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Investigating *Rickettsia africae* infection in *Amblyomma hebraeum* ticks in Mnisi, Bushbuckridge Municipality, South Africa

By

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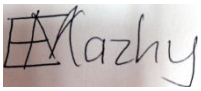
Declaration

I, Estere Mazhetese, declare that this dissertation hereby presented to the University of Pretoria for the Master of Veterinary Science degree is my own work and I have not presented it for any degree or award in any other university. All secondary material used was acknowledged and referenced as required by the University of Pretoria.

This research project was approved by the Animal Ethics Committee of the University of Pretoria on 02/08/2018 and by the Department of Agriculture, Forestry and Fisheries on the 17/07/2018.



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List of Abbreviations

ATBF	African tick bite fever
AEC	Animal Ethics Committee
BG	Bellii group
bp	Base pairs
CG	Canadensis group
cPCR	Conventional Polymerase Chain Reaction
DAFF	Department of Agriculture, Forestry and Fisheries
DEET	Diethyl-meta-toluamide
DNA	Deoxyribonucleic acid
DVTD	Department of Veterinary Tropical Diseases
ERD	Eschar rickettsial disease
gltA	Citrate synthase encoding gene
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kDa	Kilodalton
kb	Kilobyte
MIF	Micro immunofluorescence
MSF	Mediterranean Spotted fever
NaCl	Sodium chloride
NERD	Non-eschar rickettsial disease
NICD	National Institute of Communicable Diseases
ompA	Outer membrane protein A
ompB	Outer membrane protein B
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length Polymorphism
PS120	Intra cytoplasmic protein
qPCR	Quantitative Polymerase Chain Reaction
rOmpA	Rickettsial outer membrane protein A
rOmpB	Rickettsial outer membrane protein B

rRNA	Ribosomal ribonucleic acid
sca4	Surface cell antigen 4
SFG	Spotted fever group
STG	Scrub Typhus Group
TG	Typhus group
TRG	Transitional group
USA	United States of America

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DISSERTATION SUMMARY

Rickettsia africae is a gram-negative bacterium which causes African tick bite fever (ATBF) in humans. ATBF is a febrile disease mainly affecting travellers to Southern Africa. This bacterium is known to be transmitted by *Amblyomma hebraeum* and *Amblyomma variegatum* ticks. In Southern Africa, the principal vector is *A. hebraeum*. This project was performed in a rural community in Mpumalanga province and aimed at addressing knowledge gaps of *R. africae* infection in *A. hebraeum* ticks. Infection rates in adult ticks and larvae as well as transovarial transmission efficiency of *R. africae* from the tick to its offspring were determined. To accomplish this, 106 adult *A. hebraeum* ticks were collected from cattle from Utah A and 106 from Welverdiend A. Larvae (n=1060) were collected by dragging at each of the two targeted dip tanks. Engorged female *A. hebraeum* ticks (n=53) were also collected from cattle from each of the two dip tanks and they were incubated in a humidity tank to oviposit and egg masses were collected from each tick.

DNA was extracted from the engorged ticks and the egg masses as well as from the adult ticks and the larvae. After DNA quantification, a real-time quantitative PCR targeting *Rickettsia gltA* gene was performed to screen all samples for *Rickettsia* DNA. The *gltA* gene is common in all *Rickettsia* species. Samples positive for the *gltA* gene were subjected to conventional PCR targeting the *ompA* gene, which is specific for the Spotted Fever Group to which *R. africae* belongs.

The samples positive for amplicons of *ompA* gene were sequenced and all the sequenced samples were found to be 99.98% identical to *R. africae* sequences from GenBank. From the sampled adult ticks, 13.43% tested positive for *R. africae* and 13.20% of the larvae also tested positive. The infection rate for larvae and adult ticks collected from Utah A was 15.09% and those from Welverdiend A was 11.79%. From these results, there were no notable differences in the infection rates of ticks at different stages of development. *R. africae* infection rates for the two study sites were found to be similar which can be an indication of even distribution of *R. africae* in this area. Transovarial transmission was found to be 100% in engorged female ticks collected from Utah and 71.43% in ticks from Welverdiend, which indicates a high transmission rate of the pathogen to the offspring of the vector. The presence of *R. africae* in *A. hebraeum* ticks from this area is a cause of concern since there are chances of people getting ATBF after bites by these ticks.

CHAPTER 1

GENERAL INTRODUCTION

Tick-borne rickettsiosis is amongst the oldest discovered vector-borne zoonotic diseases. Studies have shown that intracellular Gram-negative coccobacilli which fall under the genus *Rickettsia*, order *Rickettsiales* are responsible for causing rickettsial diseases (Falcão, 1966; Raoult et al., 2005). Most of the *Rickettsia* species which fall under this genus are transmitted vertically by arthropods, which suggest that their vectors are also their reservoirs in the environment (Parola et al., 2013).

The *Rickettsia* pathogens which are responsible for illnesses in humans are grouped into Spotted Fever Group (SFG), Typhus Group (TG) and the Scrub Typhus Group (STG) which was reclassified as *Orientia* (Tamura et al., 1995; Toutous-Trellu et al., 2003). From the medical perspective, rickettsiosis has been divided into two groups which are eschar rickettsial disease (ERD) and non-eschar rickettsial disease (NERD) (van Eekeren et al., 2018). An eschar is an area of cutaneous necrosis resulting from rickettsial vasculitis at the tick-bite site and it is called a “tache noire” in French, which means a black spot. This eschar is usually pathognomonic of the ERD.

The *Rickettsia* which is most reported amongst those isolated in the African continent is *Rickettsia africae* and it causes African tick bite fever (ATBF) in humans which is a febrile disease (Ndip et al., 2004). The first discovery of this illness in South Africa was in the 1930s, and was described as a disease which affects people who live in the rural areas who have contact with cattle ticks (Tomassone et al., 2016). Pijper isolated the disease-causing pathogen in the 1930s but his colleagues did not agree with his findings (Althaus et al., 2010). Later on, *R. africae* was found in a case of rickettsiosis in a woman in Zimbabwe, who was reported to have spotted fever at a hospital in Chiredzi, a small town located in the south-eastern part of the country (Mediannikov et al., 2010). The pathogen was detected by serotyping, PCR restriction fragment length polymorphism analysis, protein profiling, comparison of

16S rRNA gene sequences, and genomic macro-restriction analysis (Kelly et al., 1996). This pathogen was formally named *R. africae* in 1996 (Kelly et al., 1996).

Mediterranean spotted fever (MSF), caused by *Rickettsia conorii,* presents with more severe clinical signs compared to ATBF. Despite this, these two diseases need to be carefully differentiated. *R. conorii* is transmitted by *Rhipicephalus sanguineus* (the brown dog tick). Both ATBF and MSF belong to the SFG and they are prevalent in the same parts of the African continent (Toutous-Trellu et al., 2003). In MSF, a single black spot appearing as a black crust with a red halo, which is an inflammatory border, is usually present. The red halo or eschar corresponds to the site of the tick bite (inoculation site). In ATBF, there will be several similar spots which resemble those of MSF (Toutous-Trellu et al., 2003).

The main tick-vector of *R. africae* in South Africa is *Amblyomma hebraeum* which is widely distributed in this country. The host range of this tick is diverse, including cattle, sheep, goats, wildlife and birds for the larvae and nymphs (Jensenius et al., 2003). The wide distribution of *A. hebraeum* in the communal areas of South Africa increases the chances of infections in the general population. According to Kelly (2006), *R. africae* infection rates in ticks across the African continent are around 16-75%. Maina et al*.* (2014) and Tomassone et al. (2016) reported that *R. africae* infection rates of *Amblyomma* tick vectors in endemic areas can be as high as 100%, so the chances of becoming infected by the pathogen after a tick bite are very high. All the active tick stages can transmit the pathogen to humans. Transovarial and transstadial transmission of this *Rickettsia* within the tick vector has been proved and these tick vectors also act as reservoirs of this pathogen (Fournier et al., 2009; Socolovschi et al., 2009). There is limited data on transovarial transmission efficiency of *R. africae* in *A. hebraeum*.

Cases of ATBF in humans usually occur in clusters because of the feeding habits of *Amblyomma* ticks. These ticks hide in their microhabitats and attack hosts as they appear, hence the probability that many people can be bitten by these ticks in tick-infested areas is high. ATBF cases usually present with several eschars since one person can be bitten by several *A. hebraeum* ticks at the same time (Althaus et al.,

2010). *R. africae* infections were detected in almost all of the 376 people who got tick-bite rickettsial diseases after visiting endemic areas in Southern Africa (Raoult et al., 2001, Maina et al., 2014). From the data available on ATBF, mainly middle-aged men who are more active in outdoor activities like hunting and sporting are affected and most of the patients were from Europe (Jensenius et al., 2003). Visitors to South Africa have been reported to contract ATBF where international tourism is very high. Visits to popular wildlife attractions like Kruger National Park where *R. africae* infection is endemic is the major source of infection for travellers and game hunters (Jensenius et al., 2003). Almost all the acute ATBF cases have been reported in travellers from Europe and America after visiting sub-Saharan Africa (Parola, 2006). Reports on seroprevalence rates indicate that *R. africae* has a very wide geographical distribution in the African continent although there are hardly any clinical cases of the rickettsial disease that have been documented in the native population which could be due to misdiagnosis of ATBF. Some ATBF patients do not seek treatment due to the mild symptoms leading to distorted epidemiological data. The threat of ATBF in Southern Africa is real; however, its epidemiology is not clear.

The relationship between *R. africae* infections and its tick vector continues to stimulate study interest due to its importance in travel medicine. The infection rates in tick vectors in areas where the disease is prevalent have been reported to be as high as 100% (Parola et al., 2001); however, infection rates of the ticks in South Africa are still poorly defined. Data on the *Rickettsia* in its tick vector is also scarce in South Africa hence the need for further studies on this aspect (Kelly et al., 1992). This stimulates the need to use molecular tools to detect the *Rickettsia* from different developmental stages of the tick (larvae and adults) to determine the stages that have the highest infection rates and to determine the efficiency of transmission of the pathogen from infected engorged females to their offspring. Transovarial and transstadial transmission of *R. africae* in *A. hebraeum* ticks has been proved (Socolovschi et al., 2009), but its efficiency is unknown.

1.1 Research aim

The aim of this research was to investigate *R. africae* infection rates in *A. hebraeum* ticks in a rural community in the Bushbuckridge Municipality of Mpumalanga province, South Africa.

1.1.1 Research objectives

Objective 1: To determine *R. africae* infection rates in larvae and adult *A. hebraeum* ticks.

Objective 2: To determine efficiency of transovarial transmission of *R. africae* in *A. hebraeum* ticks

1.2 Benefits arising from the research project

This project provides information about *R. africae* infection rates in *A. hebraeum* ticks at different developmental stages, thus adding to the knowledge base on the pathogen and its tick vector. The results will be shared with the scientific community through publication of papers in peer-reviewed journals allowing verification of the findings and peers in this field to continue research into other areas concerning ATBF, *R. africae* and the vector.

The information about levels of infection at different developmental stages of the tick is also crucial for public awareness since this determines the chances of contracting ATBF after being bitten by the tick vector. It is also important for tourists because they are not aware of the disease and they get sick after visiting Southern African countries. The information may also be helpful to local clinics and to the National Institute of Communicable Diseases (NICD) since they should also consider ATBF as a differential diagnosis on febrile patients, thus contributing to public health.

CHAPTER 2

LITERATURE REVIEW

2.1 History and classification of *R. africae*

The family Rickettsiaceae is named after Howard Taylor Ricketts who was the first to discover spotted fever rickettsia and he died after contracting typhus fever while performing research work (Oberoi & Singh, 2010). Pathogenic *Rickettsiae* were grouped into two groups, which are the Typhus group (TG) and the Spotted fever group (SFG). Most of these *Rickettsia* species cause illnesses in animals and humans after transmission by their arthropod vectors such as fleas, lice, mites and ticks (Parola et al., 2005). The TG consists of *Rickettsia prowazekii,* and *R. typhi,* transmitted by lice and fleas respectively. The SFG consists of about 24 species and the *Rickettsiae* in this group are transmitted mainly by ixodid ticks (Raoult & Roux, 1997). The SFG *Rickettsiae* consists of several pathogenic *Rickettsiae* which cause febrile diseases in humans in many parts of the world (Parola et al., 2005).

The main disease causing *Rickettsiae* in sub-Saharan Africa are *R. conorii, R. africae*, *R. aeschlimannii*, *R. sibirica,* and *R. massiliae* and all of them belong to the SFG and all of the SFG *Rickettsiae* are transmitted by ticks (Parola, 2006).

Worldwide distribution of the SFG *Rickettsiae* has been documented (Beninati et al., 2005). The SFG *Rickettsia* transmission to animals and humans occurs via tick saliva and their maintenance in the arthropod vectors is by transovarial and transstadial transmission or by horizontal acquisition after feeding on infected blood. The life cycle of Rickettsiae is as indicated by Figure 2.1 below. When animals are infected, they become rickettsemic and there can be infection of new tick lineages when the ticks feed on blood of such animals (Parola et al., 2005; Socolovschi et al., 2009).

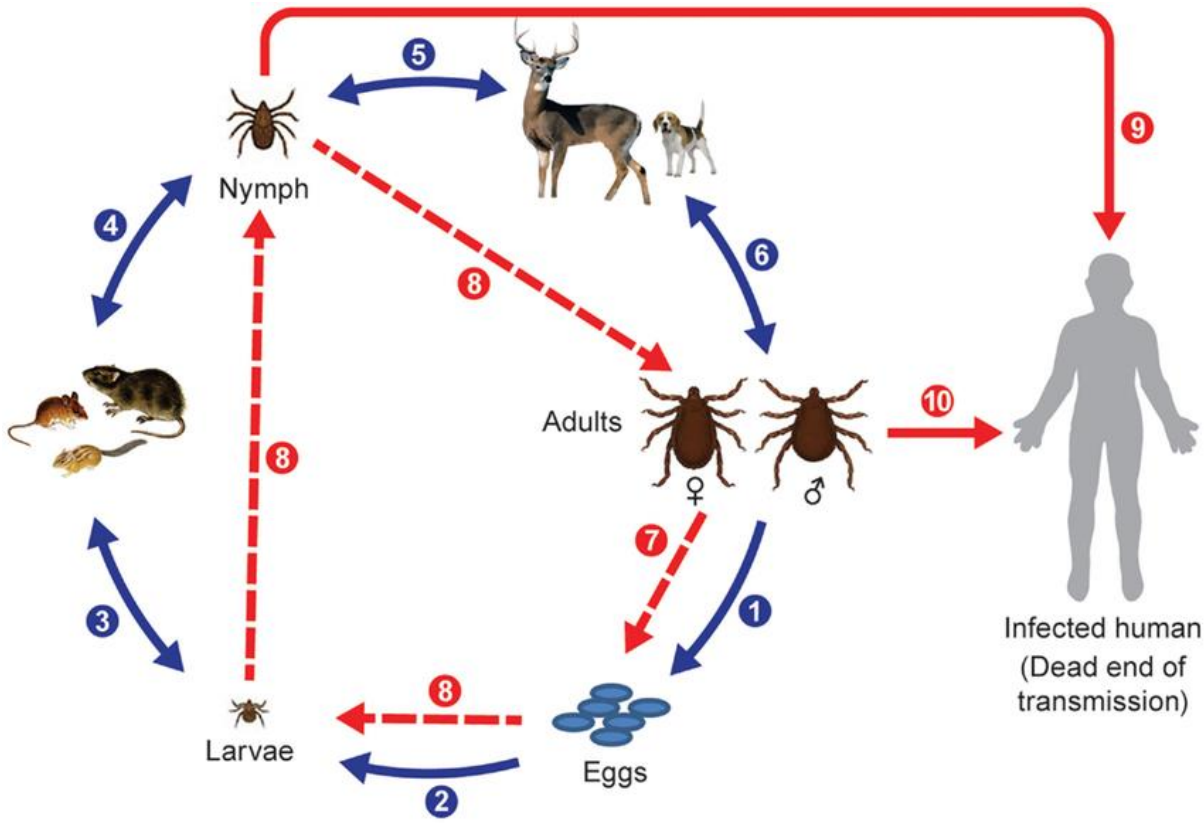

Figure 2.1: *Rickettsiae* life cycle (Eremeeva & Dasch, 2015)

Genetic diversity in *R. africae* has been reported which points to various *R. africae* variants that are documented in many different studies across Africa (Macaluso et al., 2003; Maina et al., 2014; Kimita et al., 2016). Macaluso et al. (2003) reported genotypic variation of *R. africae* in A. *variegatum* ticks that were obtained from the same herd and it was speculated that the variation was also possible for ticks collected from the same cow.

2.2 Scientific classification of *R. africae*

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Alphaproteobacteria

Order: Rickettsiales

Family: Rickettsiaceae

Genus: *Rickettsia*

Species: *R. africae*

Group: Spotted Fever Group

Classification of *R. africae* (Todar, 2006)

The relationship of *R. africae* with other *Rickettsia* species is as shown by the phylogenetic tree below (Figure 2.2).

