.96%)	1 (2.6%)	0	0	3 (1%)
Single infections (SUBTOTAL)	4 (8%)	18 (17.6%)	6 (15.8%)	2 (2.1%)	3 (21.4%)	33 (11%)
MIXED INFECTIONS						
<i>Theileria parva</i>	1 (2%)	0	0	0	0	1 (0.4%)
<i>Theileria equi</i>	4 (8%)	0	1 (2.6%)	3 (3.1%)	0	8 (3.5%)
<i>Theileria mutans</i>	43 (86%)	26 (25.5%)	10 (26%)	73 (77%)	11 (78.6%)	163 (54.5%)
<i>Theileria sp.(buffalo)</i>	0	1 (0.98%)	0	33 (35%)	0	34 (11.4%)
<i>Theileria buffeli</i>	0	0	1 (2.6%)	0	0	1 (0.4%)
<i>Theileria sp. (sable)</i>	4 (8%)	0	7 (18.4%)	0	0	11 (3.7%)
<i>Theileria sp. (kudu)</i>	0	0	0	20 (21%)	0	20 (6.7%)
<i>Theileria velifera</i>	45 (90%)	26 (25.5%)	8 (21%)	64 (67.4%)	11 (78.6%)	154 (51.5%)
<i>Theileria taurotragi</i>	0	5 (4.9%)	0	29 (30.5%)	0	34 (11.4%)
<i>Babesia bovis</i>	2 (4%)	8 (7.8%)	1 (2.6%)	10 (10.5%)	2 (14.3%)	23 (7.7%)
<i>Babesia bigemina</i>	0	0	0	10 (10.5%)	0	10 (3.3%)
<i>Babesia caballi</i>	0	16 (15.7%)	0	0	0	16 (5.3%)
<i>Babesia sp. (sable)</i>	0	38 (37%)	0	0	0	38 (12.7%)
<i>Babesia gibsoni</i>	0	38 (37%)	0	0	0	38 (12.7%)
<i>Anaplasma bovis</i>	1 (2%)	0	0	1 (1%)	0	2 (0.67%)
<i>Anaplasma marginale</i>	8 (16%)	38 (37%)	0	29 (30.5%)	2 (14.3%)	77 (25.7%)
<i>Anaplasma centrale</i>	0	0	0	3 (3.2%)	0	3 (1%)
<i>Anaplasma (Ehrlichia) sp. Omatjenne</i>	4 (8%)	3 (2.9%)	0	14 (14.7%)	1 (7%)	22 (7.3%)
<i>Ehrlichia canis</i>	0	2 (1.96%)	0	32 (33.7%)	0	34 (11.4%)
<i>Ehrlichia chaffeensis</i>	0	0	0	12 (12.6%)	0	12 (4%)
<i>Ehrlichia ruminantium</i>	1 (2%)	0	0	0	0	1 (0.33%)
Mixed infections (SUBTOTAL)	46 (92%)	66 (64.7%)	11 (28.9%)	92 (97%)	11 (78.6%)	226 (75.6%)
Negative/below detection limit	0	12 (11.8%)	15 (39.5%)	1 (1.0%)	0	28 (9.4%)
<i>Theileria/Babesia</i> genus-specific only	0	6 (5.9%)	0	0	0	6 (2%)
<i>Ehrlichia/Anaplasma</i> genus-specific only	0	0	6 (15.8%)		0	6 (2%)

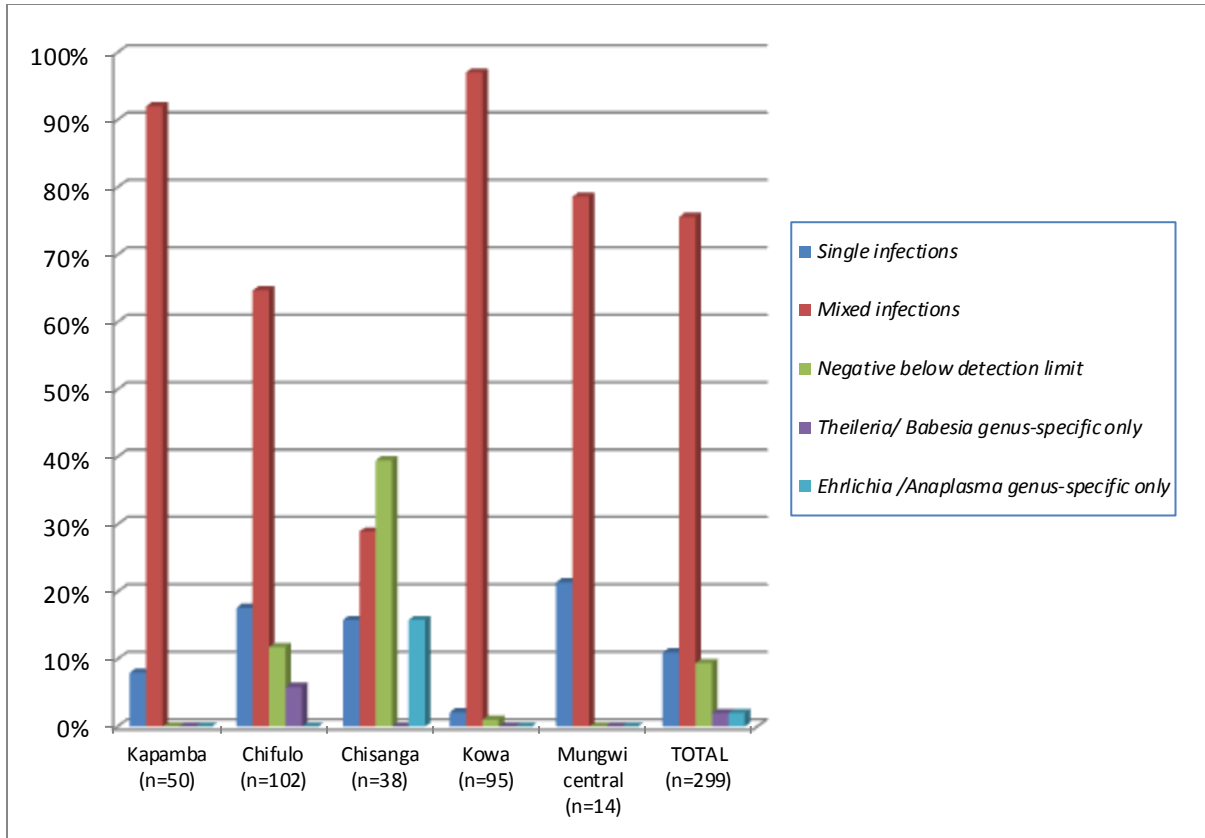


Fig. 2: Schematic representation of the occurrence of tick-borne haemoparasites in bovine blood samples from five localities in Mungwi, Zambia as determined by the RLB hybridization assay.

