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**The distribution of *Amblyomma variegatum* and
A. hebraeum in Zimbabwe and their infection with
Ehrlichia ruminantium and *Rickettsia africae***

by

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Declaration

I declare that this dissertation submitted to the University of Pretoria for the degree of Magister Scientiae (Tropical Animal Health) has not been previously submitted by me for the degree at this or any other University, that it is my own work in design and in execution, and that all material contained therein has been duly acknowledge.

Signed: ... 

Date: 2018.11.07

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Dedication

To my wife, Memory, and son, Lincoln Stephen.

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List of Abbreviations

DNA	Deoxyribonucleic acid
GPS	Global Positioning System
OIE	World Organization for Animal Health
<i>ompA</i>	outer membrane protein A
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction-restriction fragment length <i>polymorphism</i>
ng	Nano grams
NCBI	National Centre for Biotechnology Information

Thesis Summary

The aim of this study was to give an update on the spatial distribution of the *Amblyomma* tick species in Zimbabwe and their infection rates with *Ehrlichia ruminantium* and *Rickettsia africae* pathogens. A total of 183 ticks were collected at 10 sites across Zimbabwe based on previous records of absence or presence of *Amblyomma* ticks. Morphological keys were used to identify the ticks using a stereo microscope. Infection of the ticks with *Ehrlichia ruminantium* was investigated using PCR targeting pCS20 (*E. ruminantium*), *gltA* (*Rickettsia* spp.) and *ompA* (*R. africae*) genes. *Amblyomma* ticks were found widespread in southern, central and north-western Zimbabwe. The species found were *Amblyomma hebraeum*, *A. variegatum* and *A. gemma*, which had previously not been reported in Zimbabwe. *Amblyomma hebraeum* was found in all the areas surveyed while *A. variegatum* and *A. gemma* were only found in some of the places. A limited spread in *A. hebraeum* and *A. variegatum* distribution in Zimbabwe was noted. It also showed that they are infected with rickettsial pathogens at relatively high rates (average 15.8% for *E. ruminantium*). The real-time PCR for *R. africae* *gltA* gene showed an average infection rate of 95% and for *ompA* it was 40%. DNA sequence analysis showed a high similarity (98-99%) with three other *R. africae* published sequences. The widespread distribution of *Amblyomma* ticks and their infection with rickettsial pathogens means heartwater and rickettsial infections will continue as problems for livestock and humans in Zimbabwe.

KEY WORDS: Ticks, Rickettsial pathogens, heartwater, Zimbabwe

CHAPTER 1: INTRODUCTION

The tick species *Amblyomma variegatum* and *Amblyomma hebraeum* are the cyclical vectors of *Ehrlichia ruminantium*, (Bournez *et al.* 2015) the bacterial agent that causes heartwater in Zimbabwe (Norval *et al.* 1994). Heartwater is a fatal disease of ruminants. The adult ticks cause significant declines in milk production and weight gain, (Norval *et al.* 1991). Secondary effects include lesions that attract the larvae of the screw-worm fly, *Chrysomya bezziana*. *Amblyomma variegatum* has also been implicated in the spread of a skin disease, bovine dermatophilosis, via immunosuppression (Chatikobo *et al.* 2009). These ticks are of great significance both from a veterinary and public health perspective.

The distributions of these vectors of heartwater in Zimbabwe were poorly documented prior to 1975. Available knowledge is based on occasional collections and largely assumed from reports of heartwater outbreaks (Norval *et al.* 1994). The distributions of these two *Amblyomma* species were separate from around 1930 until the war for independence halted cattle dipping for tick control in 1975 (Bournez *et al.* 2015). They are geographically separated at the central highveld plateau of Zimbabwe, on which neither of the two species has been permanently established (Norval 1983), *A. variegatum* occupies the north while *A. hebraeum* predominates in the south. Tick surveys conducted later revealed a parapatric distribution with some locations in western Zimbabwe having a rare phenomenon of having both species. (Bournez *et al.* 2015).

Since the year 2000, Zimbabwe conducted a land re-distribution exercise that caused major changes in agriculture and land use patterns across the country (Ndhlovu 2014). This may also have affected the distribution of animal parasites such as ticks due to livestock movements. Thus there is a need for continuous updating of information on ticks due to political and socio-economic events that influence their spatial distribution over time.

Ehrlichia ruminantium is transmitted biologically by several tick species that belong to the genus *Amblyomma*. *Amblyomma variegatum* is the most important of twelve *Amblyomma* species shown to transmit *E. ruminantium* because it is the most widely distributed species and is an efficient heartwater vector (Peter *et al.* 1998). In southern Africa including Zimbabwe, *A. hebraeum* is the predominant *Amblyomma* tick species making it the most important heartwater vector in this region (Allsopp *et al.* 2005).

Heartwater seriously affects livestock production development wherever it occurs, especially in most areas of the sub-Saharan Africa region (Walker and Olwage 1987). Traditionally, the disease has been managed by targeting the tick vector, by use of acaricides, (Mukebhi *et al.* 1998). The distribution of heartwater in Zimbabwe often follows that of the *Amblyomma* vectors and it is taken to be endemic in many regions (de Vries *et al.* 1993).

Ticks of the genus *Amblyomma* are also responsible for transmitting *Rickettsia africae* in humans, the causative organism of African tick-bite fever (ATBF). This condition is the most prevalent tick borne rickettsiosis affecting the people of and travellers to rural areas in Africa (Maina *et al.* 2014). African tick-bite fever is an acute illness with signs and symptoms such as fever, headaches, myalgia and many small eschars at the site of the tick bite. Regional lymphadenitis is also a common sign (Jensenius *et al.* 2003). *Rickettsia africae* has previously been detected in *Amblyomma* ticks using the polymerase chain reaction (PCR), (Kelly *et al.* 1996). Adult *A. variegatum* ticks taken from cattle in west African countries of Mali and Niger were found to contain three strains of the pathogen (Jensenius *et al.* 2003). Transovarial and transstadial routes are responsible for maintaining infection in the two species, *A. hebraeum* and *A. variegatum* (Jensius *et al.* 2004). Therefore, all life stages of the ticks are capable of transmitting the infection to humans. The infection rate in ticks in some endemic places has been found to be high, reaching 100% or closer (Nakao *et al.* 2013).

During the liberation war in Zimbabwe in the 1970s, army medics reported having treated many thousands of cases of tick typhus. Although both soldiers of European and those of African origin got sick with the disease, prevalence was reportedly higher in the former than the latter (Parola *et al.* 2001). The cases in Zimbabwe were mostly reported among the soldiers who originated from urban areas and deployed to serve in the rural areas, probably due to lack of immunity (Jensius *et al.* 2004). Whereas cases of ATBF in the local indigenous African populations are not common, they have increased significantly among visitors and tourists from Europe, America and elsewhere outside Africa in recent times, (Jensenius *et al.* 2003).

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The *Amblyomma* ticks are taxonomically classified in the Class Arachnida, Order Ixodida, Family Ixodidae, genus *Amblyomma* (Walker *et al.* 2003). The most widely distributed species in Zimbabwe are *Amblyomma hebraeum* and *A. variegatum*.

They transmit several blood-borne parasites that cause diseases. Among them are *Ehrlichia ruminantium*, the causative agent of Heartwater disease of ruminant animals. *Amblyomma* ticks also transmit the rickettsial pathogen, *Rickettsia africae* which causes African Tick Bite Fever in humans.

2.2 Genus *Amblyomma*

There are 126 species of ticks in the genus *Amblyomma* worldwide (Voltzit and Keirans 2003), 12 of them occurring in Africa (Walker and Olwage 1987). They make-up about 19% of the family Ixodidae (Navas *et al.* 2017). They are all characterised by a three-host life cycle. When not engorged, the tick is usually at 6 to 7mm in length, including its long mouthparts (Walker *et al.* 2003). Its body is comprised of the long mouthparts on the anterior and very ornate scuta. The basis capituli has straight lateral margins. The legs are banded and the scutum is present in females while males have a conscutum (Madder *et al.* 2010). Many of the species of the genus are ornamented with colours of the enamel varying from pink to orange to red. Fестоons and eyes are also present (Uhrquatt *et al.* 1998). The eyes are usually not housed in sockets, (Jongejan and Uilenberg 1994).