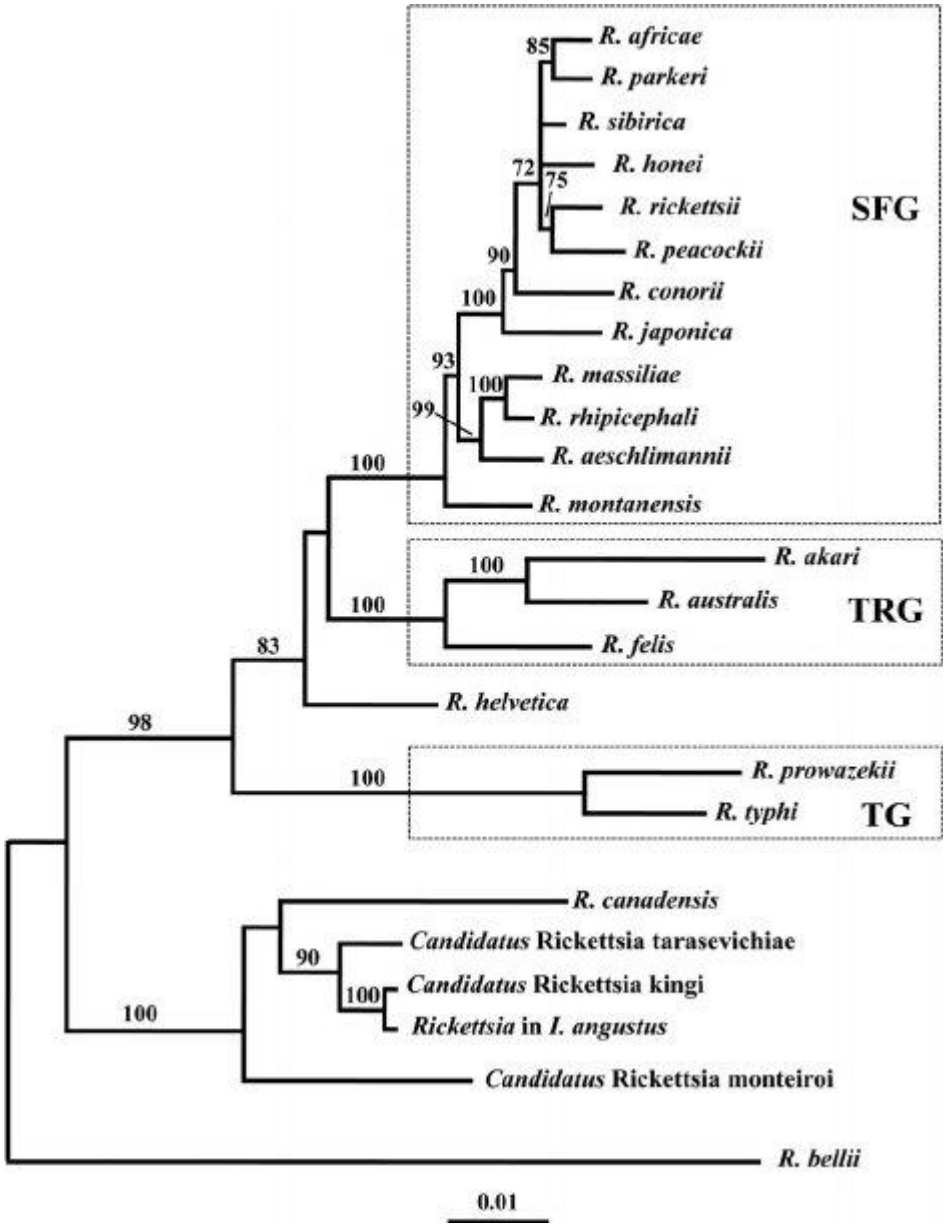

Figure 2.2: Phylogenetic tree for some *Rickettsia* species (Anstead & Chilton, 2013)

2.3 Characteristics of *R. africae*

R. africae is an obligate intracellular Gram-negative cocobacillus. It was proved by electron microscopy that this bacterium has an intracytoplasmic location and that it has an outer slime layer and a tri-laminar cell wall which has lipopolysaccharide antigens. These antigens are extremely immunogenic and are responsible for extensive cross reactivity with other SFG *Rickettsiae* (Jensenius et al., 2003). Species-specific protein antigens comprise the high-molecular-weight rickettsial outer membrane protein A (*rOmpA*) and B (*rOmpB*). The bacterium cannot be cultured in cell-free media; however, it can grow in yolk sacs of developing chicken embryos, and in cell cultures. The bacterium genome size is approximately 1228 kb (Kelly et al., 1996).

R. africae is a pathogen that causes febrile illness in its human host, referred to as ATBF. *R. africae* detection and isolation from humans previously bitten by ticks and showing signs consistent with ATBF is possible. Usually the eschar is used to culture this organism. *R. africae* was isolated from heparinised blood collected from an ATBF patient (Kelly et al. 1992). The DNA of this bacterium can also be isolated from the tick vectors.

This bacterium is of great importance in many parts of Africa, including South Africa, in terms of incidence and prevalence and also for causing ATBF which is poorly understood yet its known to affect both the indigenous population and foreign travellers (Kimita et al., 2016). No clinical signs of disease have been reported in animals although it was hypothesised that they play a major role in the maintenance of the *Rickettsiae* (Kelly et al., 1991).

2.4 Vectors of *R. africae*

2.4.1 *Amblyomma* vectors of *R. africae*

The ticks known to be vectors of *R. africae* in Africa belong to the genus *Amblyomma* of hard ticks (Ixodidae family). *A. hebraeum* (the Southern African bont tick) and *A. variegatum* (the tropical bont tick) are the main vectors transmitting this rickettsial pathogen (Jensenius et al., 2003). These two tick species are vectors and

reservoirs of *R. africae* where the infection is sustained through transovarial transmission, a process whereby the pathogen is transmitted from an infected adult female to the offspring and transstadial transmission, a process whereby the pathogen is maintained within the tick vector from one developmental stage to the next (Socolovschi et al., 2009). It is not clear as to how many generations can maintain the *Rickettsiae* in the absence of an infected host. *R. africae* may be very successful as a bacterial pathogen and it has very high transovarial transmission. This has been speculated from the high infection rates of the ticks as compared to those of other *Rickettsiae* (Fournier et al., 2009). *R. africae* was detected in ticks that were collected from non-rickettsemic animals and this may be because this pathogen is an endosymbiont of these tick vectors (Maina et al., 2014).

R. africae was also detected in *A. lepidum*, *A gemma*, *A. cohaerens*, and *A. compressum* in Sudan and Djibouti (Socolovschi et al., 2009), in the Somali region of Ethiopia (Tomassone et al., 2016; Kumsa, 2019) and in Liberia (Mediannikov et al., 2012). Furthermore, *R. africae* was also detected in *A. loculosum,* a tick usually known for infesting marine birds in tropical islands and infected ticks of this species have been found outside Africa, in countries like New Caledonia (Eldin et al., 2011; Dietrich et al., 2014, Parola et al., 2013). *R. africae* DNA was found in *A. ovale* ticks collected from dogs in Nicaragua, Central America, in 2013. *R. africae* was previously reported in the Caribbean (Kelly, 2006), but this was the first report on *R. africae* in continental America (Vogel et al., 2018). Given the documented distribution of *R. africae* among African *Amblyomma* species, it is reasonable to infer that all African *Amblyomma* species could be competent vectors of this pathogen. However, this assumption should be confirmed.

2.4.2 Other tick species found carrying *R. africae* DNA

A study done in Union of the Comoros in 2014 reported *R. appendiculatus* ticks carrying *R. africae* DNA (Yssouf et al., 2014). The detection of *R. africae* in these areas can be due to importation of cattle from countries like Tanzania into the Comoros which facilitates movement of ticks and associated pathogens into new areas (Yssouf et al., 2014). The *Rickettsia* positive *R. appendiculatus* ticks were

collected from animals infested with known vectors of the *Rickettsia*, *A. variegatum* or *A. hebraeum,* which also tested positive for *R. africae.*

Kolo et al*,* (2016) detected *R. africae* DNA in *Haemaphysalis elliptica* ticks collected from dogs in Mpumalanga province and another study reported presence of *R. africae* in ticks collected from dogs and cats from Free State, KwaZulu-Natal and from North West provinces (Mtshali et al., 2017). *Rhipicephalus (Boophilus) decoloratus* ticks collected from oryx in Botswana were also found carrying this pathogen (Portillo et al., 2007). *R. africae* was also detected in *Haemaphysalis paraleachi* ticks from Guinea (Mediannikov et al., 2012), *Hyalomma dromedarii* and *Hyalomma marginatum* ticks from camels in Algeria and Egypt (Abdel-shafy et al., 2012; Kernif et al., 2012); as well as in *R. (Boophilus) decoloratus* (Hornok et al., 2014), *R. (Boophilus) geigyi* (Mediannikov et al., 2012) and from *R. sanguineus sensu lato* (Ogo et al., 2012).

Available data does not give clear evidence on vector competence of any other tick species besides *A. hebraeum* and *A. variegatum* for *R. africae.* It was hypothesised that the other ticks that tested positive for *R. africae* acquired the pathogen during a blood meal and probably the *Rickettsia* DNA would have degraded after a few days if the tick vectors could not support the *Rickettsia* biological cycle (Portillo et al., 2007; Yssouf et al., 2014). Further investigation to ascertain vectorial competence of the other ticks is needed.

2.5 Life cycle of *A. hebraeum*

A. hebraeum is a three-host tick and has three active stages, the larva, nymph, and adult as shown in Figure 2.3 below. All three stages attack humans and if they are infected with *R. africae*, they then transmit it to humans. The tick vectors usually attach to people in the groin and axilla regions as well as behind the knees. The larvae are very small hence are difficult to see on skin and clothes. According to Mediannikov et al. (2010), adult *A. hebraeum* ticks do not usually feed on humans; however, nymphs and larvae attach more frequently and larvae are abundant and aggressive. The adult stages of the tick are very easy to see since they are highly

ornate with brownish and reddish colours. The engorged adult female tick may be as big as a grape. All the larval and nymphal stages need a blood meal for them to moult into the next developmental stage (Jensenius et al., 2003). The females should also feed on blood and become fully engorged for them to lay eggs and they die after they oviposit.

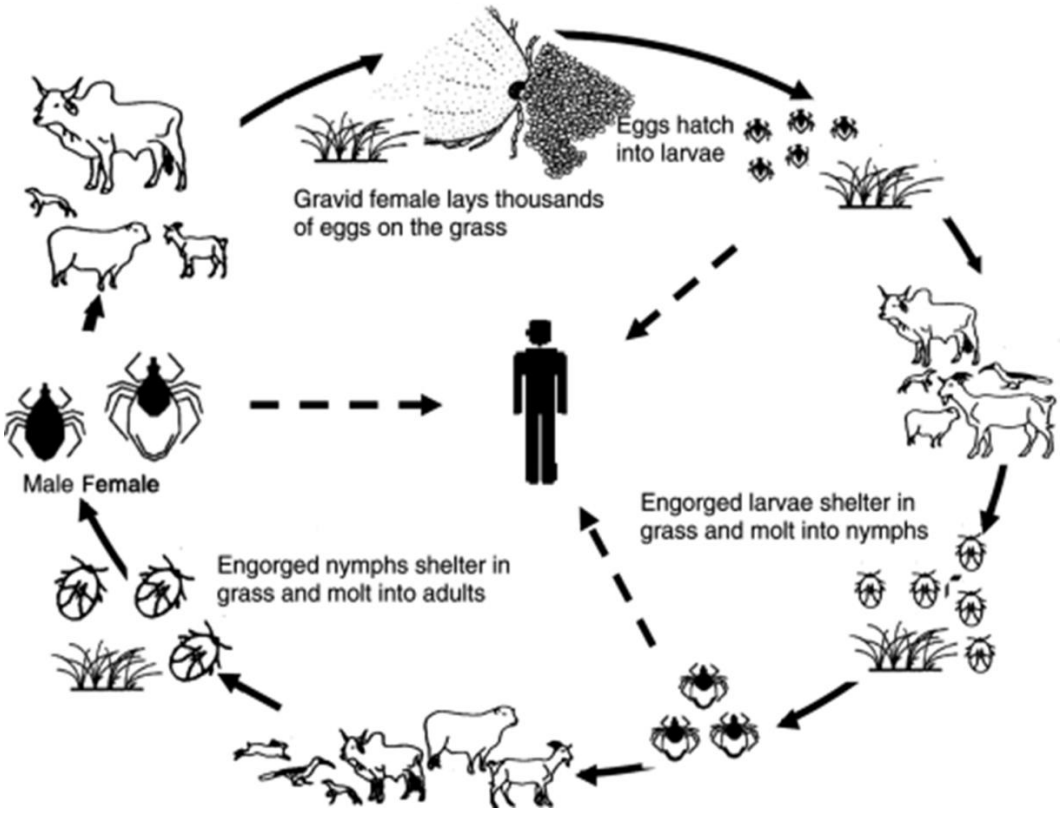

Figure 2.3: Life cycle of *A. hebraeum* (*R. africae* vector in Southern Africa) (Parola et al., 2001)

The adult ticks are most predominant during the rainy season and the nymphs during winter; however, the adult stage can be active throughout the year, although their numbers vary seasonally. From a study done in Zimbabwe in 1991, it was reported that *A. hebraeum* adult male ticks usually attach to hosts for long periods of up to four months, unlike the larval and nymphal stages and the adult females (Norval et al., 1991).

Unfed adult ticks and nymphs seek hosts despite harsh weather conditions. These stages will be hiding in their protected microhabitats and they respond to specific stimuli which can be carbon dioxide and the aggregation-attachment pheromone

produced by males whilst attaching to hosts (Norval et al., 1991). Just like *Hyalomma* ticks, *Amblyomma* ticks are quite aggressive and they actively seek out hosts (Althaus et al., 2010). This behaviour arguably explains the presence of *A. hebraeum* ticks on hosts throughout the year in different parts of the African continent. However, ATBF was reported to be seasonal, with high incidences in November to April. This may be due to the peak in the activity of the ticks (larvae and adults), during the rainy season (Tsai et al., 2009).

2.5 Hosts of *A. hebraeum*

Cattle among other domestic livestock are considered to be an important host for *A. hebraeum*. These ticks also feed on sheep, horses, donkeys, and pigs (Jensenius et al., 2003). The adult stages of *A. hebraeum* ticks also feed on wild ungulates such as giraffes, buffalo, antelope, and warthogs. These wild ungulates possibly play a role in the maintenance of the *Rickettsia* in areas where domestic animals are dipped intensively or where they are not available (Jensenius et al., 2003). Hosts for larvae and nymphs are the same as those for adults; however, they can also feed on lizards, small mammals and ground-feeding birds. Humans are accidental hosts for these ticks.

There are reports of *R. africae* DNA being amplified in ticks from areas where the pathogen is not known to exist. This has been attributed to livestock movement from disease endemic areas to disease free areas resulting in the spread of infected ticks as well as introduction of rickettsemic animals since the mammalian host competency of *R. africae* has also been proposed (Kelly et al., 1991). Migratory birds also introduce infected ticks to disease free areas since some ticks like *A. loculosum* that are known to infest birds were found carrying *R. africae* DNA (Eldin et al., 2011; Dietrich et al., 2014).

2.6 Transmission and distribution of *R. africae* and its vectors

R. africae has been reported to be found mainly in Africa and in the West Indies. In the West Indies, it was found in Guadelope, Martinique, St. Kitts, Neves and in the Antigua islands (Fournier et al., 2009). The distribution of *R. africae* corresponds with

the distribution of the tick vectors, so the pathogen is reported to be found in all those areas where the tick vectors are found (Scarpulla et al., 2016). However, there are several other tick species other than the known vectors that were also found carrying *R. africae* DNA but their competence as vectors still remains to be proved.

A. hebraeum is found in tropical and subtropical environments. *A. hebraeum* and *A. variegatum* ticks have preference for similar habitats with tall grass or shady bushes. Both tick species were found to be present the whole year in most infested areas and they are found in great numbers throughout the rainy season (Jensenius et al., 2003). *A. hebraeum* geographical distribution is different from that of *A. variegatum*. The distribution of *A. variegatum* is mainly in the communal areas of west, central, and east Africa, south of the Sahara desert, Yemen, several islands in the Indian Ocean, on the Cape Verde Islands in the Atlantic Ocean, and in the eastern Caribbean, whereas *A. hebraeum* is only distributed in Southern Africa.

In South Africa, *A. hebraeum* has widespread distribution and this tick vector has been reported along the coast of the Indian Ocean, KwaZulu-Natal province, and north eastern parts of the country where many popular wildlife tourist resort areas are located (Walker & Olwage, 1987). *R. africae* was detected by PCR and sequencing in *A. hebraeum* ticks collected from dogs at a household in Mnisi community, Mpumalanga province, South Africa (Kolo et al., 2016). This is a clear indication that the *Rickettsia* is prevalent in this rural area of South Africa. Furthermore, *A. hebraeum* has been found in the eastern part of Botswana, southern part of Mozambique, and in the Lowveld of Zimbabwe (Peter et al., 1998). These two tick species have similar requirements; however, they are found in different geographical locations as shown by the map (Figure 2.4) below (Jensenius et al., 2003).