1.1 Specific detection of *Babesia* and *Theileria* species

Nine *Theileria* species were detected namely *T. parva*, *T. mutans*, *T. velifera*, *Theileria* sp. (sable), *T. equi*, *T. taurotragi*, *Theileria* sp. (buffalo), *T. buffeli* and *Theileria* sp. (kudu). The most prevalent *Theileria* species present were *T. mutans* (4.7% single infection; 54.5% mixed infections) and *T. velifera* (4% single infection; 51.5% mixed infections). They were detected in all of the five sampled areas. Interestingly, only one sample from Kapamba tested positive for *T. parva* (Table 1).

Single *Theileria* species infections were detected in 1.3% of animals from Kapamba, 6% from Chifulo, 2% from Chisanga, 0.7% from Kowa and 1% from Mungwi central. The most common combination of mixed *Theileria* species infections which was detected in 22% of the samples was *T. mutans* and *T. velifera* reported from Kowa (Fig. 3).

From Kapamba, the most prevalent *Theileria* species were *T. velifera* (90%) and *T. mutans* (86%) (Fig. 3). The most prevalent *Theileria* species in Chifulo were *T. velifera* (25.5%) and *T. mutans* (25.5%).

Theileria taurotragi was detected in 4.9% of the samples from Chifulo (Fig. 3.3). Samples from Chisanga tested positive for *T. mutans* (26%), *T. velifera* (21%) and *Theileria* sp. (sable) (18.6%) (Fig. 3). From Kowa, the most prevalent *Theileria* species were *T. mutans* (77%) and *T. velifera* (67.4%). *Theileria* sp. (buffalo) was detected in 35% of the samples and *T. taurotragi* in 30.5% (Fig. 3). The most prevalent *Theileria* species in Mungwi central were *T. mutans* (78.6%) and *T. velifera* (78.6%) (Fig. 3).

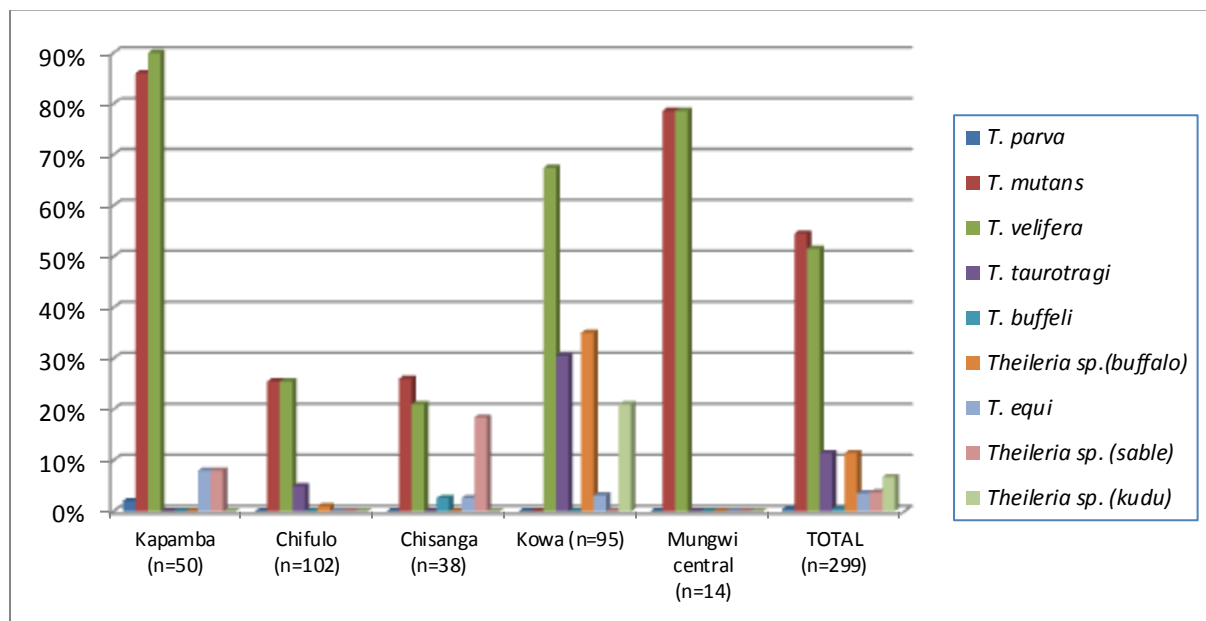


Fig. 3: Mixed *Theileria* species infections in samples from the five areas of Mungwi as screened using the RLB hybridization assay.

Five *Babesia* species were detected and these were *B. bovis*, *B. bigemina*, *B. gibsoni*, *B. caballi* and *Babesia* sp. (sable). From all the sampled areas, a single *Babesia bovis* infection was detected in one of the sampled animals (0.3%) from Chisanga. Mixed *Babesia* species infections were detected in samples from Chifulo (33.8%) and Kowa (6.7%). The most common combination of mixed *Babesia* infections was detected in 33% of samples being *B. bovis*, *Babesia* sp. (sable), *B. gibsoni* and *B. caballi* and occurred in Chifulo (Fig. 4).

From Chifulo, the most prevalent *Babesia* species was *B. gibsoni* (37%) and *Babesia* sp. (sable) (37%). *Babesia bigemina* (10.5%) and *B. bovis* (10.5%) were detected in samples from Kowa. *Babesia bovis* was detected in samples from Kapamba (4%), Chisanga (7.8%) and Mungwi central (14.3%) (Fig. 4).

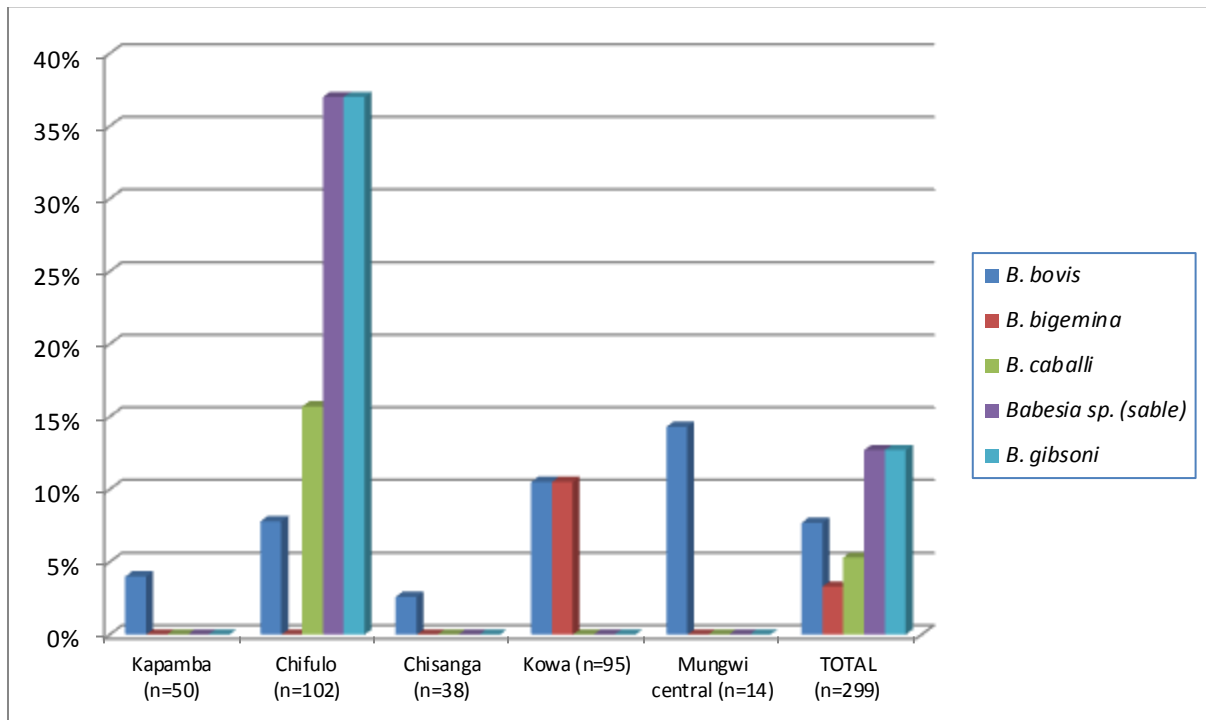


Fig. 4: Mixed *Babesia* species infections in samples from five areas of Mungwi as determined using the RLB hybridization assay.

