Occurrence of *Ehrlichia ruminantium* and *Rickettsia africae* in *Amblyomma variegatum* ticks in selected regions of Zambia

By

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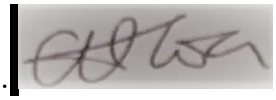
Declaration

1. I understand what plagiarism is and am aware of the University's policy in this regard.
2. I declare that this dissertation is my own original work. Where other people's work has been used, it has been properly acknowledged and referenced in accordance with the departmental requirements.

Signature: ……  ……

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Dedication

I dedicate this work to my mother, Bessie, my husband, Ernest and my children, Mapalo and Kasonde.

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List of Abbreviations

ATBF	African tick bite fever
DNA	Deoxyribonucleic acid
dd	doubly distilled
DVTD	Department of Veterinary Tropical Diseases
DVS	Department of Veterinary Services
ECF	East Coast fever
GPS	Global Positioning System
ng	nanograms
ompA	outer membrane protein A
PCR	Polymerase Chain Reaction
rpm	revolutions per minute
TAD	Transboundary Animal Diseases
UP	University of Pretoria

Abstract

This study was conducted in Zambia and the main objective was to determine the prevalence of *Ehrlichia ruminantium* and *Rickettsia africae* in adult *Amblyomma variegatum* ticks in selected regions of Zambia. A total of 567 adult *A. variegatum* ticks were collected from three regions of Zambia (eastern, central and western), following an east-west transect across the country. Ticks were most abundant in the western region, less abundant in the eastern region, and the lowest abundance was found in the central region. Ticks were identified morphologically using a stereoscopic microscope. In all three regions, only *A. variegatum* was found. The ticks' infection rates of *E. ruminantium* and *R. africae* were determined using polymerase chain reaction (PCR) targeting pCS20 fragment for *E. ruminantium*, and *ompA* gene for *R. africae*. Overall prevalence of *E. ruminantium* was determined to be 18.52% (9.4% western, 35.4% eastern, and 18.8% central regions). The overall prevalence of *R. africae* was found to be 36.07% (23.3% western, 56.1% eastern and 41.7% central regions).

The presence of the vector and its associated disease pathogens is indicative of the occurrence of heartwater and African tick bite fever (ATBF) in Zambia, with the prevalence of both pathogens probably being underestimated before, and not accurately associated with disease and/or mortality in livestock and humans, respectively. In light of the results obtained in the present study, it would be advisable for measures to be put in place by veterinary and medical authorities in order to appropriately address these important issues. Given the veterinary importance of heartwater and the medical pertinence of ATBF, this seems to be a perfect example, where a One Health framework approach may produce synergic beneficial results.

KEY WORDS: Heartwater, *Amblyomma*, *Ehrlichia ruminantium, Rickettsia africae*, ticks, Zambia.

CHAPTER ONE: INTRODUCTION

Livestock production is very important not only for the livelihoods of the populations but also contributes greatly to the economy of the country (Randela, 2007, Lubungu and Mofya-Mukuka, 2012). In Zambia livestock production has huge potential of contributing towards a country's economic growth and help with poverty reduction, but animal diseases have continued to be a constraint (Lubungu and Mofya, 2012). Diseases transmitted by ticks (tick-borne diseases) are some of the major constraints for livestock production in many developing countries like Zambia (Simuunza et al., 2011).

Tick-borne diseases (TBDs) prevent the introduction of highly productive breeds of cattle to areas where they occur (Kivaria, 2006). Tick borne diseases do not only cause losses through morbidity and mortality, but they also cause a reduction in the production of meat, milk, animal draught power and other animal products. Besides their role of disease transmission, ticks cause physical damage, including injury to hides and loss of blood as they feed (Smith et al., 1991, Rajput et al., 2006).

Theileriosis caused by *Theileria parva*, babesiosis caused by *Babesia bovis* and *Babesia bigemina*, anaplasmosis caused by *Anaplasma marginale* and heartwater caused by *Ehrlichia ruminantium* are TBDs of great importance in sub-saharan Africa (Makala et al., 2003).

Heartwater or ehrlichiosis (formerly known as cowdriosis) is a disease that is caused by an obligate intracellular bacterium, *Ehrlichia ruminantium*, and transmission is by ixodid ticks, predominantly of the genus *Amblyomma*, and this disease affects livestock such as cattle, sheep, goats and some wild ruminants (Allsopp, 2009). According to Allsopp (2009) the disease distribution in Africa is dependent on that of the vector ticks. There are a number of known *Amblyomma* species in Africa that have the ability to transmit the organism, but the most important and predominant vectors are *Amblyomma variegatum* and *Amblyomma hebraeum* (Bezuidenhout, 1987). *Amblyomma hebraeum* is the major vector of heartwater in southern Africa, while *A. variegatum* is the vector that is most widely distributed in Africa, including Madagascar. It has also spread to the Caribbean and it is a vector of the disease on three 3 islands namely; Guadeloupe, Antigua and Marie Galante (Molia et al., 2008).

In Zambia, all the major TBDs, theileriosis (East Coast fever/Corridor disease), heartwater, babesiosis and anaplasmosis are present (Tembo et al., 2018). However, East Coast fever (ECF

=*Theileria parva* infection) receives the most attention, thus neglecting the other TBDs such as heartwater, which is arguably also of significant economic importance. East Coast Fever has been contained through infection and treatment method (ITM) which is done in the areas where the disease occurs (Marcotty et al., 2002), but livestock have continued dying which could be hypothesized to be from TBDs such as heartwater. Heartwater has a high mortality rate of up to 82% in adult cattle (Prozesky and Du Plessis, 1987). In Zambia heartwater mainly affects cattle (Makala et al., 2003), but there have been some reports of the disease in small ruminants (goats and sheep). Heartwater is very difficult to diagnose in live animals based on history and clinical signs. A definitive diagnosis is confirmed by identification of heartwater colonies in the capillary endothelial cells of a brain crush smear, which can only be done after an animal has died. It is very easy to miss a number of cases because of lack of definitive diagnosis of the disease in live animals.

In the livestock farming areas of Zambia, the only vector of heartwater is *Amblyomma variegatum* (Makala et al., 2003). *Amblyomma variegatum* is found in most parts of the country with a single generation occurring per year (Pegram et al., 1989). In addition to *E. ruminantium*, *A. variegatum* also transmits *Rickettsia africae*, the causative agent of African tick bite fever in humans, which makes it of public health importance. African tick bite fever is not well documented in Zambia.

Epidemiological surveillance studies of disease pathogens in ticks are rarely done in Zambia, thus the aim of this study was to establish the prevalence of *Ehrlichia ruminantium* and *Rickettsia africae* in *Amblyomma variegatum* from different regions of Zambia, which in turn will help support the future veterinary and public health interventions.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

Tick borne diseases (TBDs) are of great significance not only in Zambia but also in many parts of Africa such as the eastern, central and southern Africa regions and the world at large (Makala et al., 2003). Ticks do not only affect the livestock production activities, they also cause high morbidity and mortality and require a large financial input to control them (Hurtado and Giraldo-Ríos, 2018). Although there are several tick genera in the world (Walker, 2003), in this particular study, interest is limited to the genus *Amblyomma.*

Amblyomma variegatum is known to transmit disease-causing pathogens such as *Ehrlichia ruminantium* which causes heartwater (ehrlichiosis) in ruminants, as well as *Rickettsia africae* which causes African tick bite fever in humans. This tick has great significance in the veterinary and public health sectors because it transmits disease to both animals and humans.