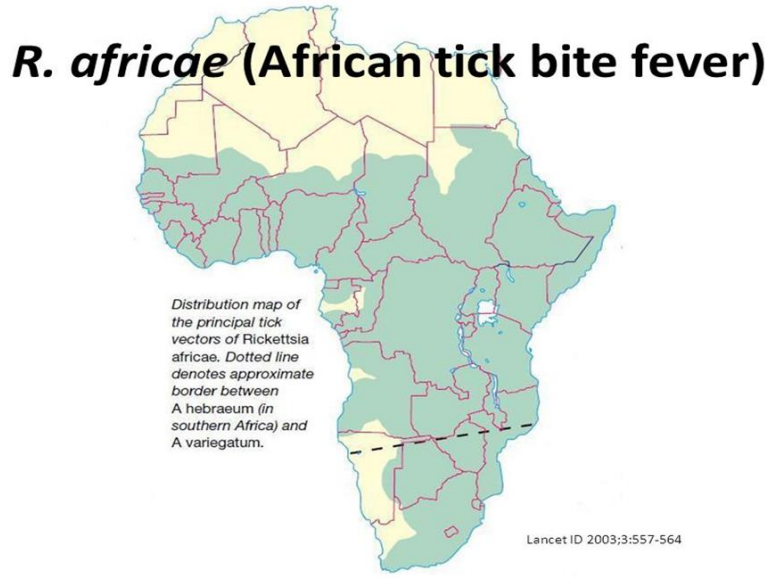

Figure 2.4: Distribution of the principal tick vectors of *R. africae* (*A. hebreaum* and *A. variegatum*) (Jensenius et al., 2003)

Cases of ATBF associated with visits to east African countries are few. This could be due to the less virulent *R. africae* variants that are found in those areas (Harrison et al., 2016) and also to the lower infection rates of *R. africae* in *A. variegatum* ticks which are the vectors in that part of the continent.

The rates of *R. africae* infection in the tick vectors have been documented to be approximately 30% in *A. variegatum* ticks and it can be as high as 80-100% in *A. hebraeum* ticks (Parola et al., 2001; Jensenius et al., 2003; Nakao et al., 2013). On average, *R. africae* infection rates in these tick vectors across Africa has been reported to be around 16-75% (Kelly, 2006). Halajian et al. (2018) reported an infection rate of 13% in ticks collected from donkeys in Limpopo province, South Africa. A recent study performed in Zimbabwe reported *R. africae* infection rate of 42.4% in *A. hebraeum* ticks collected from different parts of the country (Mandara, 2018). In a study done in Uganda and Nigeria, an infection rate of 45% was reported in *A. variegatum* ticks (Lorusso et al., 2013). *R. africae* infections across Africa have been found to vary from area to area (Kelly, 2006). The high infection rates indicate higher chances of these ticks transmitting the pathogen to humans and animals.

2.7 Clinical signs in animal hosts

No clinical signs of disease have beenreported in animals, although there are some indications for their possible role as reservoirs of the *Rickettsia*. The role of cattle and goats in *R. africae* epidemiology was investigated (Kelly et al., 1991; Kelly et al., 1991) .

Kelly and colleagues performed a study in Zimbabwe to determine the pathogenicity of SFG in domestic cattle in 1991. Cattle experimentally infected with cultured *Rickettsia*-like organisms showed mild clinical signs such as regional lymphadenitis, dermal erythema, edema, and tenderness at the inoculation site. This could be an indication that the *Rickettsia* only cause subclinical infection in cattle (Kelly et al., 1991), but this assumption still needs to be confirmed. Further investigation was performed by inoculating blood of the experimentally infected cattle into seronegative guinea pigs to determine if the cattle were rickettsemic after inoculation. Infection in the guinea pigs was determined by measuring scrotal circumference and body temperature. The guinea pigs had scrotal swelling and fever, and therefore seroconversion was assumed to have occurred. Rickettsemia was detectable in experimentally infected cattle for at least 32 days post-infection. The results from this study could mean that cattle could be possible reservoirs for *R. africae.* It was hypothesised that infection of new tick lineages could arise if ticks feed on such animals (Kelly et al., 1991). However, further investigation into this issue is necessary considering that Maina et al. (2014) also reported the presence of *R. africae* in *A. variegatum* ticks that were obtained from non-rickettsemic animals. This could be an indication that this *Rickettsia* is mainly maintained in the tick vector in the environment. The common use of antibiotics in animals has been proposed as a reason for rickettsemia not being detected in the animals (Maina et al., 2014). A similar study reported a brief detection of rickettsemia in goats and it is not certain if susceptible ticks can pick up the *Rickettsia* within that period (Kelly et al., 1991).

There is limited information on the ecology of *R. africae* as far as the reservoir hosts are concerned. Some studies have reported high seroprevalence rates of SFG *Rickettsia* in cattle, goats and sheep from endemic areas and this is an indication of previous exposure to the *Rickettsia*. In a study done by Kelly in 1991, it was reported

that 80-100% of cattle in affected areas have antibodies against SFG *Rickettsia*. In another study performed in the southern part of Zimbabwe where *R. africae* is also endemic, a 100% presence of antibodies for SFG *Rickettsiae* in sampled cattle was reported (Parola et al., 2005). Maina et al., (2014), also reported presence of antibodies against SFG *Rickettsia* in cattle, sheep and goats from Kenya. The high seroprevalence rates could also be due to cross-reaction of the SFG so it cannot be attributed to *R. africae* alone; however, this hypothesis should be investigated. It is not yet clear if these animals are competent reservoirs for this pathogen.

2.7 Clinical signs in human hosts

2.7.1 African Tick Bite Fever (ATBF)

Clinical cases of ATBF are mostly associated with foreigners visiting Southern Africa for leisure activities such as safari and game hunting (Mediannikov et al., 2010), hence ATBF is referred to as a travel associated disease (Jensenius et al., 2003). The incidence rates of ATBF are high because *R. africae* is transmitted to humans by all three active stages of the tick (Kelly & Mason, 1991). ATBF is known to be a self-limiting disease; however, it has been reported that complications also occur especially in elderly ATBF patients (Nilsson et al., 2017).

2.7.2 Risk factors of ATBF

Reports have indicated that the feeding behaviour of *A. hebraeum* has a great effect on the occurrence of ATBF. These tick vectors are found in several environments and they usually prefer humid environments where there are tall grasses and bushes. ATBF has been reported to be affecting people from other countries especially travellers from European countries when they visit endemic areas and almost all the clinical cases have been documented in international travellers. Information on the habitat types that are associated with this disease is very limited (Ndip et al., 2011).

In addition to their environmental preferences, the leisure activities that the tourists engage in usually increases their chances of getting the infection, considering that the ticks respond to several host stimuli. In a research done on 940 people from

Norway who were on a hunting expedition in South Africa, the distribution of infection by ATBF were reported to be different depending on the activities they were doing and hunting was associated with most of the ATBF cases. This was hypothesised to be due to the association of the hunting activity with substantial exposure of the individuals to ground vegetation, grasses and shrubs as well as with ungulates and their hides. Therefore, the hunting activity plays a major role in exposing people to ATBF (Ndip et al., 2011).

In 1997, there was an outbreak of ATBF in tourists from France (n=13) after they visited Lesotho and South Africa for adventure racing (Fournier et al., 1998). From a cohort study, which was done in 2011, it was concluded that these rickettsial infections are directly linked to outdoor activities like safari and hunting. About 50% of cases of affected tourists who visited endemic areas in sub-Saharan Africa or the West Indies, were microbiologically confirmed as ATBF (Jensenius et al., 2003).

2.7.3 Pathogenesis of ATBF

The incubation period for this disease is about 5-7 days, but it can extend up to 10 days (Raoult et al., 2001). *R. africae* is transmitted to humans via tick bites and the pathogen is deposited in the blood vessels by the tick whilst they feed. It then multiplies in the endothelial cells which also damages the local skin cells thereby causing dermal and epidermal necrosis resulting in eschars. The destruction of endothelial cells also results in the increased production of endothelium-associated mediators, which include the von Willebrand factor and the soluble E-selectin. The *Rickettsia* spread via blood throughout the body resulting in many endothelial cells being infected. Further destruction of the vascular endothelial cells and infiltration of perivascular mononuclear cells often lead to increase in leakage of fluid into the interstitial space and also results in perivascular infiltration of T-cells and macrophages. The perivascular infiltration results in a lymphohistiocytic vasculitis which is the histopathological hallmark of the rickettsiosis (Jensenius et al., 2003). Eruptive fevers associated with SFG *Rickettsia* are due to the ability of the pathogen to multiply in the endothelial cells (Scarpulla et al., 2016).

2.7.4 Clinical signs of ATBF

ATBF is a febrile illness hence it is mainly characterised by fever and other flu-like clinical signs such as nausea, fatigue, headache and myalgia, which usually affects the neck muscles. ATBF is a self-limiting disease, meaning it can clear without treatment, hence usually the clinical signs are not very prominent (Jensenius et al., 2003; Maina et al., 2014).

Tick bite sites are noticeable and these have been described as the inoculation eschars, consisting of a central black crust surrounded by a red halo (Jensenius, et al., 2003). The eschars are usually located on the legs, however, vulvar mucosal eschars have also been reported (Consigny et al., 2009). Generalised cutaneous rash is also common and it appears in two to three days (Toutous-Trellu et al., 2003). These characteristic lesions are often multiple and are associated with regional lymphadenopathy. Up to 34 eschars were found in a single ATBF patient, after he went on a safari in eSwatini (formerly known as Swaziland) (Caruso et al., 2002). The reason for the multiple eschars on ATBF patients is due to the feeding habits of *A. hebraeum*. They hide in their microhabitats and attack hosts as they appear, resulting in a single host being infested by several ticks at a time. The aggressive nature of these ticks has been reported to result in clustered cases (Caruso et al., 2002; Jackson et al., 2004; Oostvogel et al., 2007). In these reports, all the travellers on safari were diagnosed with ATBF on returning home.

Reactive arthritis has been recorded in some ATBF cases (Jensenius et al., 2003). From a recent report by Nilsson et al. (2017) , ATBF patients presented with a mild transient thrombocytopenia. Elevation of C-reactive protein and liver enzymes was also reported by laboratories. Complications like chronic fatigue, reactive arthritis, encephalitis, myocarditis and cellulitis after ATBF were also reported and usually associated with old age (Nilsson et al., 2017). Bellini et al. (2005) reported a case of ATBF which was complicated with cardiac disease. Zammarchi et al. (2014) reported a case of complicated ATBF in a 40-year-old Italian traveller returning from Zimbabwe. The woman had a painful sacral syndrome characterised by severe pain on the leg, urinary and faecal incontinence and rectal tenesmus which was mainly

attributed to immune mediated mechanisms. Neurological complications were also reported in two Swiss travellers (Althaus et al., 2010).

The disease can also be misdiagnosed for other diseases which present with fever such as malaria and typhoid, especially in developing countries of Africa where proper diagnostic laboratories and facilities are limited. A 31-year-old South African man got sick a few days after arriving in Minnesota, USA, where he had gone to work on a farm. He was working at a farm in KwaZulu-Natal where he was looking after sheep and cattle before leaving the country for the USA. He presented with fever, headaches and myalgia but tested negative for malaria. He was treated with amoxicillin but he did not recover. Several tests were done on second presentation. The tests that were done include lumbar puncture, HIV and dengue virus serology. On physical examination, an eschar was noticed just below his knee. ATBF was diagnosed based on history of tick bites in South Africa and the eschar that was noticed as well as response to doxycycline treatment (Johnson et al., 2019). The report of ATBF in a South African citizen soon after leaving South Africa can be an indication that several ATBF cases are being misdiagnosed in most African countries. ATBF cases are rare among indigenous people yet high seroprevalence rates are reported (Jensenius et al., 2003). According to Maina et al. (2014), seroprevalences of up to 55% in eastern and Southern Africa and up to 51% in western and central Africa have been reported. They speculated that the high seroprevalence rates could be due to the fact that *R. africae* and other SFG *Rickettsia* share common antigenic determinants. Thus, the high reactivity described in the available literature can be as a result of other *Rickettsia* species as well and not due to *R. africae* only.

It is suspected that the disease affects the indigenous population when they are still young and clinical signs are very mild symptoms or subclinical such that they usually do not go to hospitals to seek medical attention, hence the infection goes unnoticed (Kelly & Mason, 1991). Furthermore, it is difficult to diagnose the disease in the indigenous population because the inoculation eschars are not easily noticeable on pigmented skin; hence, such pathognomonic features of the disease are easily missed, which can lead to a misdiagnosis. Thorough examination and history taking

on previous exposure to ticks is always recommended if this disease is to be correctly diagnosed. The diagnostic tests which are recommended for ATBF are scarce in most of the disease endemic areas due to their costs hence the disease is poorly diagnosed (Jensenius et al., 2003).

2.7.5 Diagnosis of ATBF in humans

2.7.5.1 Serology

Currently, the routine laboratory diagnosis of this disease is based on serology; in particular microimmunofluorescence (MIF). The MIF will detect an increase in IgG or IgM antibodies in the bloodstream. Immunofluorescence assays are recommended for the diagnosis of ATBF but seroconversion is usually delayed and it may take up to three weeks in these patients, thus limiting the effectiveness of this test (Jensenius, et al., 2003). Seroconversion may not occur in mild cases and in cases that get early treatment with the recommended antibiotics (Fournier et al., 2002). The other drawback of serology is the issue of cross-reaction with other SFG *Rickettsiae,* hence it will be difficult to really ascertain if the illness was due to *R. africae* by merely using serology (Socolovschi et al., 2012).

Some of the serological tests which are more reliable and specific are very expensive and they may only be found at reference centres. For example, the multiple-antigen immunofluorescence assay in which reactions to several SFG rickettsial antigens, including *R. africae*, can be compared directly (Jensenius et al., 2004). Western blotting, a process whereby species-specific antibodies targeting high-molecular-weight proteins of the pathogen, which are *rOmpA*, *rOmpB* and PS120, are detected can be used as well as the cross-adsorption assays; a process whereby the infecting species may be determined by removing non-specific antibodies.

2.7.5.2 Culture and antigen detection

Biopsies or cultures of a person's eschar are also used to diagnose ATBF because as the *Rickettsia* multiplies at inoculation sites, eschars become the reliable place for collection of biopsy specimens for *Rickettsia* isolation and genomic detection.

Culturing of the *Rickettsia* is done in laboratories with biohazard protection level three (BSL-3). This is the commonly used method for primary isolation of this organism in fibroblasts. The samples should be transported on dry ice and it should be done within a very short period because the recommended duration between sample collection and inoculation of cell culture is 24 hours (Jensenius et al., 2003). The sensitivity of isolation of *R. africae* in ATBF is generally poor, and it is usually below 15% in heparinised blood and approximately 44% in skin-biopsy specimens.

Detection of *R. africae* antigen can be done by immunohistochemistry using monoclonal antibodies or by Polymerase Chain Reaction (PCR). PCR can be used on a wide range of samples such as blood, skin biopsy, and tick tissues (Jensenius et al., 2003). The target genes by the PCR for *R. africae* are the citrate synthase gene (*gltA)* and *ompA* (Sambou et al., 2014). Detection strategies based on recognition of sequences within the genes encoding the 16S rRNA, outer membrane proteins *rOmpA*, *rOmpB*, and PS120 have also been effectively used for the detection of this pathogen.

For identification of group, genus and species of *Rickettsia* organisms, six genes are usually used. These include sequences of the 16S rRNA (*rrs*) gene, the *gltA* gene (385 bp), *ompA* (632 bp)*, ompB (*444 bp) and *sca4* (gene D) genes (Raoult et al., 2005; Kimita et al., 2016; Scarpulla et al., 2016). Currently, the genotypic identification of the SFG *Rickettsia* is based on restriction fragment length polymorphism (RFLP) analysis of PCR-amplified genes coding for the enzyme citrate synthase and the surface proteins *rOmpA* and *rOmpB* (Regnery et al., 1991; Roux et al., 1996).

2.7.6 Treatment of ATBF

Doxycycline is used in the treatment of ATBF. The recommended dose is 200 mg per day for seven to ten days (Althaus et al., 2010). ATBF also responds well to quinolones and chloramphenicol. Macrolides such as azithromycin can be used as a treatment option especially for patients with mild clinical signs (Johnson et al., 2019). The flu-like symptoms and the dermal symptoms such as the rash responds

efficiently to the medication in a few days. The eschars take a little bit longer to resolve. Toutous-Trellu et al. (2003) and Roch et al. (2008) reported slow recovery of the eschars in patients treated with doxycycline. The eschars were reported to have resolved after a period of four weeks from the time the treatment was initiated. Rifampicin was also used in treatment of ATBF in a patient with doxycycline intolerance (Strand et al., 2017).

2.7.7 Prevention and Control of ATBF

There is no vaccine or medicine that prevents ATBF. People who travel to rural sub-Saharan Africa should be furnished with the necessary pre-travel information on this disease (Owen et al., 2006). Such awareness is necessary especially to those travelling to endemic areas (Jensenius et al., 2003). Intensive measures should be taken to prevent being bitten by ticks during their stay in communal areas of sub-Saharan Africa, although it may be difficult to completely avoid the tick bites. Humans are most prone to be bitten by the larval and nymphal stages of the tick which are also very tiny hence difficult to spot. Many people who succumb to this disease are reported to have seen ticks on their skin which necessitates the importance of preventing tick bites. Tick bites can be prevented by covering exposed skin by wearing long-sleeved shirts, long pants, and hats. Shirts and pants should be tucked in and closed shoes instead of sandals should be worn to prevent bites. Travellers should also avoid bushy areas with high grass, bush, and leaves. Walking in the centre of hiking trails is another measure of avoiding tick bites. Use of repellents like permethrin 0.5 % spray, and diethyltoluamide (DEET)-containing repellents for spraying outdoor clothing and removal of ticks as well as showering as soon as possible after coming indoors are also important preventive measures that can be implemented (Jensenius et al., 2003; Menzer et al., 2017).