1.2 Specific detection of *Anaplasma* and *Ehrlichia* species

Using the RLB hybridization assay, four *Anaplasma* species and three *Ehrlichia* species were detected in the samples. The *Anaplasma* species were *Anaplasma bovis*, *A. centrale*, *A. marginale* and *Anaplasma* (formerly *Ehrlichia*) sp. Omatjenne. There were no single *Anaplasma* species infections detected in the samples. Mixed *Anaplasma* species infections were detected in samples from Kowa (49.5%), Chifulo (40%), Kapamba (26%) and Mungwi central (21%). *Anaplasma marginale* was detected in 25.7% of the sampled animals, *Anaplasma* (*Ehrlichia*) sp. Omatjenne in 7.3%, *A. centrale* in 1% and *A. bovis* in 0.67% of the samples. No *Anaplasma* species infections were detected in Chisanga (Fig. 5).

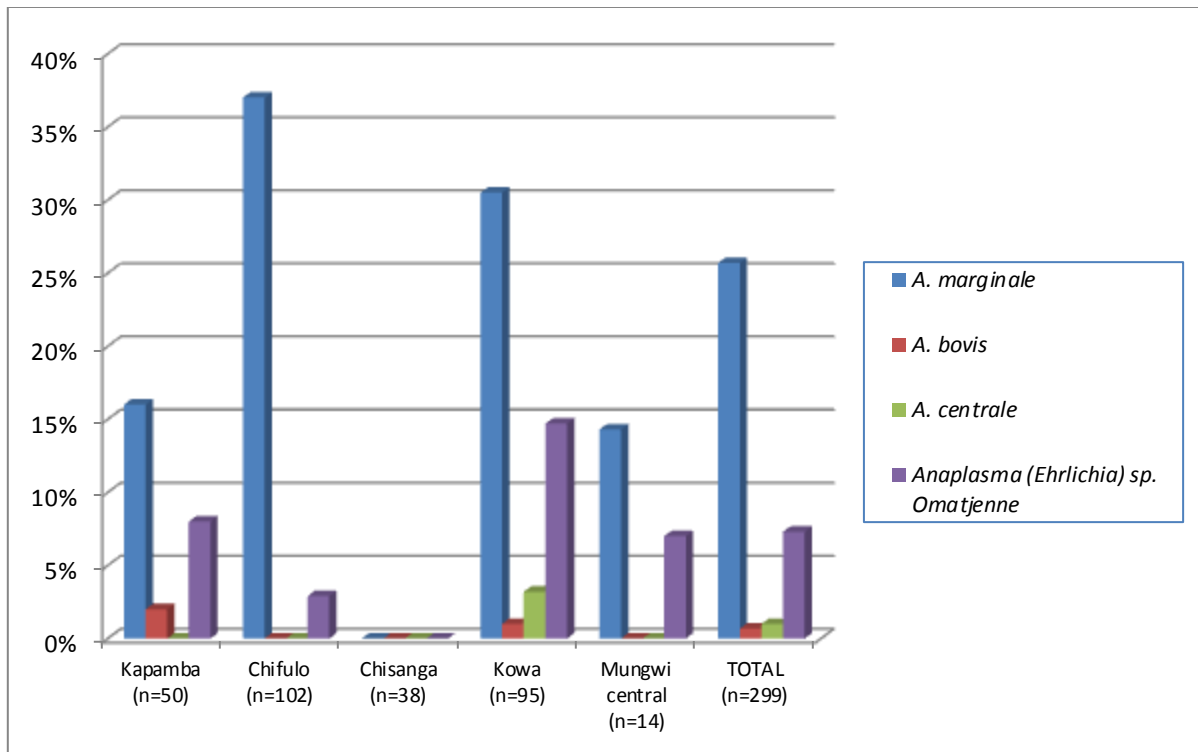


Fig. 5: *Anaplasma* species infections in samples from five areas of Mungwi as screened using the RLB hybridization assay.

The *Ehrlichia* species detected were *E. ruminantium*, *E. canis* and *E. chaffeensis*. Single *E. canis* infections were detected in samples from Chifulo (0.7%) and Chisanga (0.3%). Mixed *Ehrlichia* species infections were most prevalent in Kowa (33.7%) followed by Chifulo (1.96%) and Kapamba (2%). The most common combination of mixed *Ehrlichia* infections was *E. canis* and *E. chaffeensis* which were detected in 46% of samples from Kowa. Of all the sampled animals, only one sample from Kapamba (0.3%) tested positive for the presence of *E. ruminantium* (Fig. 6).