2.2 Genus *Amblyomma*

Amblyomma ticks are ixodid ticks belonging to the class Arachnida, order Acari, sub-order Ixodida, family *Ixodidae*, subfamily *Amblyomminae* and genus *Amblyomma* (Walker, 2003). The ticks in the genus *Amblyomma* are three-host ticks, which means that each life cycle stage of the tick leaves the host after engorgement of that stage (i.e. larval, nymphal and adult stage). Host species in each stage may vary, however, the same host species may be used by all three life cycle stages. Walker et al, (2003), describes the ticks in this genus as follows: "the unfed ticks being large ranging between 6 to 7mm in size, with long mouth parts that are placed in the anterior position, the legs are slender and have pale rings (banded). They have a scutum which has some coloration, the eyes may be flat or convex and may be difficult to see sometimes, and they have festoons which may be invisible in engorged females."

The genus *Amblyomma* has about 126 species in the world (Volcit and Keirans, 2009). The African *Amblyomma* species that are known to be vectors of heartwater are: *A. variegatum*, *A. hebraeum*, *A. pomposum*, *A. gemma*, *A. lepidum*, *A. tholloni*, *A. sparsum*, *A. astrion*, *A. cohaerens*, and *A. marmoreum* (Uilenberg, 1983).

In Zambia the most prevalent species of the genus *Amblyomma* present on cattle is *Amblyomma variegatum* and it is found throughout the country (Makala et al., 2003).

2.3 *Amblyomma variegatum*

Amblyomma variegatum are ixodid ticks with long and strong mouthparts, they have convex eyes and well-developed festoons on the dorsal-caudal region. *Amblyomma variegatum* is similar in appearance to *A. lepidum* and *A. pomposum*, however *A. variegatum* is differentiated by the presence of small to medium punctations, and the festoons of the male ticks do not have enamel (Walker, 2003). This species is amongst the most widely distributed tick on livestock in Africa (Walker, 2003).

Amblyomma variegatum is widely distributed in central, western, north-eastern, eastern and southern Africa, the tick is also found in Zambia, north-eastern Botswana, Caprivi Strip of Namibia, north-western Zimbabwe and the northern and central parts of Mozambique (Madder et al., 2014) .

Amblyomma variegatum is a three-host tick. The three life stages namely, larva, nymph and adult, need to take a blood meal on the host before they drop to the ground to molt (Barré et al., 1995). The different life stages may feed on similar or different hosts depending on which host is available at a particular time. The adult males are the ones that attach first to the host, females only attach after a few days (Barré and Garris, 1990). The mature male ticks produce pheromones that attract females, other males and nymphs to attach to the host. After engorgement the females drop to the ground and lay eggs. The hatching of the eggs may take 50 to 100 days depending on the environmental temperature, with warm weather resulting in early hatching, the larvae stay in or on the vegetation in search of, or questing, for hosts (Pegram and Banda, 1990).

The adults are usually most abundant during the rainy season while the immature stages are more abundant during the dry season (Muir). In Zambia, the adults are in abundance during the wetter months (i.e. October to February), while in Zimbabwe there is a possibility of adults being present all year round, with an increase in numbers during the months when temperatures are on the higher side , i.e. September to May (Walker, 2003). It usually takes one year to complete the life cycle.

Adult ticks prefer to feed on domesticated animals such as cattle, sheep, goats, and wild ruminants. Immature stages also feed on the same hosts as the adults but they feed in greater numbers on smaller mammals and birds which include the mongoose and cattle egret (Pegram and Eddy, 2002).

Amblyomma variegatum species is important because it transmits various disease pathogens such as *Ehrlichia ruminantium* which causes heartwater in livestock (Allsopp, 2009). The tick transmits *Theileria mutans* and *Theileria velifera* that causes benign bovine theileriosis and *Anaplasma bovis* which causes bovine ehrlichiosis (Radostits et al., 2006) . *Amblyomma. variegatum* is associated with *Dermatophilus congolensis* which causes acute bovine dermatophilosis (Madder et al., 2010). It is also of public health importance because it transmits *Rickettsia africae,* which causes African tick bite fever in humans

2.4 Rickettsial pathogens of *Amblyomma variegatum* ticks: *E. ruminantium* and *R. africae*

2.4.1 *Ehrlichia ruminantium*

Ehrlichia ruminantium is a rickettsial organism that causes heartwater, a fatal disease of domestic ruminants in sub-Saharan Africa and the eastern Caribbean (Peter et al., 2002). *Ehrlichia ruminantium* is transmitted predominantly by the ticks of the genus *Amblyomma*. The ticks acquire the pathogen as they feed on infected animals and transmit it as they feed on susceptible hosts. Transmission of heartwater is transstadial, the ticks become infected with the pathogen as larvae or nymphs and transmit it to susceptible hosts during their next blood meal as nymphs or adults, respectively (Bezuidenhout, 1987). There are a number of *Amblyomma* species known to have the capacity to transmit *E. ruminantium* and in Africa it has been reported that disease occurs where the two most important tick species are present, *A. hebraeum* and *A. variegatum*, (Allsopp, 2015) and *A. variegatum* is the most widely distributed species in Africa (Walker and Olwage, 1987).

Both domestic and wild ruminants are susceptible to infection with heartwater. The disease occurs where the vectors are present, mainly in Africa. The disease is endemic in most parts of sub-Saharan Africa and the islands of Mauritius, Reunion, Madagascar and Grande Comore (Provost and Bezuidenhout, 1987).

Heartwater can be maintained in domestic ruminants (Uilenberg, 1983), but there are several wild ruminants which are reservoirs of the disease pathogen (Allsopp, 2010). The incubation period of heartwater varies between species of animals affected and is also dependent on the virulence and amount of infective agent inoculated into an animal (Uilenberg, 1983), with an average of 18 days and 14 days in naturally infected cattle and small ruminants, respectively. Clinical signs of heartwater may not be limited to fever, respiratory distress, inappetence and anorexia. Nervous signs are usually seen in animals with peracute and acute forms and these may be confused with

signs of plant toxicity, rabies, heavy metal and acaricide poisoning (Van de Pypekamp and Prozesky, 1987).

Heartwater disease is of great importance wherever it occurs, it hinders farmers from improving their livestock by introduction of high-producing breeds (exotics) as they are more susceptible to the disease compared to the local breeds (Allsopp, 2015). The losses caused by heartwater can not only be observed through animal production loss, but they can also be through costs involved in preventing and controlling the disease, such as the cost of purchasing acaricides and drugs, and engaging veterinarians.

Heartwater control has been achieved primarily through the use of acaricides, which are administered to the animals by various methods to kill the vector ticks. However, this method has proved to be very costly making it difficult for livestock farmers to comply (Meltzer *et al*., 1996) and acaricide resistance is of concern. There is a blood-based vaccine that was developed to help with the prevention of heartwater, but its use has been limited in Africa because amongst many other limitations, it requires a strict cold chain for maintenance (Allsopp, 2009). The methods of prevention available are expensive, especially for developing countries with limited resources, thus calling for development of cheaper and more sustainable methods of control.

2.4.2 *Rickettsia africae*

Rickettsia africae is a recently discovered spotted fever group rickettsia that causes African tick bite fever (ATBF) in humans, a disease that is predominantly transmitted by ixodid ticks belonging to the genus *Amblyomma* in the sub-saharan region of Africa and the West Indies (Jensenius et al., 2003a). There are two main vectors of *R. africae*, namely *A. variegatum* and *A. hebraeum* (Kelly et al., 1996). The two vectors are also considered to be reservoir hosts because of their ability to maintain the parasite and transmit it transovarially and transstadially (Kelly and Mason, 1991).