The two predominant *Amblyomma* species in Zimbabwe are *A. variegatum* and *A. hebraeum* (Norval 1983). Among domestic livestock, their main hosts are cattle, although both *A. hebraeum* and *A. variegatum* also infest the smaller ruminants such as sheep and goats. The immature stages have a wider host-range, feeding on the smaller mammals, birds and reptiles in addition to those also fed by the adults (Walker and Olwage 1987). These ticks are often described as being nidifugous, and having a very active host finding behaviour (Kelly and Mason 1991).

2.2.1 *Amblyomma variegatum* (Tropical bont tick)

Also known as the tropical bont tick due to its coloured scutum and global distribution, *A. variegatum* is one of the commonest tick species on cattle in Africa. It is widely distributed across the continent and has spread to the Caribbean (Kelly *et al.* 2011). The tick has small to medium primary punctations on the scutum/conscutum in both sexes (Jongejan and Uilenberg, 1994). Its eyes distinctly convex and ornamentation is pink to orange. The legs are banded (Madder *et al.* 2010).

Amblyomma variegatum has a multi-component pheromone, the attraction–aggregation–attachment (AAA). Blood fed male *A. variegatum* secrete the AAA pheromone which, combined with carbon dioxide, excites host finding and formation of feeding clusters of these ticks, (Sonenshine *et al.* 2000). The host range of all the ticks' stages extends from cattle, sheep and goats to large wild ruminants. Nymphs also infest birds (Walker *et al.* 2003). The predilection sites for adults are the, hairless areas around the genitalia and udders, dewlap and also the flanks (Navas *et al.* 2017). Studies on the ecology of *A. variegatum* species revealed that the tick has a single generation in a year in Zambia, where it is indigenous, while in the Caribbean, where it was imported along with cattle, it can have two or more generations per year (Pegram 1986). Although a seasonal abundance has been observed in many countries, in Zimbabwe the adults can be found all year round with infestation increasing in the warm season while nymphs are present only in the cooler months of June to September (Walker *et al.* 2003).

Amblyomma variegatum has an exceptionally broad geographic range, a phenomenon that shows its importance as a threat to the livestock industry and public health (Walker and Olwage 1987). The species thrives in many different habitats and climates, from the savanna, rain forests through to temperate areas (Norval *et al.* 1994). It's wide geographic range spans from west to east Africa, down to central and parts of southern Africa (Allsopp *et al.* 2005). Coexistence with *A. hebraeum* in the same area is regarded as a rarity. In Africa, distribution range of *A. variegatum* includes most of sub-Saharan Africa, as far south as Zimbabwe and Mozambique and on the island of Madagascar, (Sonenshine *et al.* 2000). In addition, *A. variegatum* is also established on several Caribbean islands (Kelly *et al.* 2011).

This tick is an important vector of several pathogens of livestock. The most important being *E. ruminantium*, which is the bacterial causative agent of heartwater disease in

livestock (Allsopp 2010). In humans, it transmits *Rickettsia africae* which causes African Tick Bite Fever. It also transmits *Ehrlichia bovis* and *Theileria mutans*, causing bovine ehrlichiosis and benign bovine theileriosis respectively (Radostits *et al.* 2006). *Amblyomma variegatum* is also implicated in the skin disease, bovine dermatophilosis, which is caused by the bacterium, *Dermatophilus congolensis*. Its association with this disease is due to an immunosuppression that facilitates the bacterial disease development (Chatikobo *et al.* 2009).

2.2.2 *Amblyomma hebraeum* (South African bont tick)

Amblyomma hebraeum, called the bont tick due to the pattern of its coloured scutum, is the main vector of *E. ruminantium* in southern Africa (Walker *et al.* 2003). It is identified by the presence of the primary punctation on its scutum/conscutum. The eyes are flat and close to the margin of the scutum/conscutum. The conscutum on the male has two distinct lateral areas of colour. The festoons have a uniform yellow colour (Madder *et al.* 2010).

Amblyomma hebraeum is a three-host tick. The adults and nymphs are very active host seekers. Presence of a suitable host nearby, especially if the host animal is already infested with adult male ticks excreting an attraction pheromone further promotes this behavior (Ndhlovu 2014). The adult stage mainly feeds on large hosts such as cattle, buffaloes and other large wild animals (Norval 1994). Small ruminants like sheep and goats are also infested (Jongejan and Uilenberg 1994). The usual predilection sites for this tick are the usually hairless areas under the tail, on the udder, the genitalia and the lower perineal region. The nymphs feed on both large and small ruminants including small antelopes as well as scrub hares and tortoises (Uhrquart *et al.* 1998).

Like *A. variegatum*, this tick is also responsible for transmission of *Ehrlichia ruminantium*, which causes heartwater in ruminants. It also transmits rickettsial organisms such as *Rickettsia africae* which causes African tick bite fever in humans. *Amblyomma hebraeum* also transmits *Theileria mutans* which causes benign theileriosis in cattle (Madder *et al.* 2010). Other effects of *A. hebraeum* ticks on their host are the creation of lesions that may become infected with bacteria resulting in foot abscesses in sheep and goats, and wounds that attract the blowfly, *Chrysomya*

bezziana. The larvae of the blowfly cause myiasis. A direct effect of *Amblyomma* tick infestation on the bodyweight gain in animals has been demonstrated (Norval 1989).

2.2.3 *Amblyomma gemma*

This is an *Amblyomma* species more commonly found in east Africa. *Amblyomma gemma* female has a scutum with straight sides and a wide posterior angle. The colour of the enamel is pink to orange and the eyes are slightly convex. Only 6 out of its 11 festoons have enamel (Walker *et al.* 2003). Legs are banded with pale rings of colouration (Madder *et al.*, 2010).

Amblyomma gemma is a three-host tick that prefers large herbivores including cattle and buffaloes. Although it has been demonstrated as capable of transmitting *E. ruminantium* (Walker and Olwage 1987; Allsop *et al.* 2005), it is not known as an important pest in the health of domestic livestock (Walker *et al.* 2003).



Amblyomma gemma



Amblyomma hebraeum



Amblyomma variegatum

Figure 1: Images of *Amblyomma gemma*, *A.variegatum* and *A.hebraeum* (Dr Louwtjie Snyman, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science Onderstepoort, South Africa ©)

2.3 *Amblyomma* ticks and their distribution in Zimbabwe

There was scant knowledge of the distribution of *Amblyomma* ticks in Zimbabwe before the 1970s (Norval *et al.* 1994, Peter *et al.* 1998). Traditionally, *A. hebraeum* distribution in Zimbabwe was restricted to central and southern areas of the country, with the limit being around latitude 17° south (Peter *et al.* 1998). This was probably because this tick spread to the country from South Africa in the south (Norval 1983). Over the years, it has been reported to be spreading northwards and eastwards (Sungirai *et al.* 2015). *Amblyomma variegatum* occupies a much less wider distribution in the country (Peter *et al.* 1998).

It has been reported that the first country-wide survey of ticks, conducted between 1975 and 1980 (Norval *et al.* 1992), recorded *Amblyomma* only in restricted parts of the lowveld. Simultaneously, collections of *A. hebraeum* were made from throughout the southern lowveld, western regions of the highveld and from isolated locations in the highveld along the north-eastern border. This suggested a dramatic spread over large areas of the country (Peter *et al.* 1995).

A second country-wide tick survey (1988–1991), however, documented *Amblyomma* distributions that were essentially similar to those observed in 1980. Nevertheless, collections of both *A. hebraeum* and *A. variegatum* were made from several locations within the central region of the highveld, suggesting that spread of the ticks had occurred. These collections were associated with heartwater outbreaks in this region (Norval *et al.* 1994; Peter *et al.* 1998).