The scarcity of data on *R. africae* stimulates the need to use molecular tools to detect the *Rickettsia* in its tick vector.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study area

The Mnisi community is situated in the north-eastern corner of the Bushbuckridge municipal area in Mpumalanga province, South Africa. The study area falls within the savannah ecosystem and is situated at a wildlife/livestock interface, as it borders the Andover and Manyeleti provincial game reserves, and two private game reserves, Timbavati and Sabi Sand. The main agricultural activity in this area is livestock farming of which cattle is by far the most important species. The communal area has approximately 14 800 cattle (Musoke, 2016) and there are 15 dip tanks in the area (Figure 3.1) where government provides veterinary extension services. This area represent epidemiologic units for sampling (Musoke et al., 2015). Out of the 15 dip tanks available in this area, two of them (Hlalakane and Welverdiend B) are crush pens whereas the rest are plunge dips. Each ward in this area has got five dip tanks (Malan, 2016). Two locations (Welverdiend A and Utah A) with different vegetation and geographical locations in the study area were selected for the collection of the ticks. The cattle used for this study were infested with ticks and that were easy to restrain. The cattle in the study area are mainly mixed breed and both areas have communal cattle rearing systems.

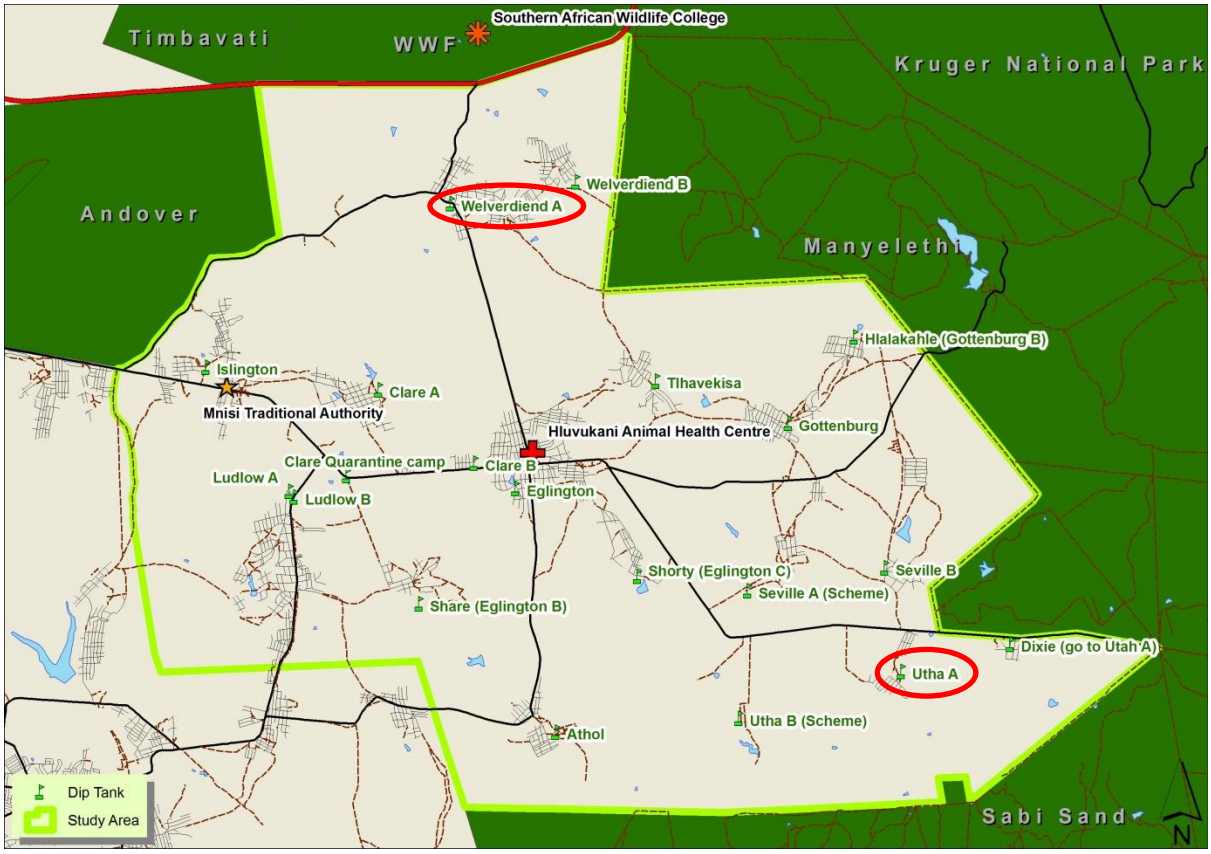

Figure 3.1: Map of the study area, showing the dip tanks, circled in red, where samples were collected for the study (map courtesy of Mnisi Community Programme, University of Pretoria).

3.2 Ethics approval and biosecurity

Approval to perform this project was granted by the Animal Ethics Committee (AEC) of the University of Pretoria (UP) (certificate number V045-18, Appendix 1.1 and 1.2) and from the Faculty Research Ethics Committee (certificate number REC066-18, Appendix 2).

Approval was also obtained from the Department of Agriculture Forestry and Fisheries (DAFF) (Appendix 3) for collection and transportation of the samples from the sample collection sites to the laboratory for processing. The collection and processing of the samples was done in line with the DAFF Section 20 application that was obtained for this project (certificate number 12/11/1/6 [811]).

3.3 Consent from community

Permission to perform research in the community was granted by the traditional leaders in the village. A consent form (Appendix 4) was drafted in English and it was translated to the local language, Xitsonga, with the help of the environmental monitors assisting with research logistics in the area. The consent forms were given to the farmers at the selected dip tanks and the aims and logistics of the research project were explained to the farmers by the environmental monitors. After obtaining the farmers consent, ticks were collected from their cattle.

3.4 Study design

A cross-sectional approach was used to collect *A. hebraeum* tick samples for *R. africae* detection. The ticks were collected over a period of 4 months (October 2018 to January 2019). Adult *Ambylomma* ticks, nymphs and larvae were selected to assess the *R. africae* infection rates at different developmental stages. Egg masses were obtained from engorged adult female ticks which were maintained in a humidity tank (relative humidity 80-85% and temperature 23-25^{o}C) in plastic tubes until they oviposited.

3.5 Calculation of sample size

The sample size for the study (at 90% CI) was estimated using the formula;

$$n_0 = \frac{\{1.645^2 \times P_{exp} \times (1 - P_{exp})\}}{d^2},$$

Where P_{exp} the expected prevalence and d is the desired precision (margin of error of prevalence).

A P_{exp}value of 50% (unknown P_{exp}) and a d value of 8% give,

$$n_0 = \frac{2.7060 \times 0.5 \times 0.5}{0.0064} = 106$$

$n_0 =$ 106 samples

(Thrusfield, 2007)

Therefore, a minimum of 106 ticks per developmental stage (106 adult ticks, and 106 pools of 10 larvae each) were collected from cattle that were brought at the selected dip tanks and from the environment where these dip tanks are situated. A total of 106 engorged female ticks were also collected from cattle at the two dip tanks and 106 eggs masses were collected from these ticks as illustrated on Table 3.1 below.

Table 3.1: Samples collected from the two sampled dip tanks

	Dip tanks	
Samples	**Utah A**	**Welverdiend A**
Adult ticks(M\F)	106	106
Larvae	1060 (106 pools of 10 larvae each)	1060 (106 pools of 10 larvae each)
Engorged females	53	53
Egg masses	53	53

3.6 Tick collection in the study area

Adult ticks were collected while the cattle were restrained in cattle crushes before dipping. Samples were collected in plastic tubes (Thermo Scientific Nunc 50 ml Conical Centrifuge tubes). Forceps were used to gently pull off adult ticks from the cattle which were then preserved in 70% ethanol. Engorged females, which were easy to see and reach, were collected from the cattle and placed in plastic tubes with holes on top to keep them alive. The tubes were lined with paper towels to make sure that the ticks were dry before putting them in the humidity tank to avoid fungal growth. The cattle from the study area had a very high tick infestation, especially those from the Utah dip tank.

Dragging cloths were used to collect larvae by dragging at ground level (using one hundred steps, equivalent to an area of one hundred square metres: 100 m^2). The immature stages were then picked from the dragging cloths using forceps and put in plastic tubes (Thermo Scientific Nunc 50 ml Conical Centrifuge tubes) containing

70% ethanol. Species identification was done under a microscope according to standard taxonomic keys (Walker, 2003).

The state veterinarian provided verbal permission to move samples from the collection sites to HHWRS laboratories for DNA extraction.

3.7 Incubation of engorged ticks and collection of egg masses

In the Hans Hoheisen Wildlife Research Station (HHWRS) laboratory, the engorged *A. hebraeum* ticks were individually placed into separate plastic tubes. Cotton wool and gauze were used as stoppers. The tubes were kept in a humidity tank at 23-25^{o}C, and at a relative humidity of 80-85%. Humidity was maintained with a saturated NaCl solution. Temperature and humidity was monitored daily and weekly inspections were done until oviposition was observed. Egg masses from individual ticks were collected separately and placed in labelled tubes.

3.8 DNA extraction from adult ticks and larvae

To avoid cross contamination all samples were processed under sterile conditions. Each tick was disinfected with 70% ethanol and washed with PBS five times to remove all the ethanol. Adult ticks and nymphs were processed individually. Each tick was cut into small pieces using a sterile scalpel blade in a sterile petri dish and DNA was extracted using Qiagen Blood and Tissue kit (Qiagen, Whitehead Scientific, South Africa 69506). DNA extraction was done according to the manufacturer's instructions. Using the same protocol, DNA was also extracted from the engorged females after they laid eggs. The total number for the larvae was 1060 and these larvae were pooled and each pool had 10 larvae and the total number of pools was 106 (n=106). Each larvae pool was considered as a single sample equivalent to one tick, before DNA was extracted from them. The DNA was eluted in a final volume of 200 µl TE buffer.

The extracted DNA was stored at -20^{o}C and later transported to the DVTD research and training laboratories for further processing. A sample movement permit (Appendix 4) was obtained from the state veterinarian for this purpose.

3.9 DNA extraction from egg masses

Egg masses were weighed before DNA was extracted from them and each egg mass was processed separately. The engorged females laid egg masses ranging from 300 mg to 450 mg, and the similar egg mass weight of 300 mg per egg mass was used for DNA extraction to maintain consistency. Egg masses were weighed using a weighing balance (Lasec, South Africa). The egg masses were washed with 70% ethanol and then with PBS. The same DNA extraction protocol as described in 3.8 above was also used for the egg masses. The egg masses were then homogenised in PBS and the homogenate was used for DNA extraction. The DNA was eluted in a final volume of 200 µl.

3.10 DNA quantification

The extracted DNA was quantified using a spectrophotometer (Xpose, Trinean, Belgium) at the research and training laboratories (DVTD, UP) to determine if the DNA extraction process was successful and to determine the concentration of DNA extracted.

3.11 *gltA* PCR for the detection of *Rickettsia* genus

Real-time or Quantitative PCR (qPCR) was used to screen the DNA for the citrate cynthase (*gltA*) gene, which is common in all *Rickettsia.* The primers for the *gltA* gene (CS-F and CS-R) and the CS-P probe used for the qPCR reactions are as indicated in Table 3.2 below.

Table 3.2: Primers and probes for the *Rickettsia* qPCR assay

Primers & probe	Sequence (5'-3')	Target gene	Reference
CS-F	TCG CAA ATG TTC ACG GTA CTT T-	*gltA* 74 bp	(Stenos et al., 2005)
CS-R	TCG TGC ATT TCT TTC CAT TGT G-		
CS-P	6-FAM-TGC AAT AGC AAG AAC CGT AGG CTG GAT G-BHQ-1		

These oligos amplify a 74 base pair fragment. The calculated primer melting temperature values for the primers and the probe were 54^{o}C for the CS-F, 55.1^{o}C for the CS-R and 65.6^{o}C for CS-P (Stenos et al., 2005). Luna Universal Probe qPCR master mix (New England Biolabs, Inc., M3004E) was used for the reaction. It is a 2x reaction mix optimised for qPCR detection and quantitation of target DNA sequences using hydrolysis probes. Three microliters of the test samples were used and the reaction set up is shown in Table 3.3 below. The probe was prepared according to the Luna Universal Probe qPCR master mix instruction manual since it is supplied as a pellet. The Luna Universal probe qPCR Master Mix and other reaction components were stored at -20^{o}C and kept on ice during sample preparation. The qPCR master mix and the other reaction components were allowed to thaw completely at room temperature. The primer solutions were vortexed gently and then centrifuged to collect all the drops at the bottom of the tubes. After adding the DNA to the capillaries, the capillaries were centrifuged at 6000 x g (800 rpm) for one minute. A positive and a negative control were included for each cycle. The positive control was obtained from samples that tested positive for *R. africae* from a previous project by Mandara (2018). The samples were confirmed to be positive for *R. africae* by sequencing of the *ompA* gene. The negative control had the same components with the other reactions but distilled water was added instead of DNA hence they were non-template negative controls. The qPCR capillaries were loaded into the Light Cycler 2.0 qPCR machine (Roche Diagnostics, South Africa) and the machine was

programmed according to the Luna Universal Probe qPCR master mix manufacturer's thermal cycling protocol (New England Biolabs Inc.) as shown in Table 3.4.

Table 3.3: Reaction set up for the *Rickettsia* qPCR reaction targeting the *gltA* gene

Component	**×1 (20 µl reaction)**
Luna Universal Probe qPCR Master Mix (*Taq* DNA Polymerase, dUTP, non-fluorescent visible dye)	10 µl
Forward primer (10 µM)	0.8 µl
Reverse primer (10 µM)	0.8 µl
Probe (10 µl)	0.4 µl
Template DNA	3 µl
Nuclease-free water	5 µl

Table 3.4: Thermal cycling conditions for the amplification of the *gltA* gene using Lunar Universal Probe qPCR Mix

Cycles	**Temperature**	**Time (s)**	**Purpose**
1	95^{o}C	60	Initial denaturation
45	95^{o}C	15	Denaturation
	60^{o}C	30	Extension

A positive control was obtained from samples that tested positive for *R. africae* from a previous project by Mandara (2018). The samples *ompA* gene sequences for these samples were found to match those of *R. africae*. Serial dilutions were done on three positive samples using TE buffer and qPCR was done on the diluted samples, which tested positive up to the10^{-5} dilution. The last significant positive dilution for these positive controls was detected at C_t value 40, which is up to 40 cycles. Samples that were processed with qPCR were considered positive if there was DNA amplification up to 40 cycles.

3.12 Conventional PCR (cPCR)

The primers used for this PCR targeted the *ompA* gene and are as indicated in Table 3.5 below.

Table 3.5: Primers for the *Rickettsia* cPCR assay

Primer	Sequence (5' -3')	Target gene	Amplicon	Reference
Rr190.70 F	ATG GCG AAT ATT TCT CCA AAA	*ompA*	632 bp	(Stenos et al., 2005)
Rr190.70 1R	GTT CCG TTA ATG GCA GCA TCT			

cPCR was used to detect presence of SFG *Rickettsia* DNA in the samples and the primer sequences are indicated on Table 3.5 above. Phusion Flash master mix (Thermo Fisher Scientific, South Africa, F548S) was used. The master mix was prepared according to the manufacturer's instructions and the volumes used are indicated in Table 3.6 below.

Table 3.6: Reaction set up for cPCR targeting the *ompA* gene

Component	x1 (20 µl reaction)
2X Phusion Flash Master Mix (Phusion Flash II DNA Polymerase, Affibody protein)	10 µl
Forward Primer (10 µM)	1 µl
Reverse Primer (10 µM)	1 µl
Nuclease free water	6 µl
Template DNA	2 µl

Nuclease free water (2 µl) served as the negative control and 2 µl of extracted DNA was added to the PCR tubes. The positive control was a known *R. africae* positive sample from a previous study confirmed by sequencing of *ompA* gene. The PCR

tubes were centrifuged and a PCR machine was programmed using conditions outlined in the Table 3.7 below.

Table 3.7: cPCR conditions for the amplification of *ompA* gene using the 2X Phusion Flash Master Mix

Cycles	Temperature (°C)	Time (s)	Purpose
25	98°C	10	Initial denaturation
	98°C	1	Denaturation
	53°C	5	Annealing
	72°C	15	Extension
	70°C	60	Final extension
	4°C	∞	Hold

The PCR products were analysed on a 1.5% agarose gel and visualized using an ultraviolet transilluminator (Bio-Rad). A 632-bp fragment (Abdel-Shafy et al., 2012) was expected. The size of the amplicons was determined by comparison with a 100 bp molecular weight marker (Thermo Fisher Scientific, 15628019, Invitrogen). The PCR products were sequenced Inqaba biotec™ laboratories and sequencing was done in both directions using primers shown on Table 3.8 below.

3.13 Sequences and phylogenetic analysis

Table 3.8: Sequencing primers

Primer	Sequence (5'-3')
Rr190.701F	5'-ATG GCG AAT ATT TCT CCA AAA-3'
Rr190.701R	5'-GTT CCG TTA ATG GCA GCA TCT-3'

Sequences obtained from the *ompA* gene were processed using CLC Genomics Workbench version 7.5.1 (CLC Bio, Boston, MA, USA). The Basic Local Alignment Search Tool [BLAST] (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for homologous reference sequences, using the BLAST algorithm. Alignments of *ompA*

gene (611 bp) sequences were constructed using the Multiple Alignment, Fast Fourier Transform (MAFFT) [version 7] program (Katoh & Standley, 2013) and manually edited using BioEdit (version 7.2.5) program (Hall, 1999). Sequence identities (number of nucleotides) were calculated by pairwise comparison using MEGA7 (Kumar et al., 2016).