The distribution of *R. africae* coincides with that of *A. hebraeum* and *A. variegatum*, a fact that is supported by the finding that the parasite has been detected in areas where the vector *Amblyomma* species are present (Ledger et al., 2022). *Rickettsia africae* has been detected in a number of countries in Africa, including Botswana, South Africa, Zimbabwe, Mozambique, Tanzania, Kenya, Burundi, Sudan, Ethiopia, Central African Republic, Mali, Gabon, Niger, Gambia and Ivory Coast (Jensenius et al., 2003a). Reports of ATBF in indigenous people are very few, even though there is a wide distribution of *R. africae* in Africa. These few reports could be attributed to

the fact that indigenous people usually get infected when they are young and the disease may be asymptomatic, thus patients never go to the hospitals for treatment (Julvez et al., 1997). Another reason for fewer reports of the disease in indigenous people could be that the inoculation site (eschar) is usually less noticeable on dark skin and diagnostic techniques of the disease are not readily available in Africa. African tick bite fever mostly affects international travelers from Europe and other continents coming to Africa for tourism, schooling or other activities. There is one case of seven patients (international travelers) who had travelled to southern Africa and presented with high fever when they returned to Europe. It was confirmed to be ATBF through clinical findings and confirmation of the disease pathogens in the endothelial cells (Brouqui et al., 1997). This finding is proof that travelers may get infected with ATBF when they visit Africa. Clinical signs of ATBF include headache, fever, muscle pain, rash and lymphadenopathy. There could be a red sore that develops a dark crust at the tick bite site known as an eschar. Currently there is no vaccine available to prevent the disease, the only way to prevent it is through tick control.

CHAPTER THREE: METHODOLOGY

This study was done in three regions of Zambia. Zambia, in the southern region of Africa, is a landlocked country surrounded by eight countries, namely Angola to the west, Malawi to the east, Mozambique to the southeast, Democratic Republic of Congo to the north, Namibia to the southwest, Tanzania to the northeast, Zimbabwe and Botswana to the south. Zambia has a total area of 752, 614 km^2 of which rivers and lakes cover 11,890 km^2.

3.1 Tick collection

3.1.1 Study sites and sampling

The officers from the Department of Veterinary Services (DVS) in the various districts identified the villages from which samples were collected. Villages were purposely selected, taking into consideration the availability of cattle and accessibility of the areas. The project was explained to the farmers and consent forms were signed before collection of ticks from the animals (see appendix F).

Adult ticks were collected from the bodies of restrained cattle, paying attention to the preferred sites of feeding of *Amblyomma* ticks, in particular: the udder, groin, dewlap and around the perineum. The ticks were grouped according to villages and were preserved in 70% ethanol.

This project was carried out after obtaining approval from the Research Ethics Committee of the University of Pretoria (REC189-21; see appendix C), as well as the Animal Ethics committee (AEC; see appendix A). Permission to conduct the study was sought from the Department of Agriculture, Land Reform and Rural Development of the Republic of South Africa (section 20: Reference no. 12/11/1/1/8/2286(HP); see appendix B). The Department of Veterinary Services (DVS) in the Ministry of Fisheries and Livestock of the government of Zambia granted permission for collection of ticks from cattle (see appendix D). The ticks were couriered to South Africa (permit no. 13/1/1/30/2/0-202204001937; see appendix E) and quarantined at Tropical Animal Diseases (TAD) for 14 days before being taken to the University of Pretoria, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort campus.

3.2 Sampling and data collection

Villages were selected by officers from DVS based on accessibility and willingness of the farmers to participate in the study. Animals observed to have *Amblyomma* ticks on their bodies were

conveniently selected from the herd. Information about the village name, GPS coordinates, date and name of district were recorded on data sheets.

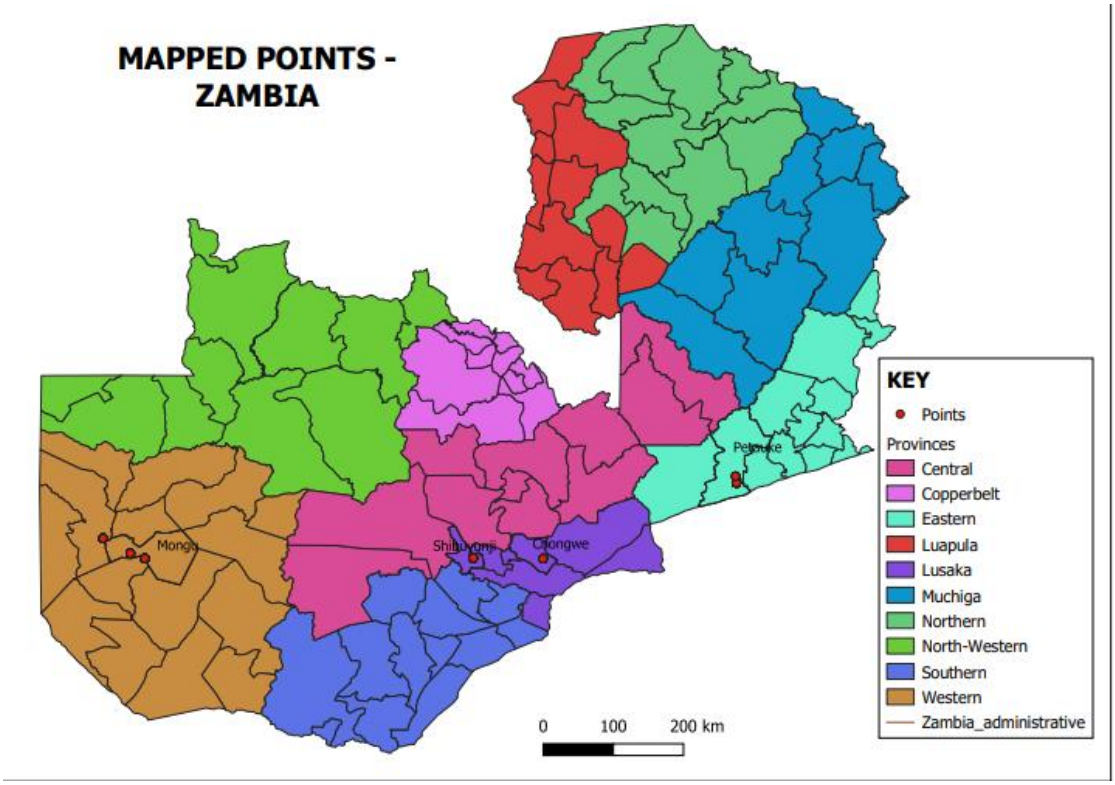

Figure 1: Map of Zambia showing the sampling sites as red dots.

3.3 Identification of ticks

The ticks were identified microscopically at the Department of Veterinary Tropical Diseases (DVTD) research and training laboratory by use of a stereomicroscope and entomological keys as described by Walker et al. (2003). Figure 2 shows images of *A. variegatum* (male and female)

Figure 2*:* photograph illustrating the morphological characteristics of *A. variegatum* (left-female, right- male) obtained from: ***commons.wikimedia.org/wiki/file:Amblyomma-variegatum-female-male-dorsal.jpg***

3.4 Molecular detection of *Ehrlichia ruminantium* and *Rickettsia africae*

DNA was extracted from the collected ticks using the Chelex 100 resin insect DNA extraction protocol: A tick was taken from the 70% ethanol rinsed off with doubly distilled (dd) water and patted dry using a piece of tissue paper. The tick was cut in half, one half was stored for other studies while the other half was placed in a 1.5 ml tube containing 200 µl of 5% chelex®100 chelating resin. Two glass beads were placed in the tube and the tick was crushed using a homogenizer for 18 seconds at 15000 rpm in each direction. The tube was then incubated using a heating block at 56°C for one hour, followed by DNA denaturation at 95°C for 30 minutes. The tube was then centrifuged at 12000 g for three minutes and frozen at -20°C until PCR analysis.