To determine whether any further spread of the ticks had occurred, another country wide survey on the distribution of the *Amblyomma* ticks was conducted in 1996. The results of the survey confirmed the findings of previous surveys of *Amblyomma* distribution and suggested that some limited spread of the ticks had occurred in recent years, (Peter *et al.* 1998). Both the 1975–1980 and 1988–1991 surveys demonstrated that *A. hebraeum* and *A. variegatum* were endemic in the lowveld and well established in the western regions of the highveld. These surveys also demonstrated that *A. hebraeum* had become increasingly established along the eastern border of the country and, by 1991, was present, together with *A. variegatum*, at several locations in the central region of the highveld, (Peter *et al.* 1998). Newly infested areas appeared restricted to the central and eastern regions of the highveld, where *A. hebraeum* seemed to have spread northwards and *A. variegatum* westwards. The occurrence of

this spread is supported by the observation that recent outbreaks of heartwater in the highveld have been mostly within these regions (Sungirai *et al.* 2015).

Amblyomma hebraeum has now been reported in the eastern highlands area of Zimbabwe. This area was previously free of the tick (Madder *et al.* 2010). The climatic conditions of this area (characterised by high rainfall, relatively low annual temperatures) is supposedly uncondusive for development of the immature stages of *A. hebraeum*, making its reported presence in the area of great significance to note, (Sungirai *et al.* 2015). *Amblyomma* spread is likely to be due to animal movements. Cattle straying and wildlife movement between farms could result in local spread, while livestock translocations from *Amblyomma*-endemic regions are believed to be responsible for long-distance introductions. *Amblyomma hebraeum* was nearly eradicated from Zimbabwe in the mid-1970s due to compulsory dipping of cattle, but it quickly spread after a disruption in the dipping programs due to the civil war for independence (Norval 1983).

2.4 Rickettsial pathogens of *Amblyomma* ticks: *Ehrlichia ruminantium* and *Rickettsia africae*

2.4.1 *Ehrlichia ruminantium* – the causative agent of Heartwater disease

Ehrlichia ruminantium is an intracellular tick-borne rickettsia organism that causes heartwater disease (Ehrlichiosis) of domestic and wild ruminants (Bezuidenhout *et al.* 1994). It is transmitted by the ticks, *A. variegatum* and *A. hebraeum*. In southern Africa, the former is the principal vector and *A. variegatum* is for much of the rest of sub-Saharan Africa and the Caribbean islands, (Peter *et al.* 1999). *Ehrlichia ruminantium* is classified under the Order Rickettsiales, Family Anaplasmatacea. They are small, gram negative obligate intracellular parasites and they stain purple-blue with Giemsa (Allsopp 2010). They are coccoid in form although horseshoes, rods and irregular shapes do occur (Taylor *et al.* 2004). *Ehrlichia ruminantium* organisms are mostly found in the endothelial cells of blood vessels (Radostits *et al.* 2006). Replication is by binary fission (Allsopp *et al.* 2005). Strains of *E. ruminantium* are very diverse and vary in virulence: while some strains are highly virulent, others appear to be less-pathogenic (OIE 2017). The development of *E. ruminantium* in the ticks is not well understood. After taking in infected blood, the organism first replicates in the tick's intestinal

epithelial cells before ending up affecting the salivary glands (Bezuidenhout *et al.* 1994). Although they may be infected with *E. ruminantium*, ticks will not be infective until after 38 hours and 75 hours after attachment for nymphs and adults respectively (Allsopp 2010).

Heartwater is a fatal disease of both domestic and wild ruminants (Radostitis *et al.* 2006). It is endemic in most areas of sub-Saharan Africa and the Caribbean islands. About 150 million domestic ruminants are at risk of *Amblyomma* challenge in sub-Saharan Africa of which 114 million (76%) are at greatest risk, (Kifle and Sori 2014). Traditionally, control of the disease has hinged on using acaricide chemicals to kill the tick vectors. This has become difficult off late, due to the increasingly high cost of the acaricide chemicals to both individual stockowners and veterinary departments of countries (Meltzer *et al.* 1996). Therefore there is a need for cheaper and more sustainable ways to control this disease. For prevention of the disease, a blood-based vaccine that contains live *Ehrlichia* organisms was developed (Allsopp 2009). Despite being generally widely available across Africa, its use on the continent has been limited due to several constraints such as the need for a strict cold chain (Allsopp *et al.* 2005). Furthermore, the vaccine requires that the vaccinated animals be monitored so that any animal that shows clinical reaction to it is treated (Mukhebi *et al.* 1999).

2.4.2 *Rickettsia africae*

Rickettsia africae is the causative agent of the increasingly important emerging rickettsiosis, African tick bite fever (Jensius *et al.* 2003). African tick-bite fever has become the most common human disease caused by a spotted fever group (SFG) rickettsia, both in terms of disease incidence and seroprevalence. (Fournier *et al.* 2009). This is partly due to increased numbers of foreign visitors to rural areas and game parks of Africa. Furthermore, the host-seeking behavior of its vectors, the *Amblyomma* tick species, and its very high prevalence (which can be up to 100%) in these ticks, have also been implicated as possible reasons for the remarkable epidemiological success of this rickettsia (Fournier *et al.* 2009). *Rickettsia africae* bacterium is regarded highly adapted to its tick vector since the vector remains fit when infected and can carry very high infection rates.

The pathogen, *Rickettsia africae*, is an obligate Gram-negative bacterium that occurs freely in the cytoplasm of cells. It measures 0.3–0.5 by 0.9–1.6µm in size, (Kelly *et*

al.1996). Its structure consists of a slime layer outside and a trilaminar cell wall. It is one of the spotted fever group (SFG) rickettsia. Transmission is via the hard ticks of the *Amblyomma* genus in Africa. *Rickettsia africae* is the etiological agent of ATBF in humans (Kelly *et al.* 1996). The type strain of *R.africae* was determined by Kelly *et al.* (1996) to be strain Z9-Hu, which is an isolate that was obtained from a human with clinical tick typhus. PCR-RFLP analysis can be used to differentiate *R. africae* from the other SFG rickettsia excluding *Rickettsia parkeri*.

African tick bite fever is a human sickness that is usually characterized by symptoms such as headaches, fever, a neck muscle myalgia and eschars at the bite site. This may occur with or without a regional lymphadenitis. (Jenselius *et al.* 2003).

2.5 Infection of *Amblyomma* ticks with *Ehrlichia ruminantium* and *Rickettsia africae* pathogens

Studies on *A. hebraeum* ticks in Zimbabwe have revealed infection rates with *Ehrlichia ruminantium* at 0-45%, and 0-14% in adult ticks and nymphs respectively, (Norval *et al.* 1990). The accuracy of these estimates is however questionable because the reliability of xenodiagnostic tests that were used have not been evaluated, (Peter *et al.* 1999). In a study to find PCR based estimates of *Ehrlichia ruminantium* in *Amblyomma* ticks, Peter *et al.* (1999) found the prevalence in the adult ticks to be within the range found for *A. hebraeum* in heartwater endemic areas of Zimbabwe (0–45%) and higher than those previously determined for *A. variegatum*. In the Caribbean, infection rates of between 15.8% and 39% were found in field adult *A. variegatum* ticks (Vachierey *et al.* 2008). It was demonstrated in the laboratory that *A. hebraeum* carries higher infection rates and numbers of *E. ruminantium* strains than *A. variegatum*. *Amblyomma hebraeum* has also been implicated in many and most severe heartwater outbreaks in the field (Allsopp *et al.* 2005).