The *ompA* nucleotide sequences were translated to amino acid sequences using EMBOSS Transeq (EMBL-EBI, 2016). ProtTest version 3.0 (Darriba et al., 2012) was used to select a JTT + F model of evolution for proteins for the *ompA* sequences, under the Akaike Information Criterion (AIC) as the model selection criterion. Phylogenetic trees for the *ompA* amino acid sequences were reconstructed using maximum likelihood (ML) as implemented in PhyML 3.1 (Guindon et al., 2010). The reliability for the internal branches was assessed using bootstrapping [1000 bootstrap replicates] (Felsenstein, 1985). Graphical representation and editing of the phylogenetic trees were performed with MEGA7 and Paint Tool for Windows 10.0.

3.14 Data analysis

Minimum Infection Rate (MIR) was calculated for larvae pooled samples using the following formula:

MIR = (number of positive tick pools / total number of ticks in pools tested) × 1000

Microsoft Excel and Statistical Package for the Social Sciences (SPSS) using the Pearson chi square test were used to analyse the results from this study. Chi-squared test (χ^2 test) at 95% CI was used to compare the different groups from the study. Z distribution was used to analyse data for the transovarial transmission efficiency.

3.15 Farmer feedback report

A report (Appendix 5) was prepared to present the research findings to the community where the samples for the research project were collected. The report

was prepared in English and was translated to Xitsonga, the local language of the people residing in the community, with the help of the environmental monitors who also helped with presenting the information to the farmers. The presentations were done on the 23rd and 24th of May 2019 at the dip tanks where the samples were collected. The translated report was distributed to the farmers together with *A. hebraeum* ticks preserved in 70% ethanol so that the farmers could familiarise themselves with the tick vector for *R. africae*.

CHAPTER 4

RESULTS

4.1 Detection of *R. africae* infection rates in *A. hebraeum* adult ticks and larvae

4.1.1 Real-time *Rickettsia* PCR (qPCR) to screen adult tick and larvae samples for *gltA* gene

A total of 106 adult male or female *A. hebraeum* ticks were collected from cattle at each of the two dip tanks. Out of the 106 samples obtained from Utah, 102 (96.23%) were positive for the *gltA* gene. From Welverdiend, 101 (95.28%) samples tested positive for the *gltA* gene. A total of 1060 larvae were collected from each dip tank and they were grouped into pools of 10 resulting in a total of 106 pools. From Utah, 68.87% (73/106) of DNA from larvae samples were positive for the *gltA* gene and for Welverdiend, the *gltA* gene was detected in 46.23% (49/106) samples.

4.1.2 Conventional PCR (cPCR) to screen DNA from adult ticks and larvae for the *ompA* gene

All samples that tested positive for the *Rickettsia* species in the qPCR were then tested in a cPCR for the presence of the *ompA* gene. The amplified products of the *ompA* gene were visualized on a 1.5% agarose gel and some of the positive samples are as indicated on the gel picture (Figure 4.1 below). From Utah, 14 out of the 106 (13.21 %) adult ticks tested positive for the *ompA* gene, whereas 15 out of 106 (14.15 %) samples from Welverdiend *gltA* screened positive for the *ompA* gene. On average, the *R. africae* infection rate for the tested adult tick samples from Utah and Welverdiend was found to be 13.68% (29/212). For the larvae that were collected from Utah, 18/106 (17%) were positive for the cPCR targeting the *ompA* gene and for Welverdiend 10/106 (9.4%) were positive. From the sampled larvae pools from Utah and Welverdiend, 13.21% (28/212) tested positive for *R. africae*.

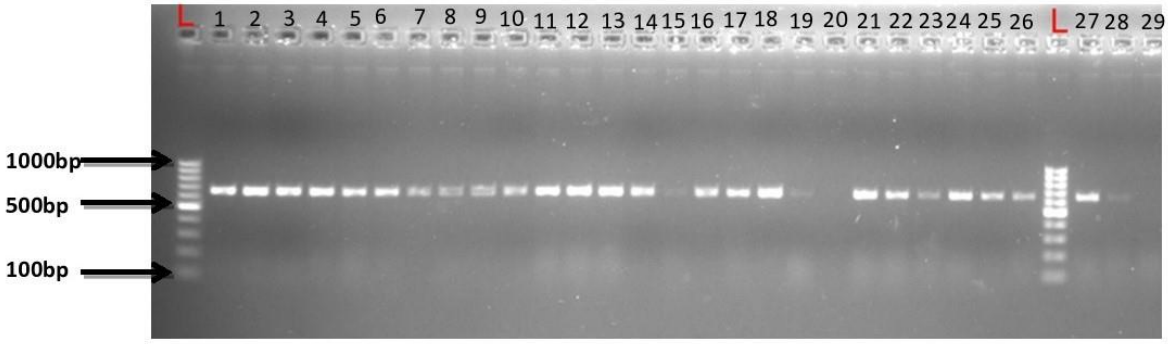

Figure 4.1: Picture of cPCR products for the *ompA* gene on 1.5% agarose gel, (L) 100 bp molecular weight marker (Thermo Fisher Scientific - Invitrogen, South Africa) on the left and far right. Lane 1-19, 21-26 and 28 are some of the *ompA* cPCR amplicons, Lane 27: positive control, Lane 29: negative control

All the *ompA* positive amplicons (adults and larvae) were sent for sequencing and the sequences from all the *ompA* positive amplicons were found to be 99.98% identical to *R. africae* sequences from GenBanik.

On average, *R. africae* infection rates for ticks collected from the study area was found to be 13.44% as indicated on Table 4.1 below.

Table 4.1: Cross tabulation of *R. africae* infection rates across the adult ticks and larvae from Utah and Welverdiend based on *ompA* sequence analysis

	Location	**Utah**	**Welverdiend**	**Utah**	**Welverdiend**	
	Sample	**Adult**	**Adult**	**Larvae**	**Larvae**	**Total**
Samples negative for R. africae	**Count**	92	91	88	96	367
	% within sample	86.79%	85.85%	83.0%	90.6%	86.6%
Samples positive for R. africae	**Count**	14	15	18	10	57
	% within sample	13.21%	14.15%	17.0%	9.4%	13.44%
Total	**Count**	106	106	106	106	424

From the observed frequencies of *R. africae* in the samples collected from cattle at the respective dip tanks, the percentage of positive samples for all the four groups tested lies in the range of 9.4% to 17.0%. This is also supported by the cross-tabulations for all larvae against all adult ticks (Table 4.2) and when samples collected from each dip tank were compared (Table 4.3). Chi-squared test was used to see if the differences in infection rates between the stages were significant.

Table 4.2: Cross-tabulation for all sampled larvae vs. all adult ticks

	Utah and Welverdiend larvae	**Utah and Welverdiend adult ticks**
Samples positive for *R. africae*	28/212 (13.20%)	29/212 (13.69%)
Samples negative for *R. africae*	184/212 (86.79%)	183/212 (86.32%)

Table 4.3: Cross-tabulation for all ticks (both stages –larvae and adult ticks) from Utah vs. those from Welverdiend

	Utah adult ticks and larvae	**Welverdiend adult ticks and larvae**
Samples positive for *R. africae*	32/212 (15.09%)	25/212 (11.79%)
Samples negative for *R. africae*	180/212 (84.91%)	187/212 (88.21%)

Table 4.4: Chi square test results from SPSS for the Welverdiend and Utah *R. africae* infection rates in *A. hebraeum* ticks

	Value	**Df**	**Asymptotic Significance (2-sided)**
Pearson Chi-Square	2.979[a]	3	.395
Likelihood Ratio	3.046	3	.385
Number of Valid Cases	424		

From the Pearson Chi-Square Test for the data illustrated on Table 4.4, the SPSS results show that $\chi(3) = 0.395$. The asymptotic difference 0.395 is > 0.05 hence we concluded that *R. africae* infection rates are the same at different stages of the tick

vector. The infection rates between the two dip tanks produced asymptotic value which is also greater than 0.05 indicating that the infection rates in ticks collected from these two dip tanks was almost the same. This is also supported by the data on Table 4.2 and Table 4.3, whereby the infection rates of all larvae is 13.20% whereas that of all adult ticks is 13.69%. The average infection rate of larvae and adult ticks from Utah was 15.09% and Welverdiend had average infection rate of 11.79%.

4.1.3 Phylogenetic analysis of the *ompA* gene isolated from adult ticks and larvae samples

A phylogenetic tree was generated for the *ompA* GenBank sequences that were found to closely match with the sequences obtained from this study. The relationship of the samples to sequences found in other areas/countries is as shown in Figure 4.2 and Table 4.5 below. The *ompA* gene sequences obtained in this study were identical to each other. Only representative sequences are indicated on the phylogenetic tree.

The DNA sequences for engorged ticks and their egg masses were identical and they were also identical to those of the adult ticks and larvae hence the same trend for their relationship with GenBank sequences was observed as indicated by the phylogenetic tree and the nucleotide differences.

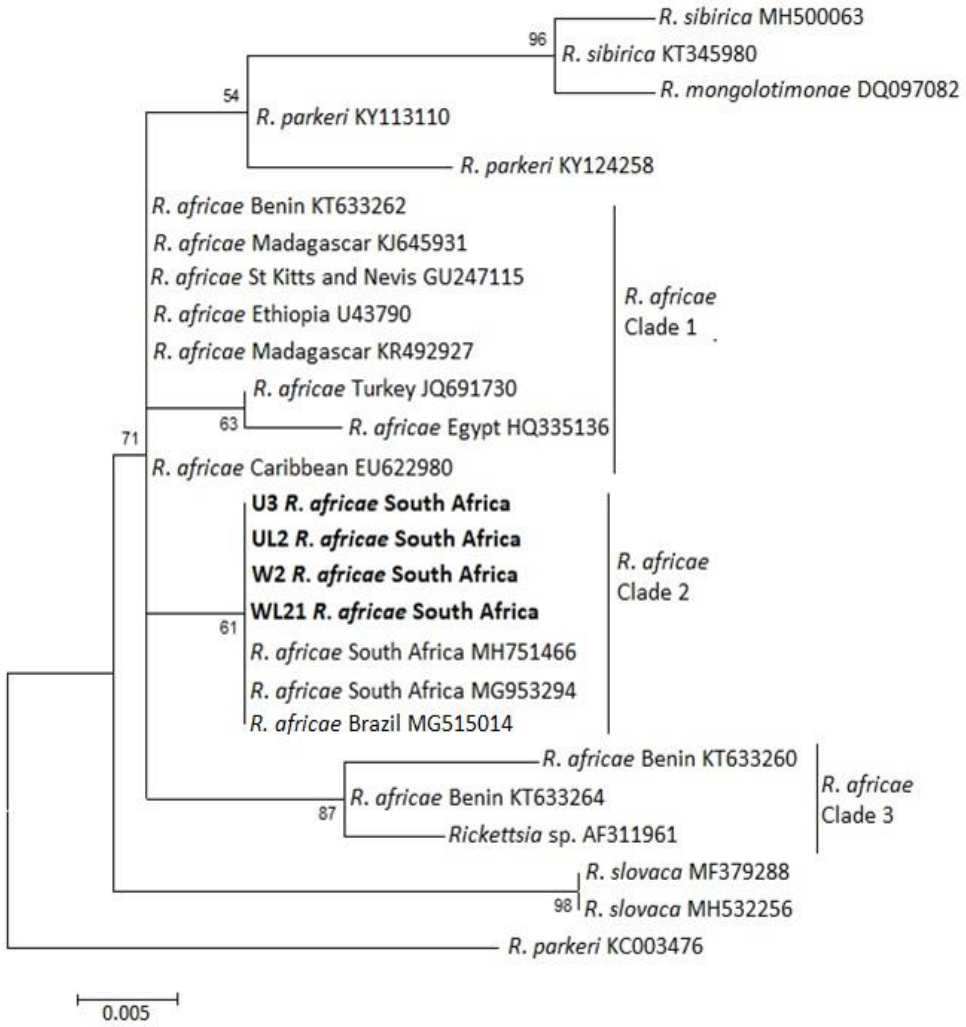

Figure 4.2: A maximum likelihood tree showing the phylogenetic relationship between the *R. africae ompA* gene sequences, identified from *A. hebraeum* samples from cattle in Mpumalanga Province, South Africa with other *Rickettsia* species. Sequences obtained in this study are highlighted in bold as follows; U3 - Utah adult tick 3, UL2 – Utah larvae 2, W2 – Welverdiend adult tick 2, WL21 – Welverdiend larvae 21. R – *Rickettsia.* The tree was rooted using the *ompA* gene sequence of *Rickettsia parkeri* (accession number KC003476). The accession number of each sequence is indicated in the sequence name. Branch lengths are proportional to the estimated genetic distance, over 203 amino acid positions between the taxa. The newly-generated *R. africae ompA* sequences grouped with isolates from ticks from South Africa and Brazil (sequence from an eschar obtained from an ATBF patient who had previously visited South Africa).

Table 4.5: Pairwise differences (number of nucleotides) among the different *ompA* sequences from *A. hebraeum* collected from cattle from Mpumalanga Province, South Africa and reference sequences from different parts of the world.

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	**U3 South Africa**										
2	**UL2 South Africa**	0									
3	**W2 South Africa**	0	0								
4	**WL21 South Africa**	0	0	0							
5	*R*. *africae* Madagascar KJ645931	1	1	1	1						
6	*R*. *africae* Turkey JQ691730	5	5	5	5	4					
7	*R*. *africae* South Africa MH751466	0	0	0	0	1	5				
8	*R*. *africae* Benin KT633264	4	4	4	4	3	7	4			
9	*R*. *africae* Egypt HQ335136	6	6	6	6	5	3	6	8		
10	*R*. *africae* Benin KT633260	7	7	7	7	6	10	7	3	11	

Sequences from this study are indicated in bold as follows; U3 - Utah adult tick 3, UL2 – Utah larvae 2, W2 – Welverdiend adult tick 2, WL21 – Welverdiend larvae 21. R – *Rickettsia.* Analysis was conducted in MEGA7. All ambiguous positions were removed for each sequence pair. There were a total of 611 positions in the final dataset. Seventeen sequences that we analyzed were identical to each other, therefore only representatives by adult tick and larvae and location are shown in this table.

The phylogenetic tree (Figure 4.2) and the pairwise difference table (Table 4.5) shows that the analysed sequences obtained from adult ticks and larvae from this study grouped together with GenBank sequences from South Africa (MH751466; MG953294) and Brazil (MG515014). The sequences were also found to be closely related to a sequence from Madagascar (KJ645931), with which they only had one nucleotide difference, and those from Benin (KT633264) with a difference of four nucleotides and Turkey (JQ691730) with a five nucleotide difference.

4.2 Determining *R. africae* transovarial transmission efficiency in *A. hebraeum* ticks

The observed average time for the engorged female ticks to oviposit was 27 days. From Utah, 92.45% (49/53) of the engorged female ticks were positive for the *gltA* gene whereas 91% (48/53) of the egg masses obtained from these females tested positive for the same gene. The *ompA* gene was detected in 9.43% (5/53) of the engorged female samples that tested positive for the *gltA* gene and 9.43% (5/53) of the egg mass samples that tested positive for the *ompA* gene. All the Utah engorged ticks samples that tested positive for the *ompA* gene laid *ompA* positive egg masses. The amplicons were sequenced and the sequences were 99.98% identical to *R. africae* resulting in transovarial transmission efficiency of 100% for the *R. africae* positive engorged female ticks collected from Utah.

Out of the 53 engorged female ticks that were collected from cattle in Welverdiend, the *gltA* gene was detected in 96.23% (51/53) of samples and 26.42% (14/53) of these samples were positive for the *ompA* gene. The obtained sequences matched those of *R. africae* sequences from GenBank by 99.98%. From the egg masses (n=53) that were laid by the engorged females collected from cattle in Welverdiend, 92.45% (49/53) tested positive for the *gltA* gene and 18.87% (10/53) of these samples tested positive for the *ompA* gene. The sequences for the *ompA* positive amplicons were found to be 99.98% identical to *R. africae*. From the 14 engorged female ticks that tested positive for *ompA*, 10 of them laid *ompA*-positive egg masses, as indicated in Table 4.6 below, which indicates 71.43% transovarial transmission efficiency. The transovarial transmission efficiency was found to be 71.43 % (10/14) for the Welverdiend engorged female ticks and 100% (5/5) for those collected from Utah as indicated above.

When considering the *gltA* gene qPCR assay results from this study, engorged female ticks from Utah had 92.45% (49/53) transmission efficiency of Rickettsia species whereas Welverdiend had 96.23% (51/53).

Table 4.6: Cross tabulation of *R. africae* infection rates (based on *ompA* gene sequence analysis) for engorged female ticks and their egg masses

	Utah	**Welverdiend**	
	Engorged female ticks	**Engorged female ticks**	**Total**
Total	53	53	106
Samples negative for *R. africae*	48 (90.57%)	39 (73.58%)	87 (82.08%)
Samples positive for *R. africae*	5 (9.43%)	14 (26.42%)	19 (17.92%)
	Egg masses	**Egg masses**	**Total**
Total	53	53	106
Samples negative for *R. africae*	48 (90.57%)	43 (81.13%)	91 (85.85%)
Samples positive for *R. africae*	5 (9.4%)	10 (18.87%)	15 (14.15%)
% within sample	100.0%	100.0%	100.0%

CHAPTER 5

DISCUSSION

5.1 *R. africae* infection rates in *A. hebraeum* ticks

ATBF cases have been reported in foreign travelers after they had visited Kruger National Park which is close to the study area (Sexton et al., 1999; Jackson et al., 2004; Consigny et al., 2009). This is to the best of our knowledge the first study conducted in a rural area of the Mnisi community to determine *R. africae* infection rates in *A. hebraeum* ticks with the aim of better understanding of the epidemiology of this *Rickettsia*. Kolo et al. (2019) reported an average of 82.35% infection rate from 348 *A. hebrauem* ticks collected from Mnisi area using a qPCR assay targeting *ompB* gene.