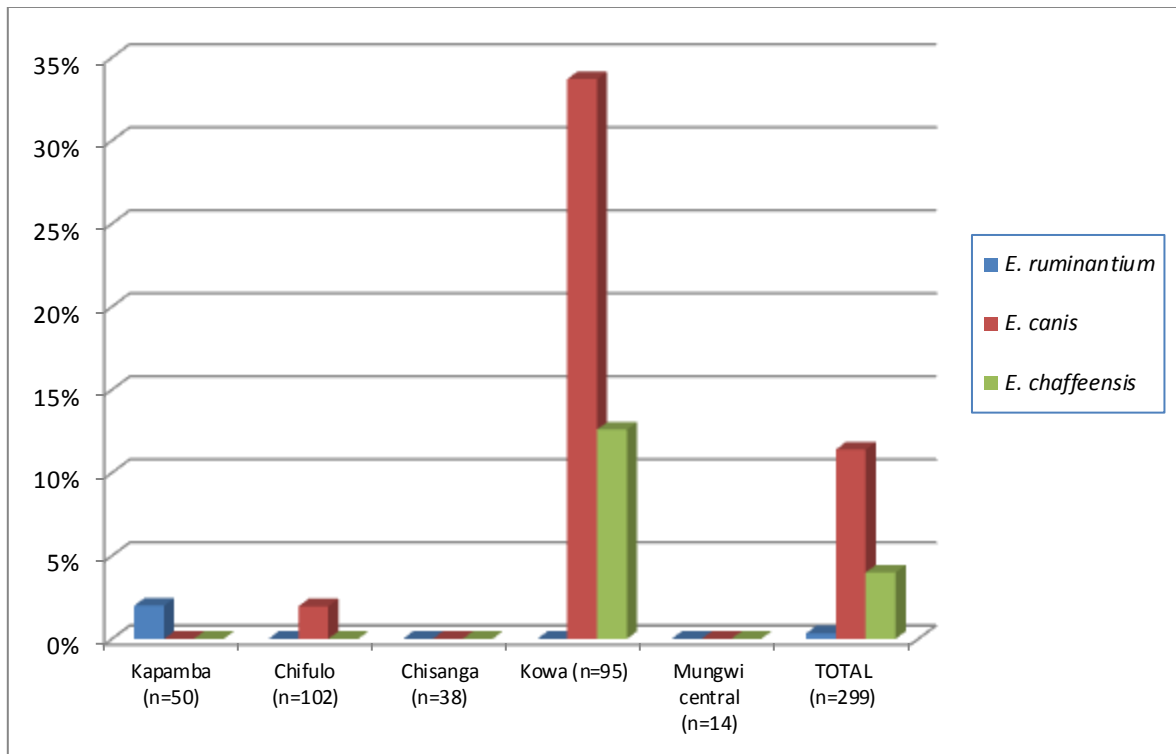


Fig. 6: Mixed *Ehrlichia* species infections in samples from five areas of Mungwi as screened using the RLB hybridization assay.

2. TICK IDENTIFICATIONS

A total of 5288 ticks were collected from 299 cattle between April and July 2011 and identified to species level. The ticks species identified were *Amblyomma variegatum*, *Rhipicephalus (Boophilus) decoloratus*, *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus appendiculatus*.

From Kapamba, the most prevalent tick species identified were *Amblyomma variegatum* (72.5%) and *Rhipicephalus (Boophilus) microplus* (13.6%) (Table 2; Fig. 7). *Amblyomma variegatum* (47.3%) and *Rhipicephalus (Boophilus) decoloratus* (33%) were most prevalent in Chifulo. From Chisanga, *Rhipicephalus (Boophilus) decoloratus* (43.3%) and *Amblyomma variegatum* (39.8%) were most prevalent. In Kowa, *Amblyomma variegatum* (40.4%) and *Rhipicephalus (Boophilus) decoloratus* (36.3%) were most prevalent. *Amblyomma variegatum* (65.7%) was most prevalent in Mungwi central (Table 2; Fig. 7).

Table 2: Occurrence of tick species on 299 cattle sampled from five areas of Mungwi district, Zambia.

	Kapamba (n=1532)	Chifulo (n=1802)	Chisanga (n=460)	Kowa (n=1313)	Mungwi central (n=181)	Total (n=5288)
<i>Amblyomma variegatum</i>	1110 (72.5%)	853 (47.3%)	183 (39.8%)	531 (40.4%)	119 (65.7%)	2796 (52.9%)
<i>Rhipicephalus (Boophilus) decoloratus</i>	192 (12.5%)	595 (33%)	199 (43.3%)	477 (36.3%)	33 (18.2%)	1496 (28.3%)
<i>Rhipicephalus (Boophilus) microplus</i>	209 (13.6%)	245 (13.5%)	39 (8.5%)	116 (8.8%)	29 (16%)	638 (12.1%)
<i>Rhipicephalus appendiculatus</i>	21 (1.4%)	109 (6%)	39 (8.5%)	189 (14.4%)	0	358 (6.8%)

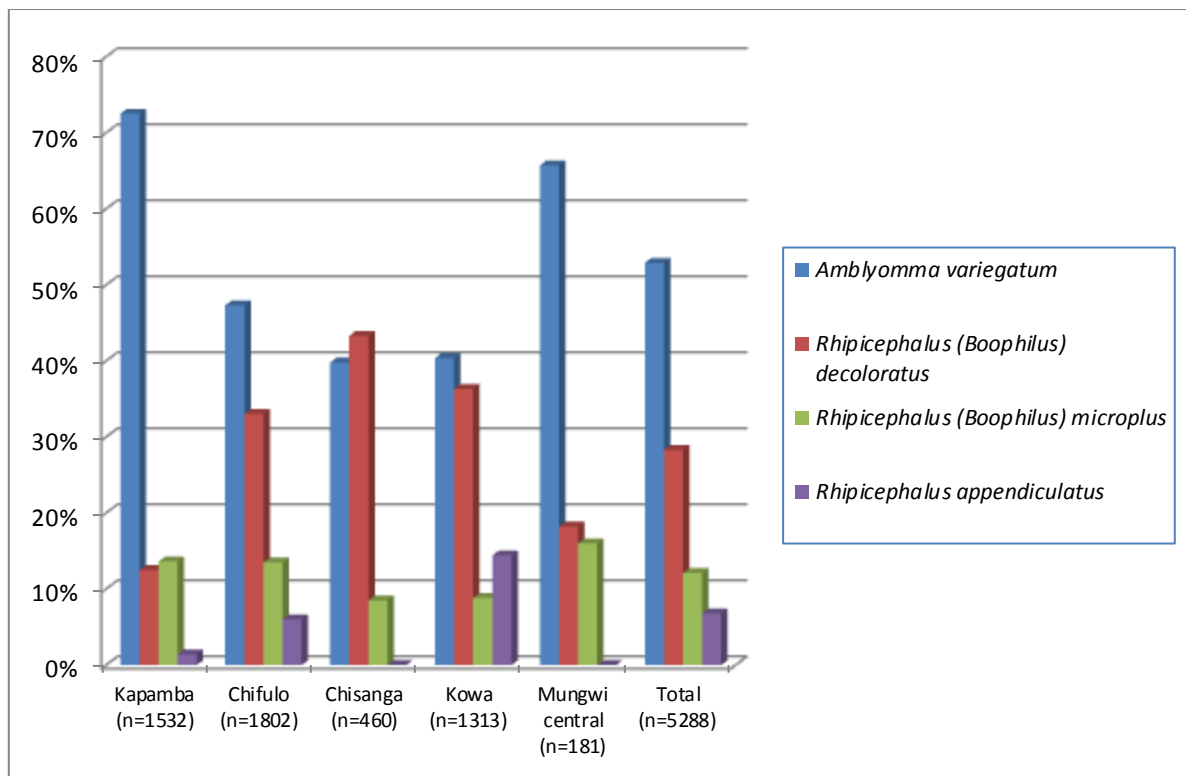


Fig. 7: Tick species identified from the cattle sampled from five areas of Mungwi district, Zambia.

DISCUSSION

In this study, the occurrence of tick-borne haemoprotozoan parasites (*Theileria*, *Babesia*, *Anaplasma*, and *Ehrlichia*) in the local breeds of cattle grazing along the banks of the Chambeshi River in Mungwi, Northern Province, Zambia was determined using the reverse line blot (RLB) hybridization assay. The most prevalent tick species present on cattle were also identified. Results demonstrated the presence of *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species in 86.6% of the samples, either as single (11%) or mixed (75.6%) infections. Some samples (9.4%) tested negative for any of the tick-borne haemoparasites from the four genera. In a number of samples (4%) the PCR products failed to hybridize with any species-specific probes but hybridized only with the genus-specific probes which could suggest the presence of a novel species or variant of a species. The ticks species identified in the study were as expected: *Amblyomma variegatum*, *Rhipicephalus (Boophilus) decoloratus*, *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus appendiculatus* which have been reported previously to be the most important ticks of cattle in Zambia (Pegram et al., 1986).