The two *Amblyomma* species, *A. variegatum*, and *A. hebraeum*, are regarded as the principal vectors of *Rickettsia africae*, (Maina *et al.* 2014). The latter, is the main vector in the southern Africa region, (Kelly *et al.* 1996). *Amblyomma hebraeum* and probably *A. variegatum* also, do not only act as the biological vectors, but also as the reservoirs of *R. africae*(Jenselius *et al.* 2003). This is done by passing on of infection from one life stage to another through transovarial and trans-stadial transmission (Jenselius *et al.*

2003). Each feeding stage of the ticks can transmit the organism, (Kelly *et al.* 1996). In Zimbabwe, the traditional *A. hebraeum* areas coincide with the highest prevalence of tick bite fever in the country and also have the highest prevalence, by sero-surveys, of rickettsial illnesses in humans, (Kelly *et al.* 1996).

The rates of infection of both species, *A. variegatum* and *A. hebraeum*, with *R. africae* have been found to be high and may be over 30% and 70%, respectively. It has been revealed that in Zimbabwe, 72% of *A. hebraeum* ticks contain different rickettsia organisms, (Beati *et al.* 1995). Ndip *et al.* (2004) also found a high infection rate of 75% in *A. variegatum* ticks in Cameroon.

CHAPTER 3: MATERIALS AND METHODS

3.1 Tick Survey

3.1.1 Study site and sampling

This study entails a cross-sectional survey on the distribution of *Amblyomma* ticks infesting cattle, in selected sites in Zimbabwe. Study locations (Figure 2) were diptanks and villages selected on previous surveys on *Amblyomma* (Noval, 1982; Peter *et al.* 1998; Sungirai *et al.* 2015) as well as the presence of cattle handling facilities and road accessibility to selected areas.

Ticks were grouped by diptank and village, and were preserved in 70% ethanol. Adult ticks were collected before cattle entered the diptank from *Amblyomma* ticks predilection sites: the ears, dewlap, groin, udder, and around the perineum.

This project has been approved by the Animal Ethics Committee of the University of Pretoria (AEC v012-17; see Appendix). Permission was obtained from the Department of Agriculture, Forestry and Fisheries, Republic of South Africa (Section 20: Reference no. 12/11/1/1/8, see Appendix). Approval for this study under Section 20 of the Animal Diseases Act (1984) was obtained from the Department of Agriculture, Forestry and Fisheries, Republic of South Africa (see Appendix). Authorisation to collect the ticks was obtained from the Division of Veterinary Service, Government of Zimbabwe (see Appendix) and ticks were couriered to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort Campus (Permit Number: 13/1/1/30/2/0-201711000677; see Appendix).

3.1.2 Sampling and Data collection

In this study, a purposive sampling technique was used (Thrusfield 2005) and five animals were selected per site. Data sheets for record keeping were completed. These sheets contained the name of the diptank, village, GPS coordinates, district and number of *Amblyomma* adult ticks collected.

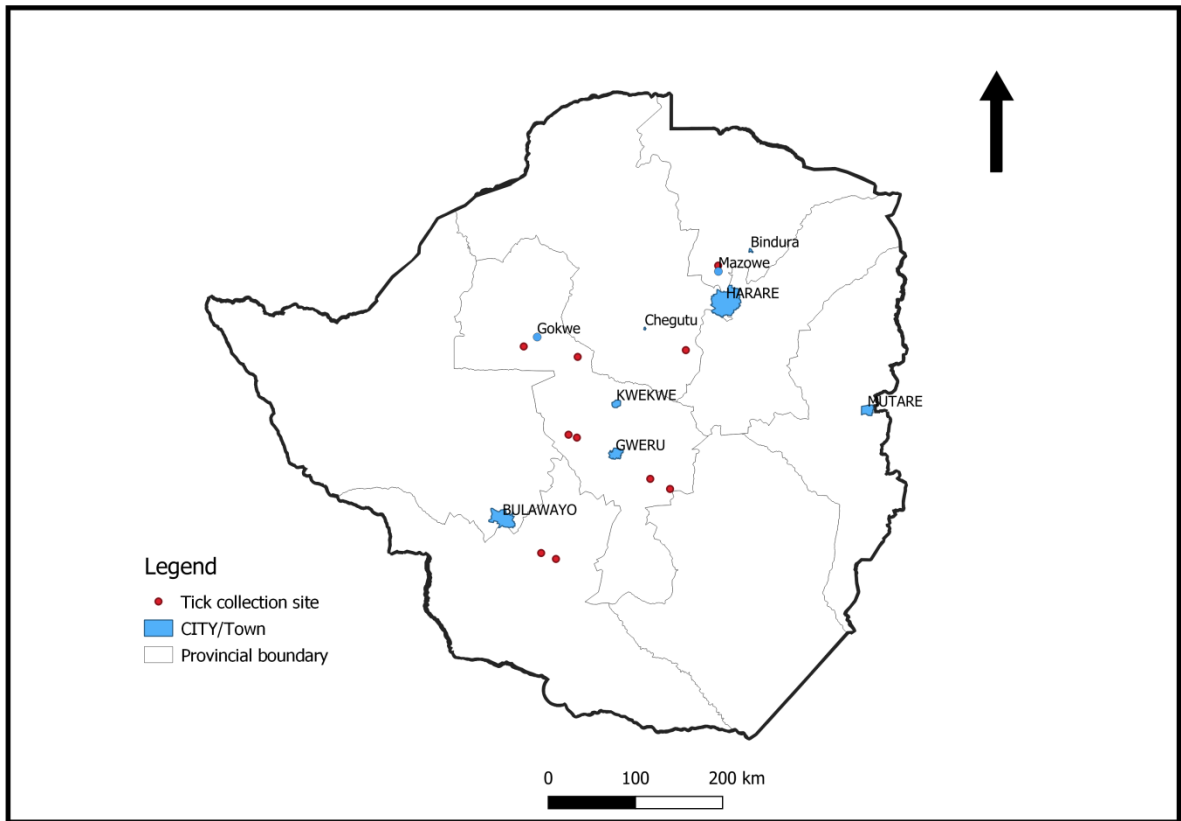


Figure 2: Map of Zimbabwe showing tick collection sites

3.1.3 Morphological confirmation of tick species

Morphological confirmation, of ticks was done at the University of Pretoria, using entomological keys as described by Walker *et al.* (2003).

3.2 Molecular detection of *Rickettsia* species

Collected ticks were also tested for presence of *Ehrlichia ruminantium* and *Rickettsia africae* nucleic acid using PCR. DNA was extracted from the ticks using a commercially available DNA extraction kit, QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's guidelines.

3.2.1 Detection of *Ehrlichia ruminantium*

A nested PCR assay, targeting the pCS20 gene region, as described by Peter *et al.* (2000) and Mahan *et al.* (2003), was used for the detection of *Ehrlichia ruminantium* in collected ticks. The first PCR consisted of primers AB128 and AB130 (Table 1), 2X PCR Master Mix (Thermo Scientific™ Phusion™ Flash High Fidelity PCR Master Mix), 2.5 µl (~ 70 ng/µl) template DNA and 10 µM of each primer to a final volume of 20 µl. PCR cycling conditions were: 98°C 10 seconds; followed by 25 cycles of 98°C for 1 seconds, 50°C for 5 seconds and extension at 72°C for 15 seconds followed by a final extension of 72°C for 1 minute. The nested PCR consists of the same master mixture as described above and the cycling conditions as the previous PCR were used with the exception of an annealing temperature of 55°C. The primers used are listed in Table 1.

In all the PCR reactions, negative (no template DNA) and a positive control of *E.ruminantium* DNA from a blood-based vaccine (Ondestepoort Biological Products Ltd.) made from sheep blood, were included. Products from the second nested PCR were analyzed by electrophoresis on a 2 % agarose gel, stained with ethidium bromide and documented using a Bio-Rad gel documentation system, (USA).

3.2.2 Detection of *Rickettsia* species and confirmation of *R. africae* in collected *Amblyomma* ticks

Collected ticks were screened for the presence of *Rickettsia* species, targeting the citrate synthase gene (*gltA*) as described by Stenos *et al.* (2015). Samples tested positive for a *Rickettsia* spp. were subjected to amplification and sequencing analysis of outer membrane protein A (*ompA*) to confirm the presence of *R. africae* nucleic acid (Kleinerman *et al.* 2013).