A few extracted DNA samples were selected to determine the quantity of DNA using a DNA spectrophotometer (BioTeK). PCR methods were used to detect the presence of *E. ruminantium* and *R. africae* in the extracted DNA.

3.4.1 Detection of Ehrlichia ruminantium

Real-time PCR targeting the pCS20 gene fragment of *E. ruminantium* (Cangi et al., 2017) was used to detect its presence in the tick DNA. The PCR reactions consisted of dd water, TaqMan Universal PCR master mix, 0.25µM of each primer sol1F (forward) and sol1R (reverse) , 0.4 µM of probe sol1P (FAM-QSY) and a DNA template. Each reaction had 8.5 µl distilled water, 12 µl TaqMan Universal PCR master mix (Applied Biosystems), 0.625 µl of each primer, 1.25 µl probe sol1P and 2.0 µl DNA template, making a total volume of 25 µl. The reactions were performed and analyzed using a StepOnePlus real-time PCR system machine (Applied Biosystems) using StepOne software v2.3, with a negative (no DNA template) and a positive control of a known *E. ruminantium* DNA template. The conditions for the reactions were: incubation at 50°C for 2 minutes, heating at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 55°C for 1 minute. The amplification curves produced during the reactions were used to determine if a sample is positive or negative for *E. ruminantium.*

Table 1: Primers used to amplify pCS20 gene fragment of *E. ruminantium*

Target region	Primer	Length	Sequence (5'- 3')	Final concentration
pCS20	Sol1 F	20-mer	ACA AAT CTG GYC CAG ATC AC	0.25 µM
	Sol1 R	20-mer	CAG CTT TCT GTT CAG CTA GT	0.25 µM
	Sol1 TM	32-mer	6-FAM-ATC AAT TCA CAT GAA ACA TTA CAT GCA ACT GG-BHQ1	0.4 µM

3.4.2 Detection of *Rickettsia africae*

The presence of *R. africae* in the extracted tick DNA was determined by analyzing samples using conventional PCR. The test targets the *ompA* gene of the pathogen (Mazhetese et al., 2022). The PCR consisted of the primers Rr190.70F (forward) and Rr190.701R (reverse), 2x Phusion flash PCR master mix (thermo scientific), dd water and DNA template. Each reaction had 6 µl distilled water, 10 µl 2x Phusion flash PCR master mix, 1 µl of each primer at 0.5 µM and 2 µl DNA template making a final volume of 20 µl. All the plates of PCR reactions had a negative (no DNA template) and a positive control of a confirmed *R. africae* DNA sample. The reactions were analyzed using a Veriti 96 well (Applied Biosystems) thermal cycler PCR machine set to the following conditions: pre-incubation at 98°C for 10 seconds, followed by 30 cycles of denaturation at 98°C for 1 second, 51°C for 5 seconds and 72°C for 15 seconds, annealing and extension at 70°C for 1 minute and holding temperature of 4°C. The PCR products were then analyzed using electrophoresis on 1.5% agarose gel, stained with ethidium bromide and viewed with UV light illumination using the Bio-Rad gel documentation system .

Table 2: Primers used to amplify *ompA* gene for *R. africae*

Target region	primer	Sequence (5'-3')	Final concentration
ompA	Rr190.70F	ATG GCG AAT ATT TCT CCA AAA	0.5 µM
	Rr190.701R	GTT CCG TTA ATG GCA GCA TCT	0.5 µM

CHAPTER 4: RESULTS

4.1 Tick collections

During the study, a total of 567 *A. variegatum* ticks were collected from the western, central and eastern regions of Zambia. All the collected ticks were identified to be *A. variegatum*, and of these 534 were males and 33 females. Tick collections were done from different villages of the selected districts. In the western region, Mongu district was selected and ticks were collected from three

villages; lyanena, Nalikwanda and Sefula villages. In the eastern region ticks were collected from three villages within Petauke district; Tumpa, Chileka and Manchinchi villages. In the central region ticks were collected from two different districts. In Chongwe district, which lies to the east of Lusaka, ticks were collected from Shipanuka and Chilyabale villages, in Shibuyunji district, which lies to the west of Lusaka, ticks were collected from Shibuyunji village.

Ticks were collected from cattle identified to be infested with *Amblyomma* ticks. The number and sex of ticks collected from each village is shown in the table below (Table 3).

Table 3: Summary of tick collections

District	Village	Number of ticks collected	Sex of tick	
			M	F
Mongu	Lyaneno	78	63	15
	Nalikwanda	197	194	3
	Sefula	32	28	4
Petauke	Tumpa	48	47	1
	Chileka	110	109	1
	Machinchi	6	6	0
Chongwe	Shipanuka	27	20	7
	Chilyabale	15	14	1
Shibuyunji	Shibuyunji	54	53	1

4.2 DNA yields

An average DNA concentration of 531.57ng/µL was measured which indicated good amounts of DNA. Table 4 shows the quantity of DNA in a few selected samples.

Table 4: DNA yields

Sample A		Sample B		Sample C		Sample D		Sample E		Sample F	
0.591	260	0.606	260	0.606	260	0.565	260	0.365	260	0.466	260
0.416	280	0.405	280	0.405	280	0.384	280	0.229	280	0.303	280
1.42	260/280	1.496	260/280	1.496	260/280	1.471	260/280	1.597	260/280	1.54	260/280
590.85 **ng/µL**		**605.782** ng/µL		**605.782** ng/µL		**564.66** ng/µL		**365.326** ng/µL		**465.817** ng/µL	

4.3 Detection of Rickettsial species by molecular methods

4.3.1 *Ehrlichia ruminantium*

All 567 *A. variegatum* ticks collected were analyzed for *E. ruminantium* infection using real-time PCR. The ticks were found to have an infection rate of 18.52%, the highest infection being in the eastern region (35.4%), followed by central region (18.8%) and the lowest being in the Western region (9.4%). Some of the positive and negative samples of pCS20 fragment amplification are illustrated in Figure 3 below.

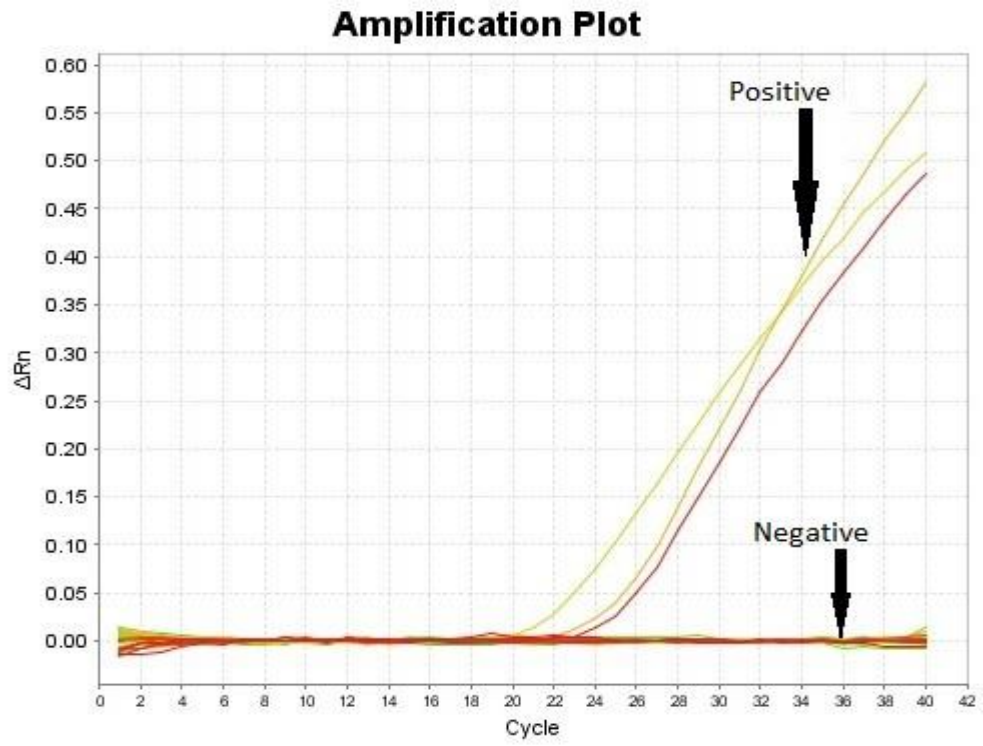

Figure 3: Real-time PCR amplification curves of pCS20 fragment for *E. ruminantium.*