Theileriosis

Four *Theileria* species are known to occur in Zambia, namely *T. parva*, *T. mutans*, *T. velifera* and *T. taurotragi* (Musisi et al., 1984; Jongejan et al., 1986b). Of these, *T. parva* is the most economically important, causing Corridor disease in the Southern, Central, Lusaka and the Copper-belt provinces, while causing ECF in the Northern and Eastern provinces of Zambia (Nambota et al., 1994; Makala et al., 2003). In our study, nine *Theileria* species were detected of which *T. mutans* and *T. velifera* were the most prevalent and detected in all of the five sampled areas. Interestingly, only one sample from Kapamba tested positive for *T. parva*. This was an unexpected finding since ECF is known to be endemic in the district of Mungwi in the Northern Province of Zambia and the tick vector, *Rhipicephalus appendiculatus*, was identified on animals from Kowa, Chisanga, Chifulo and Kapamba. The only ECF control measure employed in this province is vector control using acaricides; ITM vaccination does not take place. Increased cattle mortalities due to ECF have been observed in the Mungwi district between December to March and May to July and all age groups of cattle are affected (own unpublished observations, 2010).

In 1902 theileriosis was diagnosed in some locations of southern Africa, south of the Zambezi River. The disease originated from Dar es Salaam, Tanzania and was introduced to southern Africa through importations of cattle after the cattle population of much of southern Africa was destroyed by an epidemic of rinderpest (Lawrence, 1992). The first case of theileriosis in Zambia was recorded in 1922 in the Nakonde District, Northern Province (Nambota et al., 1994). In 1946 theileriosis was diagnosed in the Mbala District, Northern Province after which it spread within the Northern Province and is now established enzootically (Dolan, 1988). In the Eastern Province of Zambia, ECF was first diagnosed in 1947 (Nambota et al., 1994). It is currently endemic on a large part of the plateau of the province (Marcotty et al., 2002). In the Southern Province, the disease was first diagnosed in 1977–1978 (Dolan, 1988) and it continues to spread through the Central Province (Minjauw et al., 1998). In the study by Mubamba et al. (2011), it was shown that ECF spread from the eastern districts towards the western districts and that a state of endemic stability in the eastern districts appears to have been reached. Makala et al. (2003) described the *Theileria* epidemiological situation in Zambia as “endemically unstable in parts of the Eastern and Northern provinces” and it is defined by the less favourable climatic conditions for *Rhipicephalus* ticks in this part of its range. This is resulting in complex tick ecology, characterized by one or two tick generations a year and the occurrence of diapause in contrast to a year round presence of ticks in Kenya, Tanzania and Rwanda (Makala et al., 2003).

In a sero-survey done between February and March of 2010 by the smallholder livestock investment programme (SLIP), sponsored by the international food and agricultural development (IFAD), it was shown that 9% of cattle were seropositive for *T. parva* in the Mungwi district, Northern Province. Another tick and sero-survey conducted between February and March 2012 detected 29% *T. parva* sero-positive cattle in Mungwi district (Fandamu & Sinyangwe 2012, personal communication). They observed that ECF antibodies were circulating in the animals and the tick vector (*R. appendiculatus*) was present in Mungwi district. We can only speculate that the RLB hybridization assay may not have been able to detect the parasite in the animals sampled due to a too low parasitaemia. It is also possible that the RLB hybridization assay could have failed to detect *T. parva* due to mixed infection with other *Theileria* species, particularly when the other species occur at high levels compared to *T. parva*. Since the blood samples were collected at a time of very low adult tick activity, suggesting that the animals were not constantly exposed to the parasite, one might expect that the parasite load was, therefore, likely to be too low to be detected by RLB hybridization (Simuunza et al., 2010). The RLB hybridization assay has previously been shown to be able to detect *Theileria* and *Babesia* spp. at a parasitaemia of $1 \times 10^{-6}\%$, enabling detection of the carrier state of most parasites (Gubbels

et al., 1999). However, Bhoora et al. (2010) demonstrated that real-time PCR is more sensitive than the RLB hybridization assay in detecting *T. equi* and *B. caballi* infections and that *Theileria* and *Babesia* spp. infections can be detected by real-time PCR in samples that test negative by the RLB hybridization assay. Also, the *T. parva* specific real-time PCR assay developed by Sibeko et al. (2008) was shown to be able to detect *T. parva* with 100% certainty in carrier animals with a piroplasm parasitaemia as low as $8.79 \times 10^{-4}\%$, and that the real-time PCR assay was 27% more sensitive than the RLB hybridization assay. It is advised that the samples also be subjected to the *T. parva* specific real-time PCR assay to determine a more accurate *T. parva* prevalence in cattle in the Mungwi district, Northern Province, Zambia.

The most prevalent *Theileria* species detected in our study were the benign *T. mutans* (54.5%) and *T. velifera* (51.5%). *Theileria mutans* causes benign bovine theileriosis, is transmitted by different species of *Amblyomma* ticks and occurs in most parts of sub-Saharan Africa (Perie et al., 1979; de Vos and Roos, 1981; Paling et al., 1981; Uilenberg et al., 1982; Musisi et al., 1984; Walker et al., 2003; Lawrence & Williamson, 2004a). Some *T. mutans* strains have been associated with disease in cattle; invasion of the brain capillaries by *T. mutans* can result in a special form of benign bovine theileriosis known as turning sickness (Irvin et al., 1972; Young et al., 1978; Paling et al., 1981; Saidu, 1981; Uilenberg, 1981; Seifert, 1996). *Theileria velifera* is a mild pathogen of cattle (Lawrence & Williamson, 2004b) and was first described from cattle by Uilenberg (1964). It is also transmitted by ticks of the genus *Amblyomma* (Norval et al., 1992). In our study, *Amblyomma variegatum* was found to be the most abundant tick species identified and could explain the positive association in the detection of *T. mutans* and *T. velifera*. Furthermore, the high prevalence of *T. mutans* in our study is in agreement with the findings of Simuunza et al. (2010) who have shown that *T. mutans* was the most prevalent tick-borne pathogen species in the Central, Lusaka and Eastern Provinces of Zambia. It is also in concordance with similar studies in Uganda (Oura et al., 2004) and Sudan (Salih et al., 2007). This would suggest that cattle in these parts of Africa are exposed to a high and continuous *T. mutans* challenge or that *T. mutans* is harboured at detectable levels for long periods post-infection. Alternatively, but less likely, cattle may be more susceptible to *T. mutans* infection than to other tick-borne pathogens (Simuunza et al., 2010).