3.2.2.1 *Rickettsia* genus-specific real-time PCR

Real-time PCR consisted of primers CS-F and CS-R (Table 1). Each reaction contained 10 µl of each primer and probe, 2X real-time PCR master mix (Luna® Universal qPCR Master Mix, New England Biolabs Inc., USA) and 2.5 µl (~ 70 ng/µl) template DNA to a final volume of 20 µl. The reactions were performed and analysed using the Light Cycler® 2.0 Software version 4.1 (Roche, Germany). The program consisted of pre-incubation at 95°C for 1 minute followed by 45 cycles of 95°C for 10 seconds, 60°C for 15 seconds, 60°C for 1 second then cooled at 4°C for 1 second hold.

3.2.2.2 Confirmation of the presence of *R. africae* nucleic acid in collected ticks

i. Amplification of the *ompA* gene

The PCR consisted of primers Rr190.70F and Rr190.701R (Table 1). PCR consisted of the same master mixture and cycling conditions as previously described above (3.2.1) with the exception of an annealing temperature of 51°C.

In all the PCR reactions, negative (no template DNA) and a positive control of a sequence-confirmed *R. africae* DNA sample from the salivary gland pool of *A. hebraeum* collected from cattle were included. PCR products were analyzed by electrophoresis on a 1.5 % agarose gel and viewed with UV light illumination and photographed after staining with ethidium bromide, using Bio-Rad gel documentation system (USA).

ii. Sequencing analysis

Amplification products of 12 *ompA* positive samples were submitted to Inqaba Biotechnology (PTY) LTD, Pretoria, South Africa, for DNA sequencing. The sequences were aligned using BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html) (Kleinerman *et al.*, 2013) then submitted to BLASTn (www.blast.ncbi.nih.gov/Blast.cgi) (Kleinerman *et al.*, 2013) to confirm sequence similarity to *R. africae* sequences on NCBI (Yssoufet *al.*, 2014).

Table 1: Primers and probes used to amplify *Ehrlichia ruminantium*, *Rickettsia spp.* and *R. africae* from *Amblyomma ticks*

Primer name	Sequence (5'-3')	Product size (bp)	Tm (°C)	Reference
AB128	ACTAGTAGAAATTGCACAATCTAT	402	62	Mahan et al., 2003
AB130	TCTDGCWGCTTTYTGTTTCAGCTAT	402	60	Peter et al., 2000
AB129	TGATAACTTGGTGCGGGAAATCCTT	280	72	Mahan et al., 2003
CS-F	TCG CAA ATG TTC ACG GTA CTT T	74	54	Stenos et al., 2005
CS-R	TCG TGC ATT TCT TTC CAT TGT G	74	55.1	Stenos et al., 2005
CS-P	6-FAM-TGC AAT AGC AAG AAC CGT AGG CTG GAT G-BHQ-1	74	65.5	Stenos et al., 2005
Rr190.70F	ATGGCGAATATTTCTCCAAAA	632	56	Kleinerman et al., 2013
Rr190.701R	GTTCCGTTAATGGCAGCATCT	632	62	Kleinerman et al., 2013

CHAPTER 4: RESULTS

4.1 Tick Survey

A total of 194 adult *Amblyomma* ticks were collected during the study. Of these, 166 (90 males, 76 females) were identified to be *A. hebraeum*, 9 (8 males, 1 female) *A. variegatum* and 19 *A. gemma* (8 males, 11 females). The ticks were collected from 10 sites in the 7 sampled districts across the country namely; Kwekwe, Gweru, Shurugwi, Umzingwane, Gokwe, Chegutu and Mazowe. Collections were done from 5 animals with the highest infestation per collection site. An average of 19 ticks was collected per site.

Amblyomma hebraeum was found at every collection site while *A. variegatum* was found in 3 districts namely Kwekwe (Bluegum), Gokwe South (Chemawororo) and Shurugwi (Gwanza East). *Amblyomma gemma* was found in two districts namely Mazowe and Shurugwi (Gwanza East). The distribution of the tick species in the different districts and diptanks is shown in Table 2.

Table 2: Tick survey summary and molecular tests results.

District	Dip tank/ Village	Tick Species found	Number of ticks					
			Male	Female	Total	pCS20 positive	<i>gltA</i> positive	<i>ompA</i> positive
KWEKWE	Bluegum	<i>A. variegatum</i>	5	0	5	0	5	0
		<i>A. hebraeum</i>	4	0	4	1	4	0
CHEGUTU	Chivero	<i>A. hebraeum</i>	7	6	13	2	12	4
GOKWE SOUTH	Chemawororo	<i>A. hebraeum</i>	5	2	7	0	7	2
		<i>A. variegatum</i>	2	1	3	1	3	0
UMZINGWA NE	Nswazi	<i>A. hebraeum</i>	11	20	31	3	28	14
	Mkutshwa	<i>A. hebraeum</i>	21	10	31	3	24	12
GWERU	Sogwala	<i>A. hebraeum</i>	9	10	19	4	18	10
	Makhulambila	<i>A. hebraeum</i>	14	12	26	4	26	6
MAZOWE	Mazowe	<i>A. hebraeum</i>	4	1	5	2	4	4
		<i>A. gemma</i>	5	9	14	-	-	-
SHURUGWI	Nantes	<i>A. hebraeum</i>	10	9	19	7	19	2
	Gwanza East	<i>A. variegatum</i>	1	0	1	0	1	0
		<i>A. hebraeum</i>	5	6	11	0	9	2
		<i>A. gemma</i>	3	2	5	-	-	-
TOTAL :								
<i>A. hebraeum</i>			90	76	166	26	151	64
<i>A. variegatum</i>			8	1	9	1	9	0
<i>A. gemma</i>			8	11	19	-	-	-

4.2 Molecular detection of *Rickettsia* species

4.2.1 Detection of *Ehrlichia ruminantium*

A total of 166 *A. hebraeum* and 9 *A. variegatum* ticks were analysed for *E. ruminantium* infection. In the *A. hebraeum* ticks, an infection rate of 15.8% (12/76) and 15.6% (14/90) in females and males respectively, giving a combined rate of 15.7% (26/166). In *Amblyomma variegatum* ticks a rate of 11% (1/9) was found. Infected *A. hebraeum* ticks were mostly in Shurugwi district (Nantes - 7), Gweru (Sogwala and Makhulambila - 4 each) and Umzingwane district (Mkutshwa and Nswazi - 3 each). Chegutu and Mazowe had 2 infected *A. hebraeum* ticks each; Kwekwe had 1 while Gokwe South district (Chemawororo) had zero infection rate. However, the infected *A. variegatum* tick was from Gokwe South. Figure 3 shows some of the positive and negative samples of the pCS20 gene amplification and electrophoresis on agarose gel. A distinct band of the 279 bp product is shown.

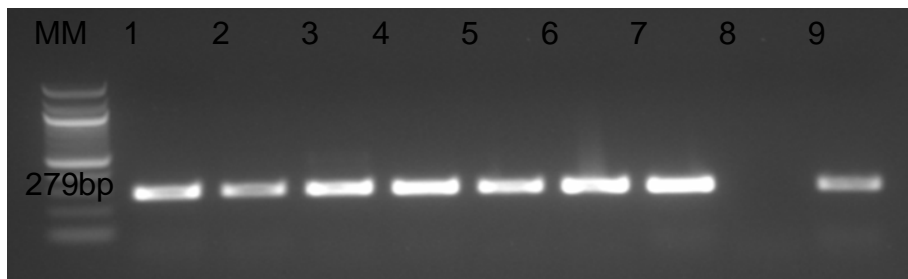


Figure 3: PCR amplified products from *Amblyomma* ticks. Amplification of a 279-basepair (bp) product of the pCS20 gene and electrophoresis on 1.5% agarose gel. Standard molecular marker (MM), negative sample (n° 8) and positive samples (n° 1-7).