Table 5: Summary of *E. ruminantium* results

District	Village	*E. ruminantium* status	
Mongu		+ve	-ve
	Lyaneno	9	69
	Nalikwanda	14	183
	Sefula	4	28
Petauke	Tumpa	33	15
	Chileka	24	86
	Machinchi	1	5
Chongwe	Shipanuka	1	26
	Chilyabale	1	14
Shibuyunji	Shibuyunji	16	38

- A sample was considered to be positive for *E. ruminantium* if its ct value was less than 38 or produced an amplication curve before cycle 38. All samples with a ct value of 38 and above were considered negative.

4.3.2 *Rickettsia africae*

All 567 *A. variegatum* ticks collected were tested for *R. africae* infection using conventional PCR. An overall infection rate of 36.07% was detected using a real-time PCR for *ompA* gene, the highest being in eastern region (56.1%), followed by central region (41.7%) and the lowest in western region (23.3%). The bands obtained from amplification of some of the samples for *ompA* gene for *R. africae* are illustrated in Figure 4 below.

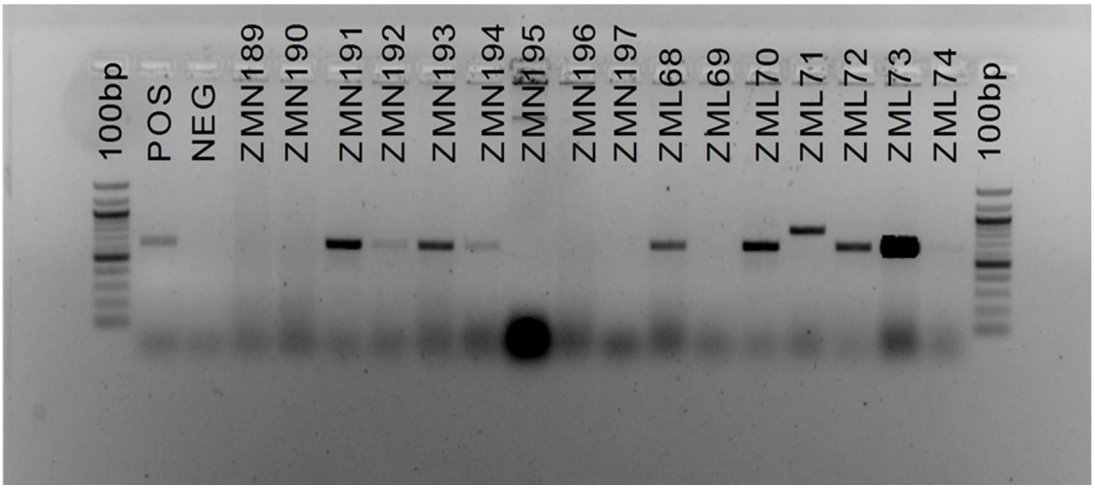

Figure 4: PCR products generated by amplification of *ompA* gene for *R. africae* visualized on 1.5% agarose gel. In lane 1 and 20 is a 100 bp ladder, lane 2 is the positive control, lane 3 is the negative control, and lane 4 to 19 are DNA samples.

Table 6: Summary of *R. africae* status

District	Village	*R. africae*	
		+ve	-ve
Mongu	Lyaneno	20	58
	Nalikwanda	40	157
	Sefula	10	22
Petauke	Tumpa	23	25
	Chileka	64	46
	Machinchi	5	1
Chongwe	Shipanuka	16	11
	Chilyabale	8	7
Shibuyunji	Shibuyunji	17	37

- Samples were considered positive by visualization of amplified *ompA* gene bands at 750 basepair using electrophoresis on 1.5% agarose gel. All samples which did not produce any bands were considered not to contain *ompA* gene thus negative for *R. africae.*

4.4 Data analysis

Data was entered into MS Excel 2013 and coded, after which it was transferred to Stata 15.0 for analysis.

Summary of analyzed data is shown in the tables below.

Table 7: Overall infection rate of *E. ruminantium*

Result	Frequency(number of ticks)	Percent
Negative	462	81.48
Positive	105	18.52
Total	567	100.00

There was no comparison in infection rates between male and female ticks because the number of female ticks was far much less than that of males (534 males, 33 females).

Table 8: Infection rates of *E. ruminantium* in three regions

Result	Province	Proportion (ticks tested negative/ positive)	Std. Err	Logit [95% Conf. Interval]	
Negative	Western	.9055375	.0167195	.8671984	.933655
	Eastern	.6463415	.0374481	.569844	.7160145
	Central	.812500	.0400452	.7211223	.8789622
Positive	Western	.0944625	.0167195	.066345	.1328016
	Eastern	.3536585	.0374481	.2839855	.430156
	Central	.1875	.0400452	.1210378	.2788777

INTERPRETATION

1. In the western region, there was a 9.4% infection rate of *E. ruminantium.* This means that approximately 9.4% of the samples tested positive for the presence of the bacterium. The 95% confidence interval (CI) provides a range of values within which we can be 95% confident that the true infection rate lies. In this case, the CI is 6.6% to 13.3%, indicating that the actual infection rate in the western region is likely to fall within this range. The standard error of 1.7% indicates the precision of the estimate.

2. In the eastern region, there was a higher infection rate of *E. ruminantium* at 35.4%. This suggests that a larger proportion of samples tested positive for the bacterium compared to the western region. The 95% confidence interval (CI) for the infection rate is 28.4% to 43%, indicating the range within which we can be 95% confident that the true infection rate lies. The standard error of 3.7% reflects the precision of the estimate.

3. In the central region, there was an 18.8% infection rate of *E. ruminantium*. This indicates that a moderate proportion of samples tested positive for the bacterium in this region. The 95% confidence interval (CI) for the infection rate is 12.1% to 27.9%, representing the

range within which we can be 95% confident that the true infection rate lies. The standard error of 4% reflects the precision of the estimate.

Therefore, these results provide insights into the prevalence of *E. ruminantium* infection in different regions, with the eastern region showing the highest infection rate, followed by the central and western regions. The confidence intervals give us an understanding of the range within which the true infection rates are likely to fall. The standard errors indicate the level of uncertainty associated with the estimates.

Table 9: Overall infection rate of *R. africae*

Result	Frequency (number of ticks)	Percent
Negative	358	63.93
Positive	202	36.07
Total	560	100.00

Table 10: Infection rates of *R. africae* in three regions.

Result	Province	Proportion (ticks tested negative/positive)	Std. Err	Logit [95% Conf. Interval]	
Negative	Western	.7666667	.02446	.7152481	.8112506
	Eastern	.4390244	.0388707	.3646739	.5162168
	Central	.5833333	.0505814	.4819344	.6781403
Positive	Western	.2333333	.02446	.2847519	.4819344
	Eastern	.5609756	.0388707	.4837832	.6353261
	Central	.4166667	.0505814	.3218597	.5180656

INTERPRETATION

1. In the western region, there was a 23.3% infection rate of *R. africae*. This indicates that approximately 23.3% of the samples tested positive for the presence of the bacterium. The 95% confidence interval (CI) specifies a range of values within which we can be 95% confident that the true infection rate falls. In this case, the CI is 28.5% to 48.2%, suggesting that the actual infection rate in the western region is likely to fall within this range. The standard error of 2.4% reflects the precision of the estimate.