Theileria taurotragi, a mildly pathogenic species of cattle in Africa, was detected in 11.4% of the cattle sampled in our study. *Theileria taurotragi* was first described from eland (*Taurotragus oryx*) in Kenya (Martin and Brocklesby, 1960), but it has also been shown to infect cattle, sheep and goats (Uilenberg et al., 1982; Stagg et al., 1983). It is transmitted by *R. appendiculatus* and *R. zambeziensis*

(Uilenberg et al., 1982; Lawrence et al., 1983) and has been isolated from cattle together with *T. parva*, *T. annulata*, *T. mutans*, *T. velifera* and *T. buffeli*, from different parts of eastern and southern Africa (De Vos and Roos, 1981; Lawrence et al., 1994b; Oura et al., 2004; Bazarusanga et al., 2007; Salih et al., 2007; Sibeko et al., 2008). Fatal infections have not been reported; infection in cattle is characterized by a transient fever and small numbers of microschorizonts and piroplasms (de Vos and Roos, 1981). In our study, we expected an association in the detection of *T. parva* and *T. taurotragi* as both parasites are transmitted by *R. appendiculatus* and *R. zambeziensis*. That was, however, not the case. *Theileria taurotragi* was detected in Chifulo (4.9%) and Kowa (30.5%), whereas *T. parva* was only detected in one sample from Kapamba. The importance of *T. mutans*, *T. velifera* and *T. taurotragi* in terms of causing morbidity and mortality in cattle in Zambia is unknown.

Other *Theileria* species detected in our study included *Theileria buffeli* (n = 1), *Theileria* sp. (buffalo) (n = 34), *Theileria* sp. (sable) (n = 13), *Theileria* sp. (kudu) (n = 20) and *Theileria equi* (n = 9). We can only speculate whether these are true findings due to incidental infections, or whether they are as a result of cross-reaction of the RLB probes with previously unknown targets or contamination with other target DNA. An incidental infection could occur when a non-host is accidentally infected with a parasite through for example a tick bite. The parasite is usually eliminated by the incidental host, but can sometimes remain in the host for a short while, but it does not cause disease. The presence of previously unrecognized species or variants of existing species (with slightly different 18S genotypes) that might have cross-reacted with the RLB probes could also explain some of these results. Alternatively these results could be due to contamination of one sample with target DNA from another sample, or contamination with PCR products from a previous experiment.

Theileria buffeli has not been reported in Zambia before and our finding warrants further investigation. *Theileria buffeli* forms part of the *Theileria buffeli/sergenti/orientalis* complex, a group of benign *Theileria* species that infect cattle and buffalo in Africa, Australia, Asia, Europe and the United States of America (USA) (Chae et al., 1998; Chansiri et al., 1999; Cossio-Bayugar et al., 2002; Aktas et al., 2007; Altay et al., 2008; M'ghirbi et al., 2008; Gimenez et al., 2009; Chaisi et al., 2011; Mans et al., 2011). It is transmitted by ticks of the genus *Haemophysalis*, but other tick species are possibly involved in the transmission in Africa (Lawrence, 2004).

A total of 11.4% (n = 34) of the samples in our study tested positive for the presence of *Theileria* sp. (buffalo) of which 33 samples were collected from Kowa. In all cases *Theileria* sp. (buffalo) was present in mixed infections with other haemoparasites. *Theileria* sp. (buffalo) has previously only

been identified in buffalo and is genetically related to *T. parva* and other pathogenic *Theileria* species (Chaisi et al., 2011). This parasite does not appear to infect cattle and its vector is not known (Chaisi, 2012). There is no game park or game management area and no buffalo are known to occur in Mungwi district. Based on this, the presence of *Theileria* sp. (buffalo) is unlikely to be a true finding and we speculate that it was due to cross-reaction of the RLB probe or due to contamination with other target DNA.

In 2005 Nijhof et al. reported on fatal cases of theileriosis (cytauxzoonosis) in sable antelope (*Hippotragus niger*), roan antelope (*Hippotragus equinus*) and greater kudu (*Tragelaphus strepsiceros*) in South Africa. The parasites detected were *Theileria* sp. (sable) in sable and roan antelope and *Theileria* sp. (kudu) in the greater kudu. In our study, 13 cattle samples tested positive for the presence of *Theileria* sp. (sable). Nijhof et al. (2005) were also able to detect *Theileria* sp. (sable) DNA in African short-horn cattle from Tanzania as well as from African buffalo, blue wildebeest (*Connochaetes taurinus*), klipspringer (*Oreotragus oreotragus*), and reedbuck (*Redunca arundinum*) from South Africa, and blesbok (*Damaliscus pygargus*) from Swaziland (Nijhof et al., 2005). Our finding is very likely to be a true finding as the Mungwi district is considered a “transit district” for cattle; cattle are moved from districts close to the border of Tanzania to markets in the provincial capital of Kasama. Illegal movement of cattle could also play an important role in the spread of *Theileria* sp. (sable) into Northern Zambia. We also detected *Theileria* sp. (kudu) in 20 of the samples tested, all from Kowa. *Theileria* sp. (kudu) is not known to be pathogenic to domestic animals and the tick vector for this parasite is yet unknown. Our finding could thus well be due to a cross-reaction with other *Theileria* species and/or due to contamination with other target DNA.

There was an unexpected finding of nine samples testing positive for the presence of *Theileria equi* DNA. Equine piroplasmosis is a disease of horses caused by *T. equi* and *Babesia caballi*. The disease is endemic in many tropical and subtropical areas and is transmitted by ticks of the genera *Rhipicephalus*, *Rhipicephalus (Boophilus)*, *Hyalomma* and *Dermacentor*. Little is known about the occurrence of *T. equi* and *B. caballi* in Zambia. *Hyalomma truncatum* is present in Northern Zambia and can transmit *B. caballi* to horses (Walker et al., 2003). However, this tick species was not present from ticks collected in this study. Apart from detecting *T. equi* in our sample set, we also detected *B. caballi* in 16 of the samples tested. There are no horses in the Mungwi district; however, since there are some donkeys present in the area, we speculate that our finding could have resulted due to an incidental infection.