This cross-sectional study was performed at a human/wildlife/livestock interface in Mpumalanga province close to game reserves frequented by tourists. qPCR and cPCR tests were used to screen samples for *Rickettsia* DNA and for SFG *Rickettsia* DNA respectively. The target gene for the cPCR and sequencing in this study was *ompA*. The basis of targeting *ompA* gene for this study was because it was found to be specific for the SFG *Rickettsia*. This gene is also an essential tool for the differentiation of *Rickettsia* species that belong to SFG since it shows a marked diversity within the group unlike *gltA* and 17 kDA genes that are more conserved in these *Rickettsiae* (Roux et al., 1996; Orkun et al., 2014).

R. africae was identified in an average of 13.68% adult *A. hebraeum* tick samples and 13.20% in larvae samples collected from the study area. From the obtained results, the infection rates in the adult ticks and the larvae from both dip tanks appeared to be in the same range (Table 4.6). Based on these results, we can safely conclude that *R. africae* infection rates are almost the same in larval (an average of 13.2%) and adult ticks (an average of 13.68%) from our study area. The detection of *R. africae* DNA in both the adult ticks and the larvae from this area increases the risk of people getting ATBF since both stages feed on humans (Jensenius et al., 2003).

The larvae are small hence not easily detected on the skin and this can pose a high risk to humans.

The two dip tanks included in this study had similar results yet they have different vegetation and are located in different geographical areas in the community. Welverdiend dip tank is located within the villages and the vegetation is sparse whereas Utah is located close to game parks and the vegetation is denser. The similarity in infection rates could mean that there is an even distribution of *Rickettsia* in the tick vector independent of the location. The reservoir hosts for *R. africae* have not yet been identified; however, there may be chances that wild animals can also be reservoirs for this pathogen considering that the *Amblyomma* ticks also feed on wild ungulates, giraffes, buffaloes and antelopes (Jensenius et al., 2003). In this scenario, there is possible sharing of the ticks between domestic animals and game animals since our study area is at the human/wildlife/livestock interface. However, this assumption needs to be confirmed. From this study, it's not certain whether the ticks got the *Rickettsia* from cattle since the cattle were not screened for *Rickettsia* at the time of sampling. There is a high probability that these ticks could have carried the *Rickettsia* through their developmental life stages. Maina et al. (2014) reported detection of *R. africae* DNA in ticks that were collected from non-rickettsemic animals; so the cattle we collected the ticks from could have been non-rickettsemic. Further research on this aspect is necessary.

On average, *R. africae* infection rates in *A. hebraeum* ticks from this study were found to be 13.44%. The results obtained in this study correspond with published results from a similar study by Halajian et al. (2018) where the infection rates in ticks collected from donkeys in Limpopo province was found to be 13% using PCR targeting *ompA*, *ompB*, 16S, rRNA, *gltA* and *sca4* genes. This shows that *R. africae* infection rates in ticks from the two study areas from Limpopo and Mpumalanga provinces of South Africa are almost the same even though the ticks were collected from different animal species. The similarity in the infection rates for these two studies also corroborates the similarity in infection rates obtained from the two dip tanks included in this research.

The infection rate obtained from this study was lower than expected considering that the study area is regarded as an endemic area, which is evidenced by the high rate of ATBF cases that are being reported in tourists after visiting Kruger National Park, where *A. hebraeum* is considered the principal vector for this pathogen. Generally, ATBF is believed to be endemic in South Africa (Halajian et al., 2018). Available data from countries where ATBF is also endemic indicate that *R. africae* infection rates in tick vectors collected from endemic areas can be as high as 80-100% (Jensenius et al., 2003; Mediannikov et al., 2012). The target genes for the study by Mediannikov et al. (2012) were the *gltA* and *ompA* genes in a *R. africae* specific qPCR assay. There is limited data on the epidemiology of *R. africae* in South Africa that can be compared with these results; however, similar studies were performed in other African countries.

A study recently performed in Zimbabwe reported *R. africae* infection rates of 42.4% in *A. hebraeum* ticks (Mandara, 2018). Other studies reported infection rates of these ticks by *R. africae* but most of the studies that have been published involved *A. variegatum* ticks. An infection rate of 5.55% in *A. variegatum* ticks was reported in a study done in Mozambique (Matsimbe et al., 2017), 16% in *A. variegatum* ticks collected from Kenya (Macaluso et al., 2003), 34% in Mali and Niger, 7.6% in Burundi (Parola et al., 2001) and 75% in Cameroon (Ndip et al., 2004). *R. africae* infection rates across African countries have been reported to be around 16-75% (Kelly, 2006). The results obtained from this study are in line with infection rates reported in other studies although they are lower than expected. Other studies reported very high infection rates of *R. africae* in the tick vector. In Uganda and Nigeria, SFG Rickettsiae DNA was amplified in 67% and 65% of *A. variegatum* ticks respectively using PCR targeting *gltA* and *ompA* genes (Lorusso et al., 2013). Yssouf et al. (2014), found a *R. africae* prevalence of 65.17% in *A. variegatum* ticks in The Comoros Islands indicating that *R. africae* infection rates can be very high in some areas.

Many ATBF cases have been associated with game parks close to our study area; however, we detected low infection rates in the ticks collected from the two dip tanks. The most plausible explanation for the high incidences of ATBF in this area is the

aggressiveness and the abundance of the tick vector. *A. hebraeum* ticks are abundant in this area since the tick infestation on cattle at the two sampling sites was very high; it was noted that all the individual animals at the dip tanks had approximately twenty ticks or more on them. This is consistent with the study by Mediannikov et al. (2012) in which they reported an abundance of *Amblyomma* ticks in African countries. The infection rates may be low in ticks from this area but the high abundance of these ticks and their aggressiveness increase chances of people being attacked by *R. africae* infected ticks, resulting in ATBF cases. This is evidenced by occurrence of ATBF cases in clusters as in the ATBF cases reported by Caruso et al. (2002); Jackson et al. (2004); and Oostvogel et al. (2007). Although the infection rates in the ticks were low, the detection of *R. africae* in these ticks is a cause for concern.

We speculate that the lower than expected infection rates could be due to low sensitivity of the cPCR used in this study. A greater percentage of the samples, (>90%), tested positive for the *gltA* gene in the *Rickettsia* qPCR as compared to the samples that tested positive for the cPCR (*ompA*). In the cPCR, only 13.68% for adult ticks and 13.20% for larvae tested positive for the *ompA* gene. This can be explained by the different assays that were used for the detection of *gltA* and *ompA* genes in these samples. The high sensitivity of the probe-based *gltA* qPCR assay could have detected very low levels of Rickettsiae DNA as compared to the cPCR which could have missed some samples that had low levels of Rickettsiae DNA. This could have led to the low *R. africae* infection rates obtained from this study.

This study provided evidence that the ticks from this communal area are infected with *R. africae*. The detection of R. africae in ticks from the study area correlates reports from similar studies performed in this area. A review study showed that 21% of patients who had fever in Mpumalanga province had evidence of acute infection by SFG *Rickettsia* species (Berrian et al., 2019). A serological survey in the Mnisi community also reported 92.2% exposure to SFG Rickettsiae in samples collected from patients who presented with acute febrile illness and in 64.4% of farmers and veterinary personnel who were screened which indicated possible past, recent and current infections (Simpson et al., 2018). These studies and results from this study

correlate with the study by Kolo et al. (2016) which reported presence of *R. africae* in ticks collected from dogs in this area although in their study, the pathogen was detected in *Haemaphysallis elliptica* ticks in addition to *A. hebraeum* ticks. This study only targeted *A. hebraeum* ticks that are the known vectors of *R. africae* in Southern Africa. However, the perception of *Amblyomma* ticks being the only tick vectors of *R. africae* need to be scrutinised considering that *R. africae* has been detected in several other tick species collected from different animals, including donkeys and birds.

5.2 Transovarial transmission efficiency

Transovarial transmission occurs between an infected female tick and its egg and it can also result in infected larvae (Socolovschi et al., 2009). Therefore, including the transovarial transmission efficiency was very important since the larva is the tick developmental stage that is most abundant and it's very difficult to detect on humans since they are very small (Jönsson, 2016).

The transovarial transmission efficiency of *R. africae* for samples collected from Utah was 1f00% and from Welverdiend it was 71%. The results from both dip tanks were almost in the same range indicating that the transovarial transmission is generally high in these ticks. On average, the transmission efficiency for both dip tanks was found to be 85.5% which is very high. The high transovarial transmission noted in this research is consistent with a research by Socolovschi et al. (2009) which reported *R. africae* transovarial transmission rate of 100% in the tick vector. The high transovarial transmission efficiency points to high infection rates of *R. africae* in the tick vector from this area.

The high transmission efficiency reported in this study could be also an indication that the tick vector acts as both a reservoir and vector for *R. africae*. This is supported by the results from a study performed by Kelly et al. (1991) to determine vertical transmission of *R. africae* in *A. hebraeum* ticks. *A. variegatum* ticks were able to maintain the pathogen through transovarial and transstadial transmission over two generations (Kelly et al., 1991) and over three generations in *A. hebraeum*

(Socolovschi et al., 2009). There is no information on how many generations the *Rickettsia* can be transmitted, hence further investigation on this aspect is necessary. This speculation may support the report from a study by Maina et al., (2014) where they detected *R. africae* DNA in *A. variegatum* ticks that were collected from non-rickettsemic animals. The high transovarial transmission obtained from this study could also point to a very strong adaptability of this *Rickettsia* to its vector.

5.3 Sequence and phylogenetic analysis

The phylogenetic trees based on *ompA* gene sequences from this study were identical to sequences detected in other studies from South Africa. Our sequences grouped together with *R. africae ompA* sequence (MG953294) obtained from Limpopo province, South Africa (Halajian et al., 2018). These sequences were obtained from *A. hebraeum* and *Rhipicephalus* species that were sampled from donkeys in Limpopo (Halajian et al., 2018).

The other South African sequence that was also identical to sequences from this study was detected in KwaZulu-Natal (GenBank MH751466). This sequence was obtained from *A. hebraeum, R. evertsi evertsi* and *R. decoloratus* ticks. The fact that *R. africae* has been detected in other tick species constitutes the need for further study to investigate whether these ticks are competent vectors of *R. africae*.

The sequences from this study were also found to be identical to a sequence detected in Brazil from an eschar sample obtained from a man who had previously visited Hluhluwe-iMfolozi, a natural reserve in KwaZulu-Natal, South Africa, (GenBank accession number MG525014; Angerami et al., 2018). This shows that the source of infection was South Africa and since this variant was the same as the one from this study, we speculate that the variant detected in the study area causes ATBF. The findings from this study indicate that there is a common *R. africae* variant in the parts of South Africa where these studies were performed, since the same variant was detected in three studies that were performed in different provinces of the country.

The sequences from this study were also found to be closely related to those from Madagascar, Benin, Turkey, Egypt, St. Kitts and Neves as well as the Caribbean. *R. africae ompA* sequences from this study only had one nucleotide difference with those from Madagascar (KJ645931) (Keller et al., 2016), four nucleotide deviations with those from Benin (KT633262) (Moumouni et al., 2016) and six nucleotide differences with those from Egypt (HQ335136) (Abdel-shafy et al., 2012). The observed close relationship of these *R. africae* variants is an indication that they originated from a common ancestor. Variants of *R. africae* have been reported in various studies (Macaluso et al., 2003; Eldin et al., 2011; Maina et al., 2014; Kimita et al., 2016).. There are some other *R. africae* variants that have been reported to exist in South Africa that we did not find in this study (Halajian et al., 2018; Guo et al., 2019). The spread of *R. africae* to different parts of the world has been mainly attributed to movement of tick-infested cattle from African countries in the 1800's (Kelly, 2006) and migratory birds spreading immature stages of *Amblyomma* ticks (Eldin et al., 2011; Dietrich et al., 2014). This could also result in non-clustered or patchy distribution of *R. africae* variants across the African continent.

The farmer's feedback session was a success and all the farmers that had come for dipping were keen to know about the outcome of this research. They requested to see the ticks and we showed them the *A. hebraeum* ticks that we were talking about and they found our visual aids helpful since it would help them identify the tick vector. Farmers were grateful for the feedback since they were not aware that ticks can transmit diseases to humans.

CONCLUSION

The results obtained from this study are in line with reports from other similar studies that have been performed across the African continent. The infection rate of *R. africae* in *A. hebraeum* was found to be 13.44%, which was consistent with other reports from different African countries like Mozambique and from a study performed in Limpopo province where the pathogen is also known to occur. The detection of *R. africae* in ticks from the study area is a cause of concern since the indigenous people and tourists who visit this area are at risk of infection after getting bitten by infected ticks. This is also consistent with recent reports from studies performed in this area where screen human serum samples had SFG Rickettsiae antibodies indicating current or past infections. The *R. africae* variant detected in this study is the same as the ones reported in Limpopo and KwaZulu-Natal provinces of South Africa. The transovarial transmission of *R. africae* in engorged *A. hebraeum* female ticks collected for this study was found to be very efficient, which is consistent with reports from other investigations done by Socolovschi et al., (2009). The high transovarial transmission could mean high levels of infection in ticks from this area.

LIMITATIONS

The adult male and female ticks that were used in this study for the first objective were placed in 70% ethanol soon after collection from cattle hence not allowed time to digest their bloods meal before DNA extraction was done. This could have resulted in false positives due to detection of *Rickettsiae* DNA, which could have been present in the blood meals and not in the ticks being infected by *R. africae*.

The *gltA* gene positive samples that tested negative for the *ompA* gene were not sequenced due to time and financial constraints. If time and money allowed, it could have given us some information on the epidemiology of other *Rickettsiae* which could be present in the study area.

FUTURE RECOMMENDATIONS

We recommend further studies in other areas in South Africa to determine infection rates in these ticks and to ascertain whether the other ticks that are carrying *R. africae* DNA are competent vectors for this pathogen. This will add information to the epidemiology of this pathogen since it is one of the neglected diseases in travel medicine and yet it affects many people.

It is important to do further investigations into how many generations these ticks can maintain *R. africae* in the absence of an infected host since transovarial and transstadial transmission has already been determined and it has been found to be very efficient.

Further investigation is required to determine if the various *R. africae* variants that are being reported across Africa cause ATBF by matching the variants that are being detected in ticks to those that are being detected in ATBF patients. This will add more information to the epidemiology of *R. africae*.

We also recommend the use of more sensitive tests like the *R. africae* qPCR which is more sensitive than screening samples for the *ompA* gene using the cPCR, which may be a cause of the low infection rates that we reported in this study. The qPCR assay is highly sensitive hence it can detect very low amounts of DNA so that more accurate results can be obtained.

Screening of animals for *Rickettsia* at the time of tick collection, which can help to ascertain the source of the *Rickettsia* for the ticks, is advised since the *Rickettsia* is also an endosymbiont of the tick vector. This will provide more reliable information on the infection rates of the *Rickettsia* in the tick vector.

REFERENCES

Abdel-Shafy, S., Allam, N. A., Mediannikov, O., Parola, P., & Raoult, D. (2012). Molecular detection of spotted fever group *Rickettsiae* associated with ixodid ticks in Egypt. *Vector-Borne and Zoonotic diseases*, *12*(5), 346-359.

Althaus, F., Greub, G., Raoult, D., & Genton, B. (2010). African tick-bite fever: a new entity in the differential diagnosis of multiple eschars in travelers. Description of five cases imported from South Africa to Switzerland. *International Journal of Infectious Diseases*, *14*, 274-276.

Angerami, R. N., Krawczak, F. S., Nieri-Bastos, F. A., Santos, F., Medorima, C., Resende, M. R., & Labruna, M. B. (2018). First report of African tick-bite fever in a South American traveler. *SAGE Open Medical Case Reports*, *6*, 2050313X18775301.

Anstead, C. A., & Chilton, N. B. (2013). A novel Rickettsia species detected in vole ticks (Ixodes angustus) from Western Canada. *Applied and Environmental Microbiology, 79*(24), 7583-7589.

Bellini, C., Monti, M., Potin, M., Dalle Ave, A., Bille, J., & Greub, G. (2005). Cardiac involvement in a patient with clinical and serological evidence of African tick-bite fever. *BMC Infectious Diseases*, *5*(1), 90.

Beninati, T., Genchi, C., Torina, A., Caracappa, S., Bandi, C., & Lo, N. (2005). *Rickettsiae* in ixodid ticks, Sicily. *Emerging Infectious Diseases*, *11*(3), 509.

Berrian, A. M., Smith, M. H., Van Rooyen, J., Martínez-López, B., Plank, M. N., Smith, W. A., & Conrad, P. A. (2018). A community-based One Health education program for disease risk mitigation at the human-animal interface. *One Health,* 5, 9-20.

Caruso, G., Zasio, C., Guzzo, F., Granata, C., Mondardini, V., Guerra, E., Macri, E. and Benedetti, P. (2002). Outbreak of African tick-bite fever in six Italian tourists returning from South Africa. *European Journal of Clinical Microbiology and Infectious Diseases, 21*(2), 133-136.

Consigny, P. H., Schuett, I., Fraitag, S., Rolain, J. M., & Buffet, P. (2009). Unusual Location of an Inoculation Lesion in a Traveler with African Tick-Bite Fever Returning from South Africa. *Journal of Travel Medicine*, *16*(6), 439-440.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more

models, new heuristics and parallel computing. *Nature Methods*, *9*(8), 772.

Dietrich, M., Lebarbenchon, C., Jaeger, A., Le Rouzic, C., Bastien, M., Lagadec, E., McCoy, K.D., Pascalis, H., Le Corre, M., Dellagi, K. and Tortosa, P. (2014). *Rickettsia* spp. in seabird ticks from western Indian Ocean islands, 2011–2012. *Emerging Infectious Diseases, 20*(5), 838.