4.2.2 Detection of *Rickettsia* species and confirmation of *R. africae* in collected *Amblyomma* ticks

The real-time PCR for *R. africae* *gltA* gene showed that 90.1% (151 out of 166) of the *A. hebraeum* samples were positive and 100% (9/9) of the *A. variegatum* samples were positive. Figure 4 shows the typical standard curves obtained from the amplification of some of the samples. Positive and negative samples are shown by the arrows. All the sites had samples that tested positive for the *gltA* gene. The *ompA* gene PCR was done on the 160 *gltA* positive samples and of those, 40.0% (64/160) were positive. All the 64 positive samples were *A.hebraeum* (31 females, 33 males). All *A. variegatum* samples were *ompA* negative. Nswazi and Mkutshwa (Umzingwane district) had the highest rate of *ompA* positive samples at 45.2% (14/31) and 38.7% (12/31) respectively while Bluegum (Kwekwe district) was the only site with no positive *ompA* samples. Part of the agarose gel image showing the band of PCR products from the amplification of a 530-basepair product is shown in Figure 5.

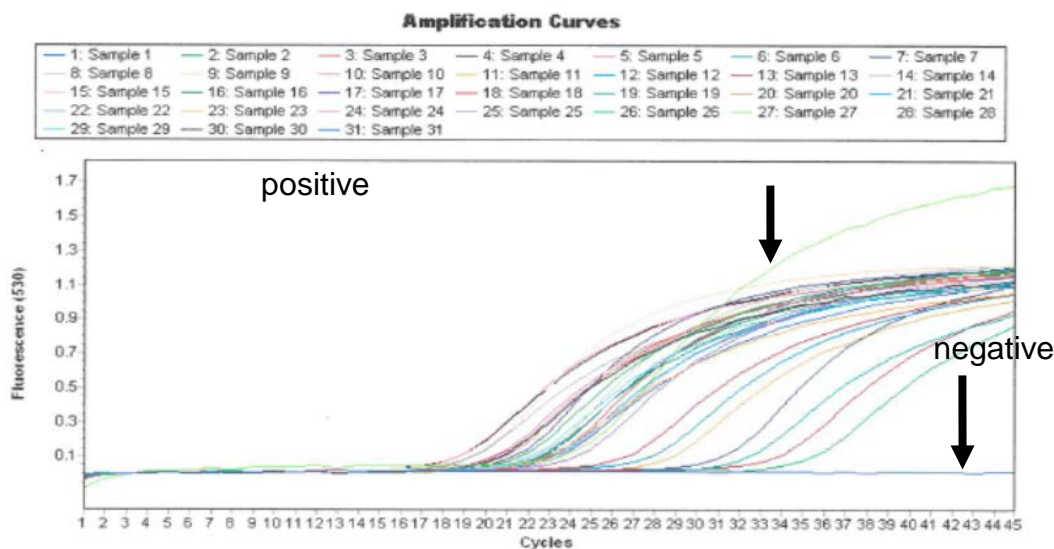


Figure 4: Real time PCR for *R. africae* *gltA* gene results showing amplification curves from 31 samples.

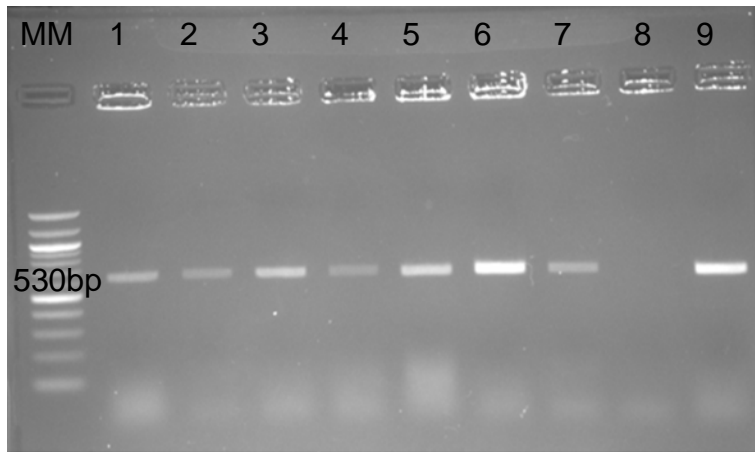


Figure 5: PCR–amplified products from *Amblyomma* ticks. Amplification of a 530bp product of the *ompA* gene on a 1.5% agarose gel.

4.2.3 Confirmation of the presence of *R. africae* nucleic acid in collected ticks

Twelve *A. hebraeum* samples (20%) (12/64) (Gweru district n=4; Umzingwane n=4; Shurugwi n=1, Gokwe South n=1, Chegutu n=1 and Mazowe n=1) positive for a *Rickettsia* spp. (*ompA*) were submitted for DNA sequencing to confirm the presence of *R. africae*. Sequence analysis showed a high similarity (98-99%) with *R. africae* sequences from GenBank - accession numbers: CP001612.1, EU622980.1 and KT 633262.1.

CHAPTER 5: DISCUSSION

This study provides an update on the distributions of *Amblyomma hebraeum* and *Amblyomma variegatum*, the important vectors of heartwater and African Tick Bite Fever in Zimbabwe. It confirms the findings of previous surveys of *Amblyomma* distribution done in the 1980s and 1990s (Norval 1983; Peter *et al.* 1998). It also suggests that some limited spread of the ticks has occurred in recent years as reported by Sungirai *et al.* (2015). Figure 6 shows the distributions of *A. variegatum* and *A. hebraeum* as revealed by a previous study by Peter *et al.* (1998) and our study.

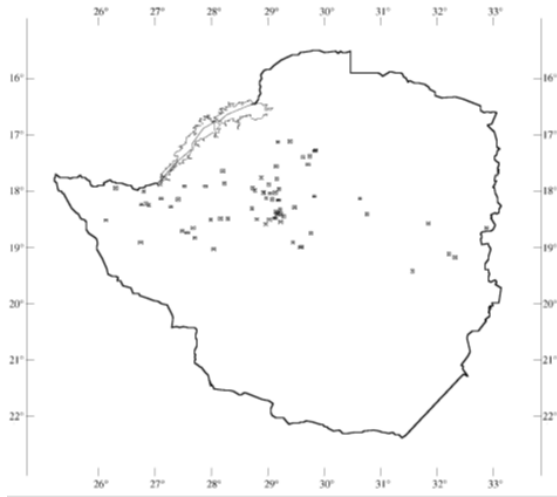
For long the distribution of *A. hebraeum* has only been to the southern areas of the country (Norval 1983), but has since been spreading to the north eastern areas of Zimbabwe (Sungirai *et al.* 2015). This was also confirmed by our study in which *A. hebraeum* was collected from cattle in Mazowe, northern Zimbabwe. Unlike the 2015 study by Sungirai *et al.*, this study did not reveal *A. variegatum* in this area. This could indicate an interspecific competition between the two species, which could have resulted in displacement of *A. variegatum* from the area.

In Mozambique, Bournez *et al.* (2015) reported that an interspecific competition prevents the spread of either species at the area of contact. It is also possible that a lack of sampling rigour (Peter *et al.* 2000) in our study prevented detection of the species. The spread of *A. hebraeum* to the northern parts of the country can be explained to be due to the anecdotal proposition that land reform programme in Zimbabwe caused changes in land ownership and farm land-use patterns (Sungirai *et al.* 2015). As a consequence, there were movements of farmers and their livestock, ultimately causing a change in tick species dynamics and distributions (Ndhlovu *et al.* 2009).