Babesiosis

Babesia bovis and *B. bigemina* are currently present in all the Zambian provinces (McCosker, 1981) and are recognized as being of economic importance in cattle and small ruminants (Luguru, 1985; Pegram et al., 1989; Pegram and Banda, 1990). In our study, we have shown that *B. bovis* was present in 7.7% of the sampled animals and *B. bigemina* in 3.3% of the animals. We detected *B. bovis* in all of the five sampled areas with the highest detection in Mungwi central (14.3%) and Kowa (10.5%). As expected, *Rhipicephalus (Boophilus) microplus* was identified from animals from all of these areas. *Babesia bigemina* was only reported from Kowa (10.5%). The most abundant ticks identified from the sampled animals from Kowa were *Rhipicephalus (Boophilus) decoloratus* (36.3%) and *Rhipicephalus (Boophilus) microplus* (8.8%). Our findings were in concordance with the results obtained by Simuunza et al. (2010). In their study, the prevalence of *B. bovis* detected in the Lusaka and Central provinces of Zambia in the wet and dry seasons was higher than that observed by Jongejan et al. (1988). The authors speculated that an increase in prevalence detected may indicate that *B. bovis* is becoming endemic in this part of the country. This could be due to uncontrolled movement of cattle that frequently occurs within Zambia. In contrast to *B. bovis*, prevalence of *B. bigemina* was close to zero in the Lusaka, Central and Eastern Provinces during the dry season but this increased to over 10% in the wet season. The authors proposed that *B. bigemina* is efficiently transmitted to susceptible cattle in Zambia in the wet season, and the ensuing parasitaemia is cleared by the immune system to levels below the detection limit of PCR during the dry season (Simuunza et al., 2010). However, our findings are in contrast to that of Jongejan et al. (1988) who reported that *B. bigemina* occurred throughout Zambia and that the infection caused by *B. bigemina* was more extensive than that caused by *B. bovis* as *B. bigemina* has a wider vector range (Makala et al., 2003). Also, in a recent study by Iseki et al. (2007) it was found that the rate of *B. bigemina* infection (23.3%) in Zambia was substantially higher than that of *B. bovis* infection (14.4%) using a multiplex loop-mediated isothermal amplification (mLAMP) assay. They also reported the mixed *B. bovis* and *B. bigemina* infection rates in cattle in Zambia to be 4.4%. Although they showed that the newly developed mLAMP assay had a higher sensitivity than the conventional PCR and nested PCR methods that they compared it with, the sensitivities of the RLB hybridization assay and mLAMP have not been compared.

Previously, *Rhipicephalus (Boophilus) decoloratus* was reported to be widespread throughout the country, whereas *Rhipicephalus (Boophilus) microplus* has only been reported from the north-eastern region (MacLeod and Mwanaumo, 1978; Norval et al., 1983). Since 1957, sporadic cases of *B. bovis* have been recorded in the annual reports of the Department of Veterinary and Tsetse

Control Services, Lusaka, all being confined to the north-eastern part of the country (Jongejan et al., 1986a). In 1986 Jongejan et al. reported on two cases of *B. bovis* infection from Central and Lusaka provinces of Zambia, which were considerable distances away from the main foci of infection. These were based on serological results, the morphology of the parasites and clinical and post-mortem findings. This was also the first record of *Rhipicephalus (Boophilus) microplus* being a vector of *B. bovis* in this part of Zambia and the authors speculated that it was possible that *Rhipicephalus (Boophilus) microplus* ticks infected with *B. bovis* were occasionally introduced into central Zambia with tick infested cattle from the north-eastern part of the country, where *B. bovis* was endemic (Jongejan et al., 1986a). Since then, *B. bovis* and *B. bigemina* have been reported to be present in all of the provinces of Zambia (McCosker, 1981). There is full endemic stability for *Babesia bigemina*.

In our study we also found that 37% of samples from Chifulo tested positive for the presence of *Babesia* sp. (sable). Oosthuizen et al. (2008) identified a novel piroplasm parasite from a sable antelope (*Hippotragus niger*) that died from an unknown illness and described it as *Babesia* sp. (sable). While the parasite was observed in blood smears, there was no direct evidence that it was the cause of death. The tick vector of this parasite also remains unknown. In a study done by Munang'andu et al. (2011), *Babesia* parasites were detected in puku (*Kobus vordanni*) in Zambia and the authors suggested that wildlife may have a role to play in the epidemiology of babesiosis in Zambia. However, as there is no game park or game management area in the Mungwi district, the presence of *Babesia* sp. (sable) is unlikely to be a true finding and we speculate that it was due to cross-reaction of the RLB probe or due to contamination with other target DNA.

Furthermore we found that 12.7% of the sampled animals tested positive for the presence of *Babesia gibsoni* and 1% for *Ehrlichia canis*. Both these are dog parasites transmitted by *Rhipicephalus sanguineus* (Walker et al., 2003; Boozer & Macintire, 2005). These parasites have not been reported in cattle before and were probably an accidental infection or false positive result. Nalubamba et al. (2011) reported that the prevalence of *B. gibsoni* in dogs in Zambia was low compared to that reported in other African countries. Although dogs usually patronize the cattle grazing areas and kraals, no *Rhipicephalus sanguineus* ticks were identified on the sampled cattle. We therefore speculate that our finding may have resulted due to cross-reaction of the RLB probe or due to contamination with other target DNA.

Ehrlichiosis

In Zambia *Ehrlichia ruminantium* causes heartwater mainly in cattle, although outbreaks in sheep and goats have been reported and recorded; *Amblyomma hebraeum* and especially *A. variegatum* are the main vectors (Makala et al., 2003). In our study, only one sample (from Kapamba) tested positive for the presence of *E. ruminantium* even though *A. variegatum* ticks were identified from 52.9% of the sampled animals from all study areas. Records of the Central Veterinary Research Institute (CVRI) for the period 1986–1997 revealed that the disease occurred throughout Zambia (Mangani, 1997; Makala et al., 2003). The disease is believed to be responsible for numerous deaths occurring throughout the year, but especially during the rainy season from March to September. The disease is mainly seen in areas where regularly dipped animals are in close proximity to indigenous kept cattle with no acaricidal treatment and also where game is frequently seen in cattle grazing areas. Heartwater also occurs in commercial farms when they have relaxed their normal tick control practices (Makala et al., 2003). The cattle sampled in our study are not regularly dipped and no game has been spotted in cattle grazing areas. It is also possible that a state of endemic stability to heartwater may have been attained in this area. One would also speculate that the RLB hybridization assay may not have been sensitive enough to detect *E. ruminantium* infections if the parasitaemia was very low. It is advised that the samples also be subjected to the *E. ruminantium*-specific pCS20 real-time PCR assay (Steyn et al., 2008) to determine more accurately the *E. ruminantium* prevalence in cattle in the Mungwi district, Northern Province.

Ehrlichia chaffeensis was also detected in 12 samples from Kowa (12.6%). The most commonly reported pathogens in the United States causing ehrlichiosis in humans are *E. chaffeensis* and *Anaplasma phagocytophilum*. *Ehrlichia chaffeensis* causes a monocytic form of illness that is referred to as human monocytic ehrlichiosis (HME) (McQuiston et al., 1999). *Ehrlichia chaffeensis* infection has also been detected in dogs (Kordick et al., 1999) and in domestic goats in the United States, but clinical illness in these animals has not been reported (Dugan et al., 2000). *Ehrlichia chaffeensis* is transmitted among reservoir species (white-tailed deer) and to accidental hosts such as humans and dogs by *Amblyomma americanum* (lone star tick) which is distributed throughout the southeastern and south-central United States (Wolf et al., 2000; Childs and Paddock, 2003). Based on this, we are confident that our finding is due to cross-reaction of the RLB probe and/or due to contamination with other target DNA from another sample. *Ehrlichia chaffeensis*, *E. canis* and *E. ruminantium* RLB probes have previously been shown to be specific for these species. However, a close phylogenetic relationship exists between *E. chaffeensis*, *E. canis* and *E. ruminantium* (Dumler et al., 2001; Van Heerden et al., 2004) which may explain why the RLB probe could cross react.