Eldin, C., Mediannikov, O., Davoust, B., Cabre, O., Barré, N., Raoult, D., & Parola, P. (2011). Emergence of *Rickettsia africae*, Oceania. *Emerging Infectious Diseases*, *17*(1), 100.

Eremeeva, M. E., & Dasch, G. A. (2015). Challenges posed by tick-borne Rickettsiae: eco-epidemiology and public health implications. *Frontiers in Public Health*, 3, 55.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*(4), 783–791.

Falcão, E. C. (1966). Henrique da Rocha Lima and the Discovery of Rickettsia prowazeki. *Revista do Instituto de Medicina Tropical de São Paulo, 8*(2), 55.

Fournier, P.E., Jensenius, M., Laferl, H., Vene, S., & Raoult, D. (2002). Kinetics of antibody responses in *Rickettsia africae* and *Rickettsia conorii* infections. *Clinical and Diagnostic Laboratory Immunology*, *9*(2), 324–328. https://doi.org/10.1128/CDLI.9.2.324

Fournier, P. E., Roux, V., Caumes, E., Donzel, M., & Raoult, D. (1998). Outbreak of *Rickettsia africae* infections in participants of an adventure race in South Africa. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *27*(2), 316–323.

Fournier, P.E., El Karkouri, K., Leroy, Q., Robert, C., Giumelli, B., Renesto, P., Socolovschi, C., Parola, P., Audic, S. and Raoult, D. (2009). Analysis of the *Rickettsia africae* genome reveals that virulence acquisition in *Rickettsia* species may be explained by genome reduction. *BMC Genomics, 10*(1), 166.

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., & Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, *59*(3), 307-321.

Guo, H., Moumouni, P.F.A., Thekisoe, O., Gao, Y., Liu, M., Li, J., Galon, E.M., Efstratiou, A., Wang, G., Jirapattharasate, C. and Ringo, A.E. (2019). Genetic

characterization of tick-borne pathogens in ticks infesting cattle and sheep from three South African provinces. *Ticks and Tick-borne Diseases, 10*(4), 875-882.

Halajian, A., Palomar, A. M., Portillo, A., Heyne, H., Romero, L., & Oteo, J. A. (2018). Detection of zoonotic agents and a new *Rickettsia* strain in ticks from donkeys from South Africa: Implications for travel medicine. *Travel Medicine and Infectious Disease*, *26*, 43-50.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic Acids Symposium Series 41*(41), 95-98. [London]: Information Retrieval Ltd., c1979-c2000..

Harrison, N., Burgmann, H., Forstner, C., Ramharter, M., Széll, M., Schotta, A.M., Stanek, G. and Markowicz, M. (2016). Molecular diagnosis of African tick bite fever using eschar swabs in a traveller returning from Tanzania. *Wiener klinische Wochenschrift, 128*(15-16), 602-605.

Hornok, S., Abichu, G., Meli, M.L., Tánczos, B., Sulyok, K.M., Gyuranecz, M., Gönczi, E., Farkas, R. and Hofmann-Lehmann, R. (2014). Influence of the biotope on the tick infestation of cattle and on the tick-borne pathogen repertoire of cattle ticks in Ethiopia. *PloS One, 9*(9).

Jackson, Y., Chappuis, F., & Loutan, L. (2004). African tick-bite fever: four cases among Swiss travelers returning from South Africa. *Journal of Travel Medicine*, *11*(4), 225-230.

Jensenius, M., Fournier, P. E., Kelly, P., Myrvang, B., & Raoult, D. (2003). African tick bite fever. *The Lancet Infectious Diseases*, *3*(9), 557-564.

Jensenius, M., Fournier, P.E., Vene, S., Ringertz, S. H., Myrvang, B., & Raoult, D. (2004). Comparison of immunofluorescence, Western blotting, and cross-adsorption assays for diagnosis of African tick bite fever. *Clinical and Diagnostic Laboratory Immunology*, *11*(4), 786–788. https://doi.org/10.1128/CDLI.11.4.786-788.2004

Jensenius, M., Fournier, P.E., Vene, S., Hoel, T., Hasle, G., Henriksen, A.Z., Hellum, K.B., Raoult, D., Myrvang, B. and Norwegian African Tick Bite Fever Study Group (2003). African tick bite fever in travelers to rural sub-Equatorial Africa. *Clinical Infectious Diseases, 36*(11), 1411-1417.

Johnson, K. R., Schupack, D. A., & Virk, A. (2019). 31-Year-Old South African Man With Fever and Headache. *Mayo Clinic Proceedings*, *94*(2), 336–340.

https://doi.org/10.1016/j.mayocp.2018.03.034

Jönsson, J. (2016). Identification of the tick-borne pathogens Anaplasma phagocytophilum, Neoehrlichia mikurensis and Rickettsia in Swedish ticks: investigation of transovarial transmission and co-infection. *Centre for Ecology and Evolution in Microbial Model Systems*.

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780.

Keller, C., Krüger, A., Schwarz, N. G., Rakotozandrindrainy, R., Rakotondrainiarivelo, J. P., Razafindrabe, T., & Hogan, B. (2016). High detection rate of *Rickettsia africae* in *Amblyomma variegatum* but low prevalence of anti-rickettsial antibodies in healthy pregnant women in Madagascar. *Ticks and Tick-borne Diseases*, *7*(1), 60-65.

Kelly, P. J., & Mason, P. R. (1991). Tick-bite fever in Zimbabwe. *South African Medical Journal*, *80(5)*, 233-236.

Kelly, P., Matthewman, L., Beati, L., Raoult, D., Mason, P., Dreary, M., & Makombe, R. (1992). African tick-bite fever: a new spotted fever group rickettsiosis under an old name. *The Lancet*, *340*(8825), 982-983.

Kelly, P. J., Beati, L., Mason, P. R., Matthewman, L. A., Roux, V., & Raoult, D. (1996). *Rickettsia africae* sp. nov., the etiological agent of African tick bite fever. *International Journal of Systematic and Evolutionary Microbiology*, *46*(2), 611-614.

Kelly, P. J., Mason, P. R., Rhode, C., Dziva, F., & Matthewman, L. (1991). Transient infections of goats with a novel Spotted fever group *Rickettsia* from Zimbabwe. *Research in Veterinary Science*, *51*(3), 268-271.

Kelly, P. J. (2006). *Rickettsia africae* in the West Indies. *Emerging Infectious Diseases*, *12*(2), 224-226.

Kelly, P. J., Mason, P. R., Manning, T., & Slater, S. (1991). Role of cattle in the epidemiology of tick-bite fever in Zimbabwe. *Journal of Clinical Microbiology*, *29*(2), 256-259.

Kernif, T., Djerbouh, A., Mediannikov, O., Ayach, B., Rolain, J.M., Raoult, D., Parola, P. and Bitam, I. (2012). *Rickettsia africae* in *Hyalomma dromedarii* ticks from sub-Saharan Algeria. *Ticks and Tick-borne Diseases, 3*(5-6), 377-379.

Kimita, G., Mutai, B., Nyanjom, S. G., Wamunyokoli, F., & Waitumbi, J. (2016). Phylogenetic Variants of *Rickettsia africae*, and Incidental Identification of" *Candidatus Rickettsia Moyalensis*" in Kenya. *PLoS Neglected Tropical Diseases*, *10*(7), 1-14. e0004788.

Kolo, A. O., Sibeko-Matjila, K. P., Maina, A. N., Richards, A. L., Knobel, D. L., & Matjila, P. T. (2016). Molecular detection of zoonotic *Rickettsiae* and *Anaplasma* spp. in domestic dogs and their ectoparasites in Bushbuckridge, South Africa. *Vector-Borne and Zoonotic Diseases*, *16*(4), 245-252.

Kolo, A. O. (2019). Detection of zoonotic bacterial pathogens in multiple hosts using a microbiome approach and molecular characterization of Anaplasma phagocytophilum (Doctoral dissertation, University of Pretoria).

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870-1874.

Kumsa, B., Socolovschi, C., Raoult, D., & Parola, P. (2015). Spotted fever group *Rickettsiae* in ixodid ticks in Oromia, Ethiopia. *Ticks and Tick-borne Diseases*, *6*(1), 8-15.

Lorusso, V., Gruszka, K. A., Majekodunmi, A., Igweh, A., Welburn, S. C., & Picozzi, K. (2013). *Rickettsia africae* in *Amblyomma variegatum* ticks, Uganda and Nigeria. *Emerging Infectious Diseases*, *19*(10), 1705.

Macaluso, K. R., Davis, J., Alam, U., Korman, A. M. Y., Rutherford, J. S., Rosenberg, R., & Azad, A. F. (2003). Spotted fever group *Rickettsiae* in ticks from the Masai Mara region of Kenya. *The American Journal of Tropical Medicine and Hygiene*, *68*(5), 551-553.

Maina, A.N., Jiang, J., Omulo, S.A., Cutler, S.J., Ade, F., Ogola, E., Feikin, D.R., Njenga, M.K., Cleaveland, S., Mpoke, S. and Ng'ang'a, Z. (2014). High prevalence of *Rickettsia africae* variants in *Amblyomma variegatum* ticks from domestic mammals in rural western Kenya: implications for human health. *Vector-Borne and Zoonotic Diseases, 14*(10), 693-702.

Malan, R. C. (2016). Acaricide resistance in *Rhipicephalus (Boophilus)* species at a communal dipping system in the Mnisi community Mpumalanga Province (MSc dissertation, University of Pretoria).

Mandara, S. (2018). The distribution of *Amblyomma variegatum* and *A. hebraeum* in

Zimbabwe and their infection with *Ehrlichia ruminantium* and *Rickettsia africae* (MSc dissertation, University of Pretoria).

Matsimbe, A. M., Magaia, V., Sanches, G. S., Neves, L., Noormahomed, E., Antunes, S., & Domingos, A. (2017). Molecular detection of pathogens in ticks infesting cattle in Nampula province, Mozambique. *Experimental and Applied Acarology*, *73*(1), 91-102.

Mediannikov, O., Diatta, G., Fenollar, F., Sokhna, C., Trape, J. F., & Raoult, D. (2010). Tick-borne rickettsioses, neglected emerging diseases in rural Senegal. *PLoS Neglected Tropical Diseases*, *4*(9), e821.

Mediannikov, O., Diatta, G., Zolia, Y., Balde, M. C., Kohar, H., Trape, J. F., & Raoult, D. (2012). Tick-borne *Rickettsiae* in Guinea and Liberia. *Ticks and Tick-borne Diseases*, *3*(1), 43-48.

Menzer, C., Fink, C., Enk, A., & Haenssle, H. A. (2017). African tick-bite fever – a tantalizing souvenir from South Africa. *JDDG: Journal of the German Society of Dermatology*, *15*(10), 1027–1028. https://doi.org/10.1111/ddg.13322

Mnisi Community Programme, University of Pretoria, *Mnisi Student Orientation Map*, accessed on 19 July 2017, at

https://www.up.ac.za/media/shared/Legacy/sitefiles/file/50/16376/mnisistudentorientationmap1.jpg

Moumouni, P.F.A., Terkawi, M.A., Jirapattharasate, C., Cao, S., Liu, M., Nakao, R., Umemiya-Shirafuji, R., Yokoyama, N., Sugimoto, C., Fujisaki, K. and Suzuki, H. (2016). Molecular detection of spotted fever group Rickettsiae in *Amblyomma variegatum* ticks from Benin. *Ticks and Tick-borne Diseases, 7*(5), 828-833.

Musoke, J. (2016). The epidemiology of tuberculosis in cattle and humans living in the wildlife-livestock-human interface in the rural Mnisi community Mpumalanga province South Africa (Doctoral dissertation, University of Pretoria).

Musoke, J., Hlokwe, T., Marcotty, T., Du Plessis, B. J. A., & Michel, A. L. (2015). Spillover of *Mycobacterium bovis* from wildlife to livestock, South Africa. *Emerging Infectious Diseases*, *21*(3), 448.

Mtshali, K., Nakao, R., Sugimoto, C., & Thekisoe, O. (2017). Occurrence of Coxiella burnetii, Ehrlichia canis, Rickettsia species and Anaplasma phagocytophilum-like bacterium in ticks collected from dogs and cats in South Africa. *Journal of the South African Veterinary Association, 88*(1), 1-6.

Nakao, R., Qiu, Y., Igarashi, M., Magona, J. W., Zhou, L., Ito, K., & Sugimoto, C. (2013). High prevalence of spotted fever group *Rickettsiae* in *Amblyomma variegatum* from Uganda and their identification using sizes of intergenic spacers. *Ticks and Tick-borne Diseases*, *4*(6), 506-512.

Ndip, L.M., Biswas, H.H., Nfonsam, L.E., LeBreton, M., Ndip, R.N., Bissong, M.A., Mpoudi-Ngole, E., Djoko, C., Tamoufe, U., Prosser, A.T. and Burke, D.S., 2011. Risk factors for African tick-bite fever in rural central Africa. *The American Journal of Tropical Medicine and Hygiene, 84*(4), 608-613.

Ndip, L. M., Bouyer, D. H., Da Rosa, A. P. T., Titanji, V. P. K., Tesh, R. B., & Walker, D. H. (2004). Acute Spotted Fever Rickettsiosis among Febrile Patients, Cameroon. *Emerging Infectious Diseases*, *10*(3), 432–437. https://doi.org/10.3201/eid1003.020713

Ndip, L. M., Fokam, E. B., Bouyer, D. H., Ndip, R. N., Titanji, V. P., Walker, D. H., & Mcbride, J. W. (2004). Detection of *Rickettsia africae* in patients and ticks along the coastal region of Cameroon. *The American Journal of Tropical Medicine and Hygiene, 71*(3), 363-366.

Nilsson, K., Wallménius, K., Rundlöf-Nygren, P., Strömdahl, S., & Påhlson, C. (2017). African tick bite fever in returning Swedish travellers. Report of two cases and aspects of diagnostics. *Infection Ecology & Epidemiology*, *7*(1), 1343081.

Norval, R. A. I., Andrew, H. R., & Meltzer, M. I. (1991). Seasonal occurrence of the bont tick (*Amblyomma hebraeum*) in the southern lowveld of Zimbabwe. *Experimental & Applied Acarology*, *13*(2), 81-96.

Oberoi, A., & Singh, N. (2010). *Rickettsiae* Infections -Classification. *JK Science*, *12*(2), 57–59. Retrieved from www.jkscience.org

Ogo, N.I., de Mera, I.G.F., Galindo, R.C., Okubanjo, O.O., Inuwa, H.M., Agbede, R.I., Torina, A., Alongi, A., Vicente, J., Gortázar, C. and de la Fuente, J, (2012). Molecular identification of tick-borne pathogens in Nigerian ticks. *Veterinary Parasitology, 187*(3-4), 572-577.

Oostvogel, P. M., van Doornum, G. J., Ferreira, R., Vink, J., Fenollar, F., & Raoult, D. (2007). African tickbite fever in travelers, Swaziland. *Emerging Infectious Diseases*, *13*(2), 353.

Orkun, Ö., Karaer, Z., Çakmak, A., & Nalbantoğlu, S. (2014). Spotted fever group

Rickettsiae in ticks in Turkey. *Ticks and Tick-borne Diseases*, *5*(2), 213-218.

Owen, C. E., Bahrami, S., Malone, J. C., Callen, J. P., & Kulp-Shorten, C. L. (2006). African tick bite fever: a not-so-uncommon illness in international travelers. *Archives of Dermatology*, *142*(10), 1312-1314.

Parola, P. (2006). Rickettsioses in Sub-Saharan Africa. *Annals of the New York Academy of Sciences*, *1078*(1), 42-47.

Parola, P., Davoust, B., & Raoult, D. (2005). Tick-and flea-borne rickettsial emerging zoonoses. *Veterinary Research*, *36*(3), 469-492.

Parola, P., Inokuma, H., Camicas, J. L., Brouqui, P., & Raoult, D. (2001). Detection and identification of spotted fever group *Rickettsiae* and *Ehrlichiae* in African ticks. *Emerging Infectious Diseases*, *7*(6), 1014-1018.

Parola, P., Paddock, C.D., Socolovschi, C., Labruna, M.B., Mediannikov, O., Kernif, T., Abdad, M.Y., Stenos, J., Bitam, I., Fournier, P.E. and Raoult, D. (2013). Update on tick-borne rickettsioses around the world: a geographic approach. *Clinical Microbiology Reviews, 26*(4), 657-702.

Peter, T.F., Perry, B.D., O'Callaghan, C.J., Medley, G.F., Shumba, W., Madzima, W., Burridge, M.J. and Mahan, S.M., 1998. Distributions of the vectors of heartwater, *Amblyomma hebraeum* and *Amblyomma variegatum* (Acari: Ixodidae), in Zimbabwe. *Experimental & Applied Acarology, 22*(12), 725-740.

Portillo, A., Pérez-Martínez, L., Santibáñez, S., Blanco, J. R., Ibarra, V., & Oteo, J. A. (2007). Detection of *Rickettsia africae* in *Rhipicephalus (Boophilus) decoloratus* ticks from the Republic of Botswana, South Africa. *The American Journal of Tropical Medicine and Hygiene*, *77*(2), 376-377.

Raoult, D., & Roux, V. (1997). Rickettsioses as paradigms of new or emerging infectious diseases. *Clinical Microbiology Reviews*, *10*(4), 694-719.