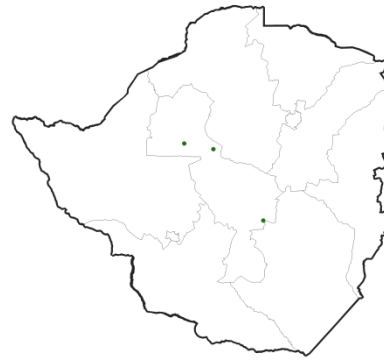
The study revealed a co-occurrence of both species in Gokwe in north-western areas and Shurugwi area in central Zimbabwe. While *A. variegatum* has previously been reported in the Gokwe area (Peter *et al.* 1998), its occurrence in Shurugwi has not been reported previously. This shows an eastward spread from its traditional areas. This overlap in the distributions of *A. hebraeum* and *A. variegatum* has been reported before in Zimbabwe, (Norval 1983; Peter *et al.* 1998). It was restricted to the northwest of the country and was persistent for over 16 years at the time of the 1996 survey (Peter *et al.* 1998). The eastward extension of this region of overlap found in this study

was also reported in the 1996 survey (Peter *et al.* 1998). It was reported in that study that there is a patchy co-existence of the two species from western Zimbabwe to eastern border with Mozambique. Thus, this study could be a further confirmation of that. Similar co-existence was observed in Mozambique in areas near the border with Zimbabwe by Bournez *et al.* (2015). It is also hypothesized that the same distribution pattern exists in Botswana to the west of Zimbabwe (Bournez *et al.* 2015). In these areas of overlap, mating between *A. variegatum* and *A. hebraeum* results in an absence of progeny (Bournez *et al.*, 2015). It has also been reported that in areas where an overlap exists, *A. variegatum* replaces *A. hebraeum* over three years (Ndhlovu 2015) although the coexistence in the western areas around Gokwe have been reported for over twenty years now. Further surveillance studies are required to monitor these and surrounding sites where both species exist so that predictions can be made about their spread, eventual distributions and interactions between them.

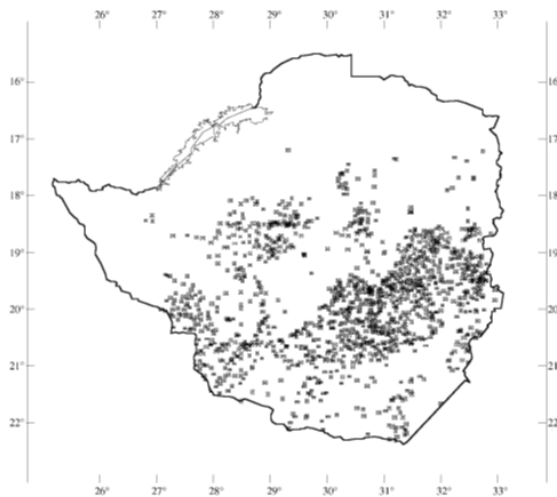
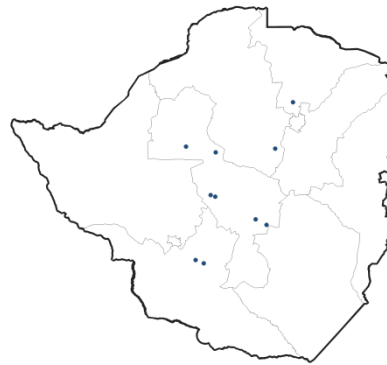
Climatic conditions have an impact on the ecology and establishment of ticks in new niches (Kleinerman *et al.* 2015). The study also found the *Amblyomma* species, *A. gemma* in two locations of Mazowe and Shurugwi. This is a novelty for Zimbabwe. This species could probably have migrated from central or east Africa where it is endemic, through cattle importations (Sungirai *et al.* 2015). This finding warrants further study to assess its geographical extent. It is thus interesting to monitor if the local climate and environment will support full establishment of this tick species in Zimbabwe. Studies to determine infections potentially spread by *A. gemma* are needed since it is a vector of heartwater in east Africa (Peter *et al.* 1999).



A. variegatum areas (Peter et al. 1998)



A. variegatum areas in our study



A. hebraeum areas (Peter et al. 1998)

A. hebraeum areas in our study

Figure 6: Maps showing the distributions of *A. variegatum* and *A. hebraeum* as revealed by our study compared to a previous study by Peter *et al.* 1998.

This study presents PCR-based estimates of *E. ruminantium* prevalence rates in adult *A. hebraeum* and *A. variegatum*. It gives an update on the infection rates of field *Amblyomma* ticks in Zimbabwe using a reliable assay (Peter *et al.* 2000). Mahan *et al.* (1998) reported infection rates of 10.5% and 12.5% in male and female *A. hebraeum* ticks respectively in Zimbabwe. In this study, the pCS20 PCR for *Ehrlichia ruminantium* revealed an infection rate of 15.7% (26/166) in the *Amblyomma hebraeum* ticks and 11% (1/9) in *A. variegatum*. This falls within the same range reported by Peter *et al.* (1999) in ticks from four Southern African countries, Zimbabwe, South Africa, Botswana and Zambia, in a previous study. Peter *et al.* (1999) also found a prevalence of 10.2% in the highveld and 11.2% in the lowveld areas of Zimbabwe. In South Africa, van der Steen (2014) found 13% of adult *A. hebraeum* ticks infected with *E. ruminantium*. Furthermore, Peter *et al.* (1999) found *Ehrlichia ruminantium* infection ranging from 6.25 to 39.3% in males and 2.1 to 14.3% in female *A. variegatum* ticks from Zambia and Zimbabwe. The recording of *E. ruminantium* infection in ticks in all the districts sampled in this study indicates that a large part of the country is potentially at risk of Heartwater. The current study showed high prevalence of *E. ruminantium* in previously non-endemic highveld areas (Norval 1983) of Mazowe and Chegutu, showing that the disease is now widespread and probably endemic in these areas too.

Several tick species, including *Amblyomma*, have been found with demonstrable *Rickettsia*-like infection before in Zimbabwe. Beati *et al.* (1995) found *Amblyomma hebraeum* to contain the highest prevalence of *Rickettsia*-like organisms among the different tick species in Zimbabwe. In this study, we found the prevalence of *Rickettsia africae* in *Amblyomma hebraeum* ticks to be 42.4%. All the *A. variegatum* ticks tested were negative. To our knowledge, the last reported prevalence of *R. africae* in ticks in Zimbabwe was 72% in *A. hebraeum* ticks by Beati *et al.* (1995). Thus this study is an update on the infection of ticks in Zimbabwe with rickettsiales.

Rickettsia africae prevalences found in this study are lower than those recorded in other African countries. In Uganda and Nigeria, SFG rickettsiae DNA was amplified in 67% and 65% of *A. variegatum* ticks respectively (Lorusso *et al.* 2013). Yssouf *et al.* (2014), found a *R. africae* prevalence of 65.17% in *A. variegatum* ticks in The Comoros Islands. It is reported that the infection of *Amblyomma* ticks with *R. africae* can even be up to 100% (Parola *et al.* 2013) indicating that the pathogen can adapt well to its host.

Twelve samples from different collection sites were selected for BLASTn analysis. In order to have a fair representation of all the sites as possible, most of the samples

were from sites that had high *ompA* positives (Gweru and Umzingwane districts). BLASTn analysis indicate a sequence identity of 98-99% to *R. africae* published sequences; from the Caribbean islands (GenBank accession number; EU622980.1) (Robinson *et al.* 2008), from Benin (GenBank accession number; KT 633262.1) (Adjou Moumuni, unpublished) and ESF-5 isolated from Ethiopia (GenBank accession number; CP001612.) (Fournier *et al.* 2009).

The findings from this study show that there is still a risk of African tick bite fever to travellers, tourists, visiting students and other foreign workers to rural Zimbabwe due to *R. africae* infection as reported in previous studies (Jensius *et al.* 2003). This is so because *Amblyomma* ticks have been proven to readily bite humans (Fournier *et al.* 1998) and have a strategy of actively seeking hosts to feed on (Sonenshine *et al.* 2000). Although this study only looked at adult ticks, there is high potential for transmission of *R. africae* to humans by the other feeding stages of the ticks (i.e larvae and nymphs) as well. This is due to an efficient transovarian (Robinson *et al.* 2009) and transstadial maintenance of *R. africae* in *Amblyomma* ticks (Ndip *et al.* 2009).