2. In the eastern region, there was a higher infection rate of *R. africae* at 56.1%. This suggests that a larger proportion of samples tested positive for the bacterium compared to the western region. The 95% confidence interval (CI) for the infection rate is 48.4% to 63.5%, indicating the range within which we can be 95% confident that the true infection rate lies. The standard error of 3.9% reflects the precision of the estimate.

3. In the central region, there was a 41.7% infection rate of *R. africae*. This indicates that a significant proportion of samples tested positive for the bacterium in this region. The 95% confidence interval (CI) for the infection rate is 32.2% to 51.8%, representing the range within which we can be 95% confident that the true infection rate lies. The standard error of 5.1% reflects the precision of the estimate.

Hence, these results provide information about the prevalence of *R. africae* infection in different regions, with the highest infection rate observed in the eastern region, followed by the central and western regions. The confidence intervals give an understanding of the range within which the true infection rates are likely to fall. The standard errors indicate the level of uncertainty associated with the estimates.

CHAPTER 5: DISCUSSION

The results of this study indicate the sole presence of *A. variegatum* from all three regions of sample collection in Zambia, confirming the presence of the main vector of *E. ruminantium* and *R. africae* in the country. This finding is supported by Makala (2003) who reported that *A. variegatum* is the main vector of heartwater in Zambia and that the ticks are found throughout the country. However, there could be other species present in other parts of the country, for example a study that was done in Shangombo district of Western Province found one *A. pomposum* (Qiu et al., 2022). Further tick surveillance studies in other regions of the country are needed to give an update of *Amblyomma* species found in Zambia.

Other countries in the southern region of Africa have records of the presence of other species besides *A. variegatum*. Zimbabwe has *A. hebraeum* and *A.variegatum* (Sungirai et al., 2015) but the recent studies done by Mandara (2018) reviewed the presence of *A. gemma* in Mazowe and Shurugwi towns of Zimbabwe. According to Walker (2003) Mozambique and Bostwana have *A. hebraeum* and *A. variegatum,* Angola has *A. pomposum* and *A. variegatum* whereas South Africa has *A. hebraeum*. More ticks were collected from the western region compared to the other two regions, namely eastern and central regions. This may be attributable to the fact that farmers in the western region do not practice tick control on their animals (Mubita J M, District veterinary officer, Mongu district, personal communication) whereas in the two other regions tick control is practiced, although it may not be intensive.

Another reason for low numbers of ticks in the eastern and central regions could be due to the timing of the sample collection which was in April at the end of the rainy season, since most ticks could have dropped off the animals at the time, which is supported by the small numbers of females that were found because they drop to the ground to lay eggs after a blood meal. This finding could be supported by a study that was done years back where it was stated that only one generation of *A. variegatum* occurs per annum and an increase in adult activity is seen during the rainy season, mainly between October and February (Pegram *et al*., 1986).

The DNA extraction method/protocol is an important step in assuring good quality DNA for reliable results from PCR techniques. One has to ensure that the method chosen for DNA extraction will yield DNA of good quality at sufficient concentrations. DNA extraction for this study was done using the Chelex 100 Resin insect DNA extraction protocol. There are other methods

available for extraction of DNA from ticks, such as commercial DNA extraction kits like the QiAmp DNA extraction kit. The QiAmp DNA extraction protocol uses more reagents making it more expensive as well as more time consuming, because, amongst other steps, samples have to be incubated for 24 hours. The Chelex method is cost effective because it utilizes only a few reagents compared to other methods, and the technique is less time-consuming, yet yields good quality DNA within two hours (Asghar et al., 2015). However, in a study by Desloire et al. (2006) it was found that the QiAmp DNA extraction kit gave results that had a DNA yield of close to 100%, compared to the 67% obtained from the Chelex method in the DNA amplifications. This could mean that the DNA extracted using the QiAmp extraction kit may be of greater purity, concentration and has less or no non-genetic material, whereas the ones extracted using the Chelex method may be of adequate concentration but with more non-genetic material.

In this study the 260/280 DNA ratio was found to be less than 1.7 which suggests that the DNA was not very pure, since pure DNA has a 260/280 ratio of 1.7-1.8 (O'neill et al., 2011). Furthermore, there is a need to take care when working with DNA samples extracted using the Chelex method, because the resin inhibits the PCR, thus possibly resulting in a number of false negatives (Desloire et al., 2006). The DNA extracted for this study was sufficient for the required tests, since it was present in adequate concentrations, and during the PCR analyses, care was taken to pipet the DNA template (sample) from the top aqueous phase, thus avoiding the Chelex resin material and preventing inhibition of the PCR by resin.

This study gives an update on the PCR-based prevalence estimates of *E. ruminantium* in adult *A. variegatum* ticks in eastern, central and western regions of Zambia. This is the second time such a study is being done in Zambia that is estimating prevalence of *E. ruminantium* in *A. variegatum* ticks. The PCR assay (pCS20) used for this study is a well validated technique (Cangi et al., 2017). In this study, the prevalence of *E. ruminantium* in *A. variegatum* ticks in selected regions of Zambia was found to be 18.52% (105/567).

Our finding is within the same range as that of Peter et al. (2000) where a prevalence range of 6.25-39.3% in male and 2.1-14.3% in female *A. variegatum* of Zambia and Zimbabwe was reported. Zimbabwe had a prevalence of 11% in *A. variegatum* (Mandara, 2018). This finding is also within the same range found in *A. variegatum* of other countries in the southern region of Africa, e.g. Angola at 52.71%, and Mozambique at 5.50%. In South Africa the range in *A.*

hebraeum was between 10-90% (Smit A, Department of Veterinary Tropical Diseases, University of Pretoria, unpublished data).

The infection rates of *E. ruminantium* for each region of study was found to be as follows; western region 9.4%, eastern region 35.4% and central region 18.6%. The western region was found to have the lowest infection rates, despite the highest number of ticks being collected there. This may be attributable to the fact that the area is covered by flood plains, the cattle usually utilizing the flood plains for grazing most of the time, and only graze the small woodland grazing areas, which is the preferred habitat of *A. variegatum* during the rainy season. This practice causes a disruption in the life cycle of the ticks, which in turn may lead to a loss of pathogens in the ticks. The high prevalence recorded in the eastern region may be an indication that heartwater disease is endemic in the area. Demonstration of the presence of *E. ruminantium* in all the sampled regions suggests that most parts of the country are at risk of heartwater disease.

Simuunza et al. (2011) reported the presence of *E. ruminantium* in cattle of Lusaka, Central and Eastern provinces of Zambia and he recorded sero-prevalences ranging between 15-38%. *Erhlichia ruminantium* is also known to infect and cause problems in goats of Zambia as demonstrated by Ahmadu (Ahmadu et al., 2010). The finding of heartwater antibodies in the cattle and small ruminants, and the presence of *E. ruminantium* in the ticks, are an indication of the widespread presence of the disease in the country. It is therefore important to investigate any clinical cases and deaths that are suspected to be heartwater, and pay attention to characteristic clinical signs, performing post-mortem examinations and analysing samples in the laboratory.

Rickettsial infections in different species of ticks have been reported in the country, but *R. africae* infections in humans have not yet been documented. The presence of the pathogen in the ticks suggests that humans are at risk of contracting the pathogen causing ATBF, especially those in close proximity to animals and tourists that visit the rural areas of the country. The finding of *R. africae* in the ticks calls for need to carry out awareness programs in the public health systems in the country. In this study the infection rate of *R. africae* in *A. variegatum* was determined by PCR, targeting the *ompA* gene for *R. africae*. In this study, the infection rate was found to be 36.1%. In a study that was done in Shangombo district of the Western Province, the infection rate of *R. africae* in *A. variegatum* was found to be 15.6% (Qiu et al., 2022), whereas in this study the infection rate for Mongu district, which is to the east of Shangombo, was found to be 23.3%.