Raoult, D., Fournier, P.E., Eremeeva, M., Graves, S., Kelly, P.J., Oteo, J.A., Sekeyova, Z., Tamura, A., Tarasevich, I. and Zhang, L. (2005). Naming of Rickettsiae and rickettsial diseases. *Annals of the New York Academy of Sciences, 1063*(1), 1-12.

Raoult, D., Fournier, P.E., Fenollar, F., Jensenius, M., Prioe, T., de Pina, J.J., Caruso, G., Jones, N., Laferl, H., Rosenblatt, J.E. and Marrie, T.J. (2001). *Rickettsia africae*, a tick-borne pathogen in travelers to sub-Saharan Africa. *New England Journal of Medicine, 344*(20), 1504-1510.

Regnery, R. L., Spruill, C. L., & Plikaytis, B. D. (1991). Genotypic identification of Rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. *Journal of Bacteriology, 173*(5), 1576-1589.

Roch, N., Epaulard, O., Pelloux, I., Pavese, P., Brion, J. P., Raoult, D., & Maurin, M. (2008). African tick bite fever in elderly patients: 8 cases in French tourists returning from South Africa. *Clinical Infectious Diseases*, *47*(3), e28-e35.

Roux, V., Fournier, P. E., & Raoult, D. (1996). Differentiation of spotted fever group *Rickettsiae* by sequencing and analysis of restriction fragment length polymorphism of PCR-amplified DNA of the gene encoding the protein rOmpA. *Journal of Clinical Microbiology*, *34*(9), 2058-2065.

Sambou, M., Faye, N., Bassène, H., Diatta, G., Raoult, D., & Mediannikov, O. (2014). Identification of rickettsial pathogens in ixodid ticks in northern Senegal. *Ticks and Tick-borne Diseases*, *5*(5), 552-556.

Scarpulla, M., Barlozzari, G., Marcario, A., Salvato, L., Blanda, V., De Liberato, C., D'Agostini, C., Torina, A. and Macri, G. (2016). Molecular detection and characterization of spotted fever group Rickettsiae in ticks from Central Italy. *Ticks and Tick-borne Diseases, 7*(5), 1052-1056.

Sexton, D. J., Corey, G. R., Greenfield Jr, J. C., Burton, C. S., & Raoult, D. (1999). Imported African tick bite fever: a case report. *The American Journal of Tropical Medicine and Hygiene*, *60*(5), 865-867.

Simpson, G.J., Quan, V., Frean, J., Knobel, D.L., Rossouw, J., Weyer, J., Marcotty, T., Godfroid, J. and Blumberg, L.H. (2018). Prevalence of selected zoonotic diseases and risk factors at a human-wildlife-livestock interface in Mpumalanga Province, South Africa. *Vector-Borne and Zoonotic Diseases, 18*(6), 303-310.

Soares, H.S., Barbieri, A.R., Martins, T.F., Minervino, A.H., de Lima, J.T., Marcili, A., Gennari, S.M. and Labruna, M.B., 2015. Ticks and rickettsial infection in the wildlife of two regions of the Brazilian Amazon. *Experimental and Applied Acarology, 65*(1), 125-140.

Socolovschi, C., Huynh, T., Davoust, B., Gomez, J., Raoult, D., & Parola, P. (2009). Transovarial and trans-stadial transmission of *Rickettsiae africae* in *Amblyomma variegatum* ticks. *Clinical Microbiology and Infection*, *15*, 317-318.

Socolovschi, C., Renvoisé, A., Brouqui, P., Parola, P., & Raoult, D. (2012). The use of eschar swabs for the diagnosis of African tick-bite fever. *Ticks and Tick-borne*

Diseases, *3*(5-6), 361-363.

Stenos, J., Graves, S. R., & Unsworth, N. B. (2005). A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group *Rickettsiae*. *The American Journal of Tropical Medicine and Hygiene*, *73*(6), 1083-1085.

Strand, A., Paddock, C.D., Rinehart, A.R., Condit, M.E., Marus, J.R., Gillani, S., Chung, I.H. and Fowler Jr, V.G. (2017). African tick bite fever treated successfully with rifampin in a patient with doxycycline intolerance. *Clinical Infectious Diseases, 65*(9), 1582-1584.

Tamura, A., Ohashi, N., Urakami, H., & Miyamura, S. (1995). Classification of Rickettsia tsutsugamushi in a New Genus, Orientia gen. nov., as Orientia tsutsugamushi comb. nov. *International Journal of Systematic and Evolutionary Microbiology, 45*(3), 589-591.

Thrusfield, M. (2007). Veterinary Epidemiology. Black Well. Oxford. UK.

Tomassone, L., De Meneghi, D., Adakal, H., Rodighiero, P., Pressi, G., & Grego, E. (2016). Detection of *Rickettsia aeschlimannii* and *Rickettsia africae* in ixodid ticks from Burkina Faso and Somali Region of Ethiopia by new real-time PCR assays. *Ticks and Tick-borne Diseases*, *7*(6), 1082-1088.

Toutous-Trellu, L., Péter, O., Chavaz, P., & Saurat, J. H. (2003). African tick bite fever: Not a spotless rickettsiosis!. *Journal of the American Academy of Dermatology*, *48*(2), S18-S19.

Tsai, K. H., Lu, H. Y., Huang, J. H., Fournier, P. E., Mediannikov, O., Raoult, D., & Shu, P. Y. (2009). African tick bite fever in a Taiwanese traveler returning from South Africa: molecular and serologic studies. *The American Journal of Tropical Medicine and Hygiene*, *81*(5), 735-739.

Todar, K. (2006). Todar's online textbook of bacteriology, http://www.academia.edu/download/48563182/Todars_Online_Textbook_of_Bacteriology.pdf.

van Eekeren, L. E., de Vries, S. G., Wagenaar, J. F., Spijker, R., Grobusch, M. P., & Goorhuis, A. (2018). Under-diagnosis of rickettsial disease in clinical practice: a systematic review. *Travel Medicine and Infectious Disease*, *26*, 7-15.

Vogel, H., Foley, J., & Fiorello, C. V. (2018). *Rickettsia africae* and novel rickettsial strain in *Amblyomma* spp. ticks, Nicaragua, 2013. *Emerging Infectious*

Diseases, *24*(2), 385.

Walker, A. R. (2003). *Ticks of domestic animals in Africa: A Guide to Identification of Species* 3-210. Edinburgh: Bioscience Reports.

Walker, J. B., & Olwage, A. (1987). The tick vectors of *Cowdria ruminantium* (Ixodoidea, Ixodidae, genus *Amblyomma*) and their distribution. *The Onderstepoort Journal of Veterinary Research*, *54*(3), 353–379.

Yssouf, A., Socolovschi, C., Kernif, T., Temmam, S., Lagadec, E., Tortosa, P., & Parola, P. (2014). First molecular detection of *Rickettsia africae* in ticks from the Union of the Comoros. *Parasites & Vectors*, *7*(1), 444.

Zammarchi, L., Farese, A., Trotta, M., Amantini, A., Raoult, D., & Bartoloni, A. (2014). *Rickettsia africae* infection complicated with painful sacral syndrome in an Italian traveller returning from Zimbabwe. *International Journal of Infectious Diseases*, *29*, 194-196.

APPENDICES

Appendix 1.1: V045-18 University of Pretoria Animal Ethics Committee approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Investigating *Rickettsia africae* infection in *Amblyomma hebraeum* ticks in Mnisi, Bushbuckridge Municipality, South Africa**
PROJECT NUMBER	**V045-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. E Mazhetese**

STUDENT NUMBER (where applicable)	U_17407380
DISSERTATION/THESIS SUBMITTED FOR	**MSc (Tropical Diseases)**

ANIMAL SPECIES/SAMPLES	**Cattle**	**Ticks**
NUMBER OF ANIMALS	212	742
Approval period to use animals for research/testing purposes	**July 2018 – July 2019**	
SUPERVISOR	**Dr. D Morar-Leather**	

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	2 August 2018
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15

Appendix 1.2: V045-18 University of Pretoria Animal Ethics Committee approval Extension 1

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

27 June 2019

Approval Certificate
Annual Renewal
(Extension 1)

AEC Reference No.: V045-18
Title: Investigating *Rickettsia africae* infection in *Amblyomma hebraeum* ticks in Mnisi, Bushbuckridge Municipality, South Africa.
Researcher: Dr E Mazhetese
Student's Supervisor: Dr D Morar-Leather

Dear Dr E Mazhetese,

The **Annual Renewal** as supported by documents received between 2019-06-07 and 2019-06-24 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-06-24.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number
Cattle	212
Ticks	742

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-06-27.
3. Please remember to use your protocol number (V045-18) 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.

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.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 3-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8463
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadirulwa

Appendix 2: Faculty Research Ethics Committee certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	**Investigating *Rickettsia africae* infection in *Amblyomma herbraeum* ticks in Mnisi, Bushbuckridge Municipality, South Africa**
PROJECT NUMBER	**REC066-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Estere Mazhetese**

DISSERTATION/THESIS SUBMITTED FOR	**MSc**

SUPERVISOR	**Darshana Morar-Leather**

APPROVED	Date **24 June 2019**
CHAIRMAN: UP Research Ethics Committee	Signature

Appendix 3: Department of Agriculture Fishery and Forestry (DAFF) approval

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8 (811)

Ms Estere Mazhetese
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Tel: 012 529 8278
E-mail: darshana.moran@up.ac.za; emazhets@gmail.com, svorpen@gmail.com

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

Dear Ms Mazhetese,

Your application sent with the email on 7 June 2016 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following 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. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. Ticks collected from cattle at diptanks in the Bushbuckridge Municipality may only be sent to the Hans Hoheisen Wildlife Research Unit (HHWRS), accompanied by the relevant state veterinary movement permit required;
4. All samples must be packaged and transported in accordance with the National Road Traffic Act, 1996 (Act No. 93 of 1996);

Appendix 4: Consent form translated into local language informing the farmers and owners of the cattle about the study. Owners signed the form to give permission to collect ticks from the cattle.

PAPILA RA MPFUMELELANO

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Hi swinile ntwanano wa leswaku swifuwo leswi vuriwaki la hansi swinga tirhisiwa hi valavisisi. Tani hi loko swi vuriwile eka nkhaluselo ya maendlelo ya kona.

1. leswi nga ta hetisisiwa hi mulavisisi.

VITO RA MULAVISISI
Estere Mazhetese

VITO RA MULAVISISI WA PROJEKE

Ku lavisisa vuvabyi bya (Ricketsia africae eku xigalana lexi vuriwaka Amblyoma hebraeum e hansi ka mfumo wa ka nxisi eka masipala wa bushbuckridge

XIKONGOMELO XA MULAVISISI WA PROJEKE

Ku tiyisisa nhlayo ya mahangalakelo ya swigalana leswi kulu na leswitsongo.

KU HUMELERISA MAENDLELO HI VUENTI

Tihomu ti ta khomiwa hi ku tirhisa xiperako ti ngasi diba
Ku susa swigalana leswi kulu swa ti amblyomma.
Ku pfimba eka le ta xisati ku nga va kona.
Myimo wa swigalana swinharhu wu ta tekiwa eka homu yinwani na yinwani ya xisati
Swigalana leswitsongo swi ta khomiwa hi ku swi koka hiku tirhisa xilo xo phasa swigalana enabyanyini.

TINGHOZI LETI NGHENELELEKE EKA MAENDLELO LAWA

Homu yi ngava na ku chavanyana ka tsongo loxo yi ri karhi yi susiwa swigalana.

MAHLAWULELO YA SWIGALANA LESWI NGATA TIRHISIWA

Swigalana swi ta tekiwa eka Tihomu, nqinqi ra Tihomu ri ta tirhisiwa ku hambanisa swifuwo

A HI XIHOXO KU HAMBANISA SWIFUWO LESWI NGA T A TIRHISIWA

Nomboro ya homu ya mufuwi yi ta tirhisiwa

2. leswi nga ta hetisisiwa hi mufuwi kumbe loyi anga yimela mufuwi a tlhela a pfumeleriwa ku sayina.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

VITO RA MUFUWI

Ku na mahungu lawa unga ma kuma mayelana na dyondzo leyi.

Unga va u hlamuseriwile hiti nghozi leti nga ta va kona eka maendlelo lawa ngava nswi twisisile hi va endi bya ti nghozi leti.

Xana wa tiyisisa leswaku hinga humelerisi maendlelo lawa

3.laha ku nga sayiniwa ku twananiwe leswaku aku na hakelo leyi ngata hakeriwa nwini wa swifuwo kumbe unwani swikexo hinkwaswo swa vulavisisi leliyi swita yimeriwa hi mulavisisi.

4. laha ku sayiniwe ntwanano wa leswaku aku ngevi na ndzho wo karhi ku suka eka yunivhesiti ya pitori mayelana na maendlelo lama nga ta endliwa eka mufuwina swifuwo swa yena.

5.laha ku sayiniwe ntwanano wa ku aku na masaka wa switirhisiwa ku kutsa mahungu, na leswi mulavisisi aswi kumeke kumbe mbuyelo wa maendlelo wu nga hundzisiwa kuya eka ntlawa wa vunharhu kumbe xikongomelo xinwani kungari leswi humelerisiweke e handle ka mpfumelelano lowu tsariweke hi nwini wa xifuwo.

6. A kuna leswi ngata humelerisiwa handle mar swinga tirhisiwa exihundleni tani hi nwini wa pfumeleriwa ku tshika loko unga ha swi lsakela.

Nsayino wa mulavisisi

Nsayino wa Mbhoni

nsayino wa mufuwi

siku

Nilokombaka ku lavisisiwa ka vuvabyi bya swigalana leswi hangalasaki mavabyi, hi xona xivangelo xa dyondzo leyi ku ya hi Nkoka wa vutshunguri va swifuwo ni vadokodela va vanhu na va ndzawulo ya swa vuendzi.
Mahungu ya mavabyi na Tiboma ma pfumaleka hikokwalaho mi fanele mi dyondzo ngopfu hi tlhelo ra mavabyi lawa .
Ku tlulela ka mavabyi lawa swi kombisiwile kambe aswi ti veki leswaku ma suka kwihi . mpimo wa mahangalakelo ya mavabyi lawa awu le rivaleni hi ndlela yoleyo hi fanele hi tirhisa switirhisiwa swo hambanahambana ku hlta riva

Appendix 5: Movement permit for moving samples from Mnisi Community Programme study area to Hans Hoheisen Wildlife Research Station.

Permit in	Permit out

DEPARTMENT OF AGRICULTURE

MPUMALANGA

Permit number: 20181025 ORC- MMP1

DEPT. LANDBOU

UMNYANGO WETEKULIMA

master

VETERINARY PERMIT TO MOVE ANIMALS / ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Dieresiektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngekulandzela lomTsetfo weTifo teTilwane No. 35 wa 1984:

Name: / Naam: / Libito:	Ester Mazhetese	ID No.:	DN698769
Address: / Adres: / Likheli:	University of Pretoria		
to move the following: / om te beweeg met:	Three hundred (amount in words)	Ticks — species: Amblyomma hebraeum	LIVE ~~ANIMALS~~
identified as follows: / geïdentifiseer as volg:	Amblyomma ticks from cattle		
from the farm/plant/area/place: / vanaf die plaas/plant/gebied/plek:	Utlah A & Welverdiend A	STOCK CARD NUMBER	in the district of: Bohlabela
to the farm/plant/area/abattoir/place: / na die plaas/plant/gebied/abattoir/plek:	Hans Hoheisen Wildlife Research Station		in the district of: Bohlabela

On the following conditions:
1. This permit: 30 days
(a) is valid for [illegible] from date of issue and for one consignment only
(b) must accompany the animals / products mentioned above;
(c) must be produced for inspection on demand by any land or animal owner or police or veterinary official;
(d) must be kept at a destination until collected by a veterinary official.
2. For game / game products a relevant conservation permit is also required.
3. Animals and products must be visibly free from external parasites.
4. Animals are to be transported in accordance with animal welfare guidelines.
5. [illegible]
6. Other conditions:

As per section 20 approval.
List of samples to be captured on accompanying MMP list.

R. Retention for / Quarantine for / Direct slaughter within: [illegible] days after arrival at destination

Seal nos:	N/A	Place / Plek:	Hans Hoheisen
Vehicle reg. nos:	FF 67 VJ GP	Date / Datum:	2018/10/25

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

Dr C.C. Davis — Issuing Veterinary Official

SIGNATURE — State Veterinarian / Staatsveearts

IMPORTANT NOTE / BELANGRIKE NOTA
[illegible]

2018/10/25 — DATE STAMP 0009

DISTRIBUTION:

Appendix 6: Farmer feedback session document

Introduction

This is a feedback session to make you aware of African Tick Bite Fever (ATBF) and how people contract it. ATBF is a disease in humans caused by *Rickettsia africae*, a bacterium. This bacterium is transmitted by *Amblyomma hebraeum* ticks (those colourful ticks – pictures shown below) and these ticks are mainly found on cattle, goats, sheep, donkeys and dogs.

Amblyomma hebraeum tick image

Fed female (*A. hebrauem*)

Symptoms of African Tick Bite Fever in humans

Headaches, fever, rash, multiple eschars at tick bite sites. After the bite one will experience symptoms after seven to ten days.

Aim of the project

From the ticks we collected from the cattle in this area, we wanted to see how many of them have the bacteria *R. africae* which causes ATBF in humans.

What was done?

Collection of *Amblyomma hebraeum* ticks from cattle at Utah A and Welverdiend A dip tanks.

Female ticks that have fed and are ready to lay eggs were also collected.

Findings

The bacteria, *R. africae* that causes ATBF, was found in some of the ticks collected from this area hence there is a risk of contracting ATBF if someone gets bitten by