Anaplasmosis

Anaplasma marginale is present in all the provinces of Zambia (Jongejan et al., 1988) and is the only *Anaplasma* species of importance to cattle in Zambia (McCosker, 1981). It is transmitted by *Amblyomma variegatum*, however, there is an overlapping distribution of *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus evertsi evertsi*, which also act as vectors (Friedhoff, 1997). Mechanical transmission by biting arthropods (biting flies) also occurs and is considered to be important as well (Makala et al., 2003). In endemic areas, the majority of the native *Bos indicus* and sanga type cattle are probably exposed to *A. marginale* infections, but do not develop overt disease. This is partly due to the existence of a state of enzootic stability, whereby the cattle become naturally infected at an early age, when there is significant passively acquired and innate immunity and are immune to challenge later in life (Makala et al., 2003). Our study revealed that 25.7% of the sampled cattle were positive for *A. marginale*; it was detected in all areas except Chisanga. *Amblyomma variegatum* was identified from 52.9% of the sampled cattle, and *Rhipicephalus (Boophilus) microplus* from 12.1% of the cattle. Since 28.3% of the sampled cattle also carried the *Rhipicephalus (Boophilus) decoloratus* ticks, we could speculate that this tick species not only transmitted *Babesia bigemina* but also *A. marginale* (Walker et al., 2003). Furthermore, three samples (from Kowa) tested positive for the presence of *A. centrale*. To our knowledge, no vaccination using *A. centrale* is being conducted in the Mungwi district of Zambia. The presence of *A. centrale* is, therefore, an interesting finding. In a study done by Oura et al. (2010), buffalo from Lake Mburo National Park in Uganda were found to be carriers of, amongst several *Theileia* spp., also *A. marginale* and *A. centrale*, using the RLB assay, indicating that buffalo have the potential to spread these parasites to cattle. Impala were found to carry only *A. centrale* and the authors speculated that since *A. centrale* was not identified in cattle in Uganda (Oura et al., 2004), it is possible that the *A. centrale*, found in both buffalo and impala, is unable to transmit to cattle (Oura et al., 2010).

In this study, 7.3% of the samples tested positive for *Anaplasma (Ehrlichia)* sp. Omatjenne. *Anaplasma (Ehrlichia)* sp. Omatjenne was described by Du Plessis (1990) as an apathogenic *Ehrlichia*-like parasite, which was isolated from a *Hyalomma truncatum* tick from Omatjenne in the Otjiwarongo district of Namibia. This is an area free of *Amblyomma* ticks and thus free of *Ehrlichia ruminantium*. However, a strain of *Anaplasma (Ehrlichia)* sp. Omatjenne has been shown experimentally to produce disease indistinguishable from heartwater in sheep (Du Plessis, 1990). There is evidence that *Hyalomma truncatum* ticks are present in the Northern Province of Zambia (Walker et al., 2003). However, *Hyalomma truncatum* ticks were not identified in our study.

Anaplasma bovis (formerly *Ehrlichia bovis*) is mainly detected in cattle, but has also been observed in small mammals which probably act as reservoir of this bacterium (Goethert and Telford, 2003). The symptoms of the disease are most visible in calves, but also in adult animals and include weakening of the body, marked reduction in weight, elevated temperature, enlargement of prescapular lymph nodes, paling of the mucous membranes, and in many cases an elevated amount of secreted mucus (Uilenberg, 1997; Santos and Carvalho, 2006). *Anaplasma bovis* has been detected in Brazil, North America, Africa (West, Central and Southern Africa) and Japan (Goethert and Telford, 2003; Kawahara et al., 2006; Santos and Carvalho, 2006). It is transmitted by *R. appendiculatus*, *A. variegatum* and ticks of the genus *Haemaphysalis* (Rymaszewska and Grenda, 2008). In our study, two samples (0.67%) tested positive for *A. bovis* and these were from Kapamba and Kowa.

Recommendation(s)

Livestock farmers in the Mungwi district, Northern Province, Zambia currently use vector control strategies (i.e. using acaricides) and chemotherapy to control TBDs. This is, however, very costly to the small scale farmer. The most cost effective way to control TBDs in cattle in communal herds is by maintaining a state of endemic stability. This can be achieved by maintaining a continuously low parasite challenge by not dipping intensively or applying strategic dipping regimes.

The Zambian Government should subsidize the cost of these veterinary inputs for the small scale livestock farmers as it does for agricultural inputs for crop production. They should also establish more infrastructure such as livestock service centres (dip tanks or spray races and crush pens) to be able to enforce the new regulation on compulsory dipping of cattle. Establishment of permanent veterinary check points will improve the control of illegal cattle movements and prevent spread of cattle diseases. The government should provide adequate and timely logistics to facilitate the continuous monitoring of livestock disease epidemiology to reduce the spread of diseases.

CONCLUSIONS

The Zambian environment and climatic conditions represent a multi-tick species vector ecology (Pegram et al., 1986). In our study, we have shown that mixed infections of different tick-borne pathogens in host populations is common. Interactions between these pathogens could affect the outcome of therapeutic intervention and may influence the severity of disease (Makala et al., 2003). The major tick-borne haemoparasites detected in Mungwi District of Northern Zambia using the RLB hybridization assay were *Theileria mutans*, *T. velifera*, *Anaplasma marginale*, *Babesia bovis* and *B. bigemina*. The tick species identified confirmed the report by Pegram et al. (1986) that the most important ticks of cattle in Zambia are *Rhipicephalus appendiculatus*, *Rhipicephalus (Boophilus) microplus*, *Rhipicephalus (Boophilus) decoloratus* and *Amblyomma variegatum*.

The results of our study also suggest that the cause of cattle mortalities in Mungwi during the winter outbreaks is mainly due to *A. marginale*, *B. bovis* and *B. bigemina* infections. This could be confirmed by the results of the RLB hybridization assay, clinical manifestation of the disease in the affected cattle (own observation) and the tick species identified on the animals. It appears that in Mungwi, babesiosis due to *B. bovis* mostly infects cattle above one year of age. Calves appear to be less affected by *Babesia bovis* infection.

Collectively, the relatively low prevalence of *T. parva*, *B. bigemina*, *B. bovis* and *E. ruminantium* found in our study could be indicative of the existence of a large pool of susceptible cattle with the potential for generation of a state of instable endemicity and the occurrence of disease outbreaks. In contrast the high prevalence of *T. mutans*, *T. velifera* and *A. marginale* could indicate endemic stability. The high prevalence of *T. mutans* compared to the other pathogens was in agreement with similar studies in Uganda (Oura et al., 2004) and Sudan (Salih et al., 2007), indicating that cattle in these parts of Africa are exposed to a high and continuous challenge or that this species is harboured at detectable levels for long periods post-infection (Simuunza et al., 2010). Alternatively, cattle may be more susceptible to *T. mutans* (and *T. velifera*) infection than the other TBD pathogens investigated in this study.

There is need for further epidemiological surveys in the Mungwi district, Northern Province, Zambia, using more specific and sensitive diagnostic tools or assays, to get a better understanding of the epidemiology of these tick-borne haemoparasites affecting cattle. We conclude that integrated control policies should be developed to take account of multi-species pathogen communities that are commonly associated with clinical and sub-clinical TBD infections in Zambia.

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