The findings from this study offer an update on the prevalence of *R. africae* in *Amblyomma* ticks in Zimbabwe. Since these ticks can feed on humans, there is a real need to consider rickettsial illnesses, like ATBF, in humans presenting with febrile illnesses that prove to be non-malarial in Zimbabwe.

Future studies on the prevalence of *R. africae* in other tick species as well as sero-surveys in humans will shade more light on rickettsial infections.

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APPENDIX



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	The distribution of <i>Amblyomma variegatum</i> and <i>A. habraeum</i> tick species in Zimbabwe and their infection with <i>Ehrlichia ruminantium</i> and <i>Rickettsia africae</i> pathogens
PROJECT NUMBER	V012-17
RESEARCHER/PRINCIPAL INVESTIGATOR	Dr. S Mandara

STUDENT NUMBER (where applicable)	U_16395540
DISSERTATION/THESIS SUBMITTED FOR	MSc

ANIMAL SAMPLES	<i>Amblyomma variegatum</i>	<i>A. habraeum</i>
NUMBER OF ANIMALS	To report tick numbers	To report tick numbers
Approval period to use animals for research/testing purposes	May 2017-May 2018	
SUPERVISOR	Prof. I Neves	

Conditions: The AEC has noted that this project will be completed in a facility outside of South Africa. Since the has not inspected the facility, please note that we cannot comment on the quality of the facility other than what provided in the study questionnaire

KINDLY NOTE:

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

APPROVED	Date	26 May 2017
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15

All communications should be addressed to
"Director Division of Veterinary Services"

Telephone: 263-4-706604
Fax: 263-4-791516
Email: nyika06@yahoo.com
Website: www.vetservices.org.zw



Reference:

DIVISION OF VETERINARY SERVICES
Ministry of Agriculture, Mechanisation and Irrigation Development
P O Box CY 56, Causeway
Harare

3 February 2017

To whom it may concern

**RE: LETTER OF PERMISSION FOR DR. S. MANDARA TO COLLECT
TICKS AT DIPTANKS FOR RESEARCH**

Dear Sir/Madam

This serves to confirm that Dr S. Mandara, a student at University of Pretoria, has been granted permission to collect ticks at communal dipping tanks run by the Division of Veterinary Services (DVS) for the purpose of his research. The division is fully aware of his research intentions and its potential benefit to the country and will help him in any way we can.

A handwritten signature in blue ink, appearing to read 'C. Njagu'.

Dr C. Njagu
Deputy Director – Epidemiology & Disease Control





agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA



IMPORTER:
PROF LUIS NEVES
UNIVERSITY OF PRETORIA
PRIVATE BAG 04
ONDERSTEPSPOORT
DVTD
0110

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
PERMIT NO: 13/1/1/30/2/0-
201711000677
Valid from: 2017-11-03
Expiry date: 2018-02-03
Valid for 3 months

**VETERINARY IMPORT PERMIT FOR TICKS TO BE INACTIVATED AT THE TAD-P
LABORATORY**

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

Authority is hereby granted for you to import 20 X 50ml BOTTLES WITH TICKS IN 70% ETHANOL:
A.HEBRAUM AND A.VARIEGATUM into Republic of South Africa:

From: ZIMBABWE
subject to the following conditions:

1. The consignment must be accompanied by this original permit and an original veterinary health certificate, complying with the conditions stipulated overleaf, duly completed and signed by an official veterinarian, authorised thereto by the Veterinary Authority of the exporting country.
2. The specimens are to be securely packed and transported in leakproof containers, sealed by an authorised official of the Veterinary Authority of the exporting country;
3. The consignment must be airfreighted through port of entry OR TAMBO INTERNATIONAL AIRPORT. **Samples may only be imported as manifest cargo under an airwaybill number and may not be imported as personal luggage.**
4. The consignment must be accompanied by this permit and its arrival reported immediately to the inspecting veterinary official: 011 393 7980 Tel: 011 973 2828, and may not be released without his/her written permission.
5. 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.
6. **The samples as stipulated above must proceed from the port of entry directly to The Foot and Mouth Disease Laboratory, TAD-P, Onderstepoort Veterinary Institute, Old Soutpan Road, Onderstepoort, 0110, RSA under guidance of a red cross permit. Dr Livio Heath must be informed of the details regarding the dispatch and estimated time of arrival of the consignment (email: HeathL@arc.agric.za; tel no. 012-5299272) well in advance of the arrival in South Africa. The treatments will be at the expense of the importer.**
7. The ticks, stored in 70% ethanol, must be placed in a water bath and subjected to a heat treatment of 57°C for 30 minutes OR any other process prescribed by TAD to render the ticks non-infectious
8. After release from TADP, the specimens must be kept and used for purposes of testing/research at the laboratories of DEPT VETERINARY TROPICAL DISEASES, UNIVERSITY OF PRETORIA under the personal supervision of PROF LUIS NEVES and may not be distributed to any other laboratory/facility without prior permission from the Director Animal Health.

SIGNATURE: _____

9. 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.
10. A copy of this import permit and the associated Veterinary Health Certificate (issued by the Veterinary Authority of the exporting country) must be retained with the laboratory submission documentation (including waybill) by the laboratory identified in condition 9 above for a period of 5 years, and made available for auditing to the Veterinary Authority upon request.
11. **This permit does not absolve the importer from compliance with the provisions of any other legislation relating to this import.**
12. This permit is subject to amendment or cancellation by the Director Animal Health at any time and without prior notice being given.
13. This permit is valid for three (3) months from date of issue and FOR ONE CONSIGNMENT ONLY.



DIRECTOR: ANIMAL HEALTH

NOTE:

- All imports for research purposes require Section 20 permission in compliance with the Animal Diseases Act.
- Any wood packing material or dunnage used in the consignment should be subjected to approved phytosanitary measures described in the IPPC: ISPM No. 15. Approved phytosanitary measures include treatment and application of official mark combined with the use of debarked wood. [Enquiries: Directorate Inspection Services Tel: 012 309 8753 Fax: 086 771 1405 or www.daff.gov.za→Branches→Agricultural production Health & Food Safety→Inspection Services→wood packaging→ISPM15]



agriculture, forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/4/1

Dr Stephen Mandara
1521 Muhacha Drive
Chiwaridzo, Bindura
Zimbabwe

Ineves17@gmail.com; Bossie.bosman@up.ac.za

Dear Dr Mandara

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

Your fax / memo / letter/ Email dated 22 August 2017, 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 *A. variegatum* and *A. hebraeum* ticks in ethanol from Zimbabwe;
4. *A. variegatum* and *A. hebraeum* ticks in ethanol must arrive in the Republic of South Africa by air and must be packaged and transported in accordance with International Air

Transport Association (IATA) requirements and/or the National Road. Traffic Act, 1996 (Act No. 93 of 1996);

5. *A. variegatum* and *A. hebraeum* ticks must be transported under red cross permit to TADD-P OVI and may only be removed from TADD-P OVI following heat inactivation;
6. Only a registered waste disposal company may be utilised for the removal of waste generated during the study.

Title of research/study: The distribution of *Amblyomma variegatum* and *A. hebraeum* tick species in Zimbabwe and their infection with *Ehrlichia ruminantium* and *Rickettsia africae* pathogens

Researcher (s): Dr Stephen Mandara

Institution: Paraclinical Building, Faculty of Veterinary Science, University of Pretoria

Your Ref./ Project Number:

Our Ref. Number: 12/11/1/1/8

Expiry date: February 2018

Kind regards



DR MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2017 -09- 28

- 2 -

CLASSIFICATION: CONFIDENTIAL

SUBJECT: THE DISTRIBUTION OF AMBLYOMMA VARIEGATUM AND A. HEBRAEUM TICK SPECIES IN ZIMBABWE AND THEIR INFECTION WITH EHRLICHIA RUMINANTIIUM AND RICKETTSIA AFRICAE PATHOGENS