Petauke in the eastern region had the highest infection rate of 56.1%, and central region had an infection rate of 41.7%. The western region had the lowest infection rate compared to the other two regions, an observation that may be attributed to the climatic differences between the regions; Mongu, for example, is on the flood plains and covered with sandy soils and sparse vegetation, ecologically not conducive for tick survival. In another study in the Southern Province of Zambia, it was estimated that the infection rate of rickettsial infections in ixodid ticks, was 18.6% (Chitanga et al., 2021). The infection rates found in the present study are in the same range as those found in other countries like Angola, where the infection rate in *A. pomposum* was found to be 41.58%, whereas in *A. variegatum* it was 10.85%. In Mozambique the infection rate was found to be in the range of 0-70% (Smit A, Department of Veterinary Tropical Diseases, University of Pretoria, unpublished data).

From the above observation, one may hypothesise that *R. africae* infections are found in most parts of the country and should be considered to be of great concern to the public health sector, since the pathogen can be readily transmitted to humans through tick bites, particularly since *Amblyomma* has been demonstrated to bite humans (Fournier et al., 1998). *Rickettsia africae* can be transmitted by all feeding stages of *Amblyomma* species since the pathogen can be maintained through transovarial and transstadial transmission in the tick population (Socolovschi et al., 2009).

The results also indicate that the prevalence of *R. africae* is higher than that of *E. ruminantium* in the ticks in all the three regions of the country, thus raising concern about the number of people who may be at risk of contracting the pathogen through tick bites. The disease could be there in indigenous people in the rural areas of Zambia especially those in close contact with animals but there is no documentation. Lack of information or reports on the disease in Zambia could be attributed to the fact that, it is not diagnosed probably not considered on the differential list when dealing with systemic febrile infections. The people in Zambia could be immune to the infection, as literature states that indigenous people may get the infection at a young age thus tend to be immune in older age (Jensenius et al., 2003b) . Lack of the diagnostic capacity also results in poor reporting of ATBF cases.

This study provides an update on the prevalence of *R. africae* in *A. variegatum* ticks in selected regions of Zambia. The fact that these ticks readily bite humans, ATBF should be considered an important differential diagnosis in febrile reactions in humans, especially when malaria is not

detected. The Ministry of Health should consider finding the means of diagnosing and keeping records of such infections.

This study adds to the little available data on the prevalence of *E. ruminantium* and *R. africae* in *A. variegatum* ticks in selected regions of Zambia. This information may prove useful in the planning of future control strategies/policies for the diseases caused by these pathogens. Further studies should be encouraged in order to generate more information for the better understanding of the impact of heartwater and ATBF in animals and humans, respectively, in the country.

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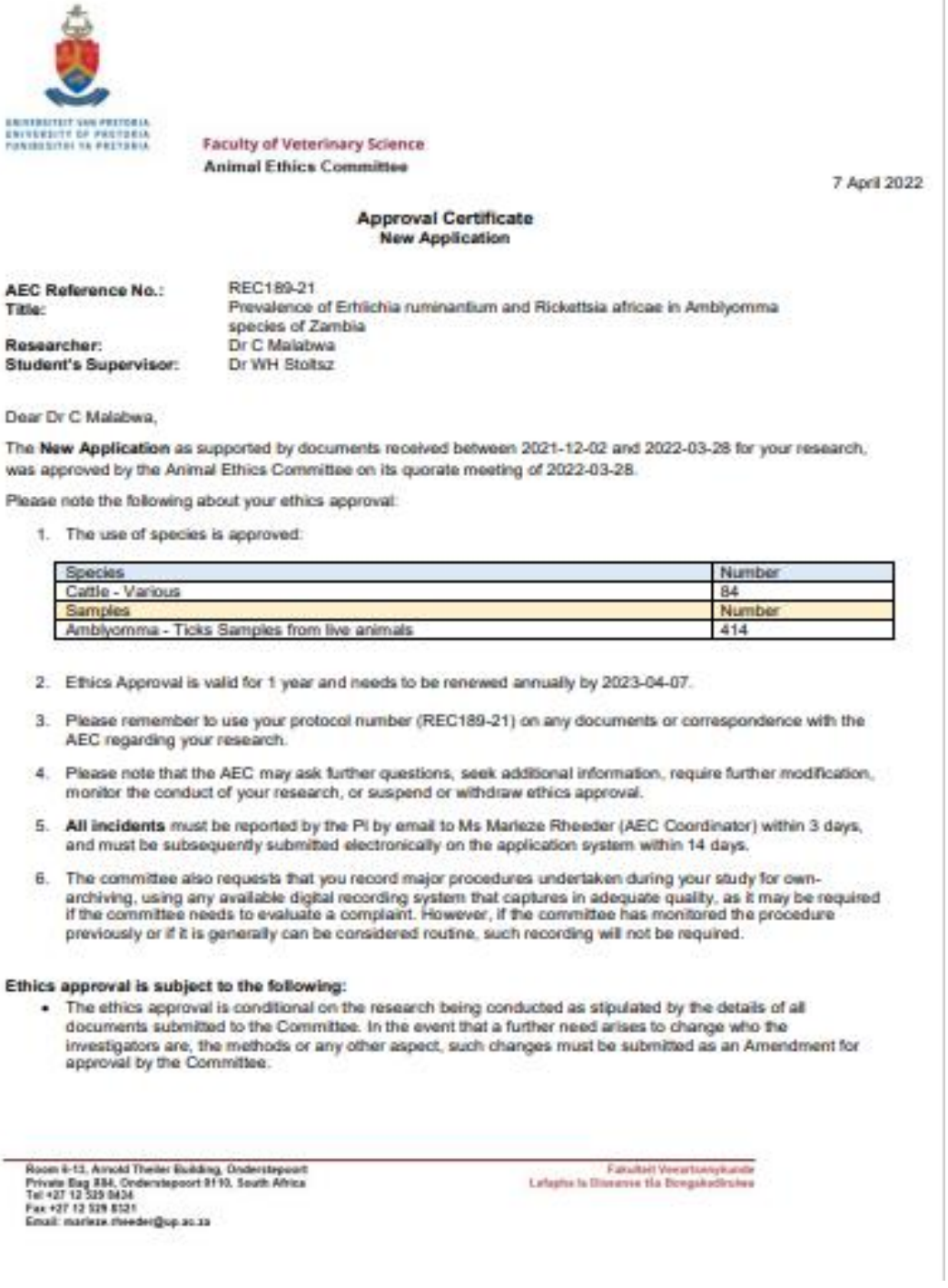
APPENDICES

APPENDIX A



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

7 April 2022

Approval Certificate
New Application

AEC Reference No.:	REC189-21
Title:	Prevalence of Erhlichia ruminantium and Rickettsia africae in Amblyomma species of Zambia
Researcher:	Dr C Malabwa
Student's Supervisor:	Dr WH Stoltsz

Dear Dr C Malabwa,

The **New Application** as supported by documents received between 2021-12-02 and 2022-03-28 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-03-28.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number
Cattle - Various	84
Samples	Number
Amblyomma - Ticks Samples from live animals	414

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-04-07.
3. Please remember to use your protocol number (REC189-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

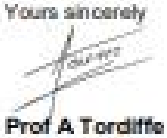
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for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

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel: +27 12 529 8424
Fax: +27 12 529 8121
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadikobo

We wish you the best with your research.

Yours sincerely



Prof A Tordiffe
DEPUTY CHAIRMAN: UP-Animal Ethics Committee

Room 4-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel: +27 12 529 8434
Fax: +27 12 529 8321
Email: marlene.meeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Dinaane tša Bongakadikobo

APPENDIX B

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X250, Pretoria 0001
Enquiries: Ms. Mama Laing · Tel: 012 319 7442 · Fax: +27 12 319 7470 E-mail: MamaL@dalrrd.gov.za
Reference: 12/11/1/1/8/2286(HP)

Dr Chooiwe Malabwa
PO Box 33980
Lusaka
Zambia
E-mail: chooiwemalabwa@gmail.com
hein.stoltsz@up.ac.za
luis.neves@up.ac.za

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

Dear Dr Malabwa

Your fax / memo / letter/ Email received 2021-12-22, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

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

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. **A veterinary import permit must be obtained prior to the importation of the *Amblyomma* ticks from Zambia.**
4. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act No. 93 of 1996);

- 1 -

5. The ticks be must be flown to OR Tambo International Airport, South Africa. The responsible State Veterinarian must issue a Red Cross Permit for the ticks to be moved to ARC-OVR Transboundary Animal Diseases (TAD).
6. The ticks must be kept in 70% ethanol at TAD for 14 days, after which they can be transported to DVTD for DNA extraction.
7. DNA extraction must be done at the DVTD BLS2 laboratory.
8. Extracted DNA samples may be stored at the access controlled BSL2 facility at the DVTD laboratory.
9. Stored samples may not be outsourced for research without prior written approval from the Director: Animal Health.
10. Should samples be used for further research, written approval from the Director: Animal Health must be obtained prior to start of project.
11. This Section 20 approval is valid until 2022-09-30.

Title of research/study: Presence of *Ehrlichia Ruminantium* and *Rickettsia africae* in *Amblyomma* species in Zambia
Researcher (s): Dr Choolwe Malatwa
Institution: DVTD, Faculty of Veterinary Science, University of Pretoria
Your Ref./ Project Number: REC198-21
Our ref Number: 12/11/1/1/8/2286(HP)

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2022 -03- 22

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

APPENDIX C

Faculty of Veterinary Science
Research Ethics Committee

9 March 2022

CONDITIONALLY APPROVAL

Ethics Reference No	**REC189-21**
Protocol Title	**Prevalence of Erhlichia ruminantium and Rickettsia africae in Amblyomma species of Zambia**
Principal Investigator	**Dr C Malabwa**
Supervisors	**Dr WH Stoltsz**

Dear Dr C Malabwa,

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC189-21) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals**: FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Conditionally approved (pending obtaining ALL other relevant approvals).

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

Room 3-8, Arnold Theiler Building
University of Pretoria, Faculty of Veterinary Science
Private Bag X04, Onderstepoort, 0110, South Africa
Tel: +27 (0)12 529 8080
Email: marie.wilson-kriek@up.ac.za
www.up.ac.za

Faculty of Veterinary Science
Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadirulwa

APPENDIX D

MEMORANDUM

To : Veterinary Research Officer- Dr. Choolwe Malabwa

From: The Chief Veterinary Officer

Date: 22nd October, 2021

SUBJECT : PERMISSION TO COLLECT AMBLYOMMA TICKS IN ZAMBIA

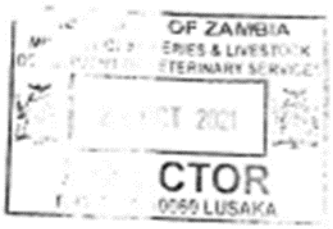
Reference is made to the above subject matter.

I am pleased to inform you that authority has been granted for you to collected 138 Amblyomma ticks from each of the following regions; Eastern, Central and Western.

The approval is under the condition that you will work hand in hand with the District Veterinary staff and the samples will be used for academic purposes during your Masters of Science Degree programme with the University of Pretoria. Further, the department should be updated on the progress of the research. If upon completion of your programme, the samples need to be used for more research, you will need to seek renewal of the mandate.

Please, ensure that you abide to the conditions of this mandate.

Dr Gift Munthali



APPENDIX E

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

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

Tel: (27)-012-319 7514
Fax: (27)-012-329 8292

IMPORTER:
DR W H STOLTSZ
DVTD
FACULTY OF VETERINARY SCIENCE
ONDERSTEPOORT
0110

PERMIT NO: 13/1/1/30/2/0-202204001937
Valid from: 2022-04-14
Expiry date: 2022-10-14

VETERINARY IMPORT PERMIT FOR PRESERVED ANIMAL MATERIAL
[Issued in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984)]

Authority is hereby granted for you to import 414 ADULT AMBLYOMMA TICKS STORED IN 70% ALCOHOL into the Republic of South Africa from ZAMBIA subject to the following conditions:

1. The importer accepts the sole responsibility of ensuring that the conditions below have been complied with, and understands his/her duty in this regard.
2. The consignment must be accompanied by this **original** permit.
3. The animal material must be securely packed in sealed, leakproof containers, filled with formalin or alcohol, and be sufficiently preserved to ensure that it is completely immersed and needs no cooling to prevent spoilage.
4. The consignment must be conveyed through port of entry O R TAMBO INTERNATIONAL AIRPORT. **Samples may only be imported as manifest cargo under an airwaybill number and may not be imported as personal luggage.**
5. The inspecting veterinary official at the port of entry must be advised timeously of the expected date and time of arrival of the consignment Tel: 011 393 7980/83/84/85 Fax: 011 973 2828, and may not be released without his/her written permission.
6. Upon arrival the inspecting veterinary official will inspect the consignment and release it to the importer only after he/she is satisfied that all the import conditions have been complied with in full.
7. On completion of tests/research the specimens, including all contaminated/infectious things or animal products (as defined by the Animal Diseases Act, 1984 [Act No. 35 of 1984]) derived/produced from or that came into contact with the above-mentioned specimens, must be destroyed by incineration. Records of the incinerations must be maintained for a period of 5 years, and made available for auditing to the Veterinary Authority upon request.
8. **This permit does not absolve the importer from compliance with the provisions of any other legislation relating to this import.**
9. This permit is subject to amendment or cancellation by the Director Animal Health at any time and without prior notice being given.
10. This permit is valid for six (6) months from date of issue and FOR ONE CONSIGNMENT ONLY.

pp **DIRECTOR: ANIMAL HEALTH**

NOTE:

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

APPENDIX F

CONSENT FORM

Section A

- There are no risks from participating in this study for you. Although plucking some ticks from the animals may cause minor trauma to the animal, efforts will be made to cause as little distress as possible.
- In case of injury to an animal during the tick collection process, it shall be treated to the best ability of the researcher at her cost.
- There are no direct benefits for participating, besides having gained knowledge into the *Ehrlichia ruminantium* and *Rickettsia africae* status of the ticks picked from your animals. Results will be shared with you through your veterinary officer.
- The study is to determine the prevalence of *Ehrlichia ruminantium* and *Rickettsia africae* in Amblyomma species of Zambia.
- This study is for school purposes to obtain a master's degree from the University of Pretoria (South Africa) and is supervised.

Section B

I confirm that I have been informed about the study and that I have been availed with the participant information and consent form.

I have read and understood the information:

- I consent voluntarily to participation in this study.
- I know that I will not receive any payment for participating in this study.
- I know that I can stop participation at any time.
- I consent to ticks being collected from my animals.
- I have had all my concerns answered to my satisfaction.

a) Mr Gilbert Mwiinde — Name of respondent

b) ..... — Signature/thumb print of respondent

c) Choolwe Malambwa — Name of researcher or clinician

d) ..... — Signature of researcher or clinician

e) Chongwe — Place

f) 28/07/2022 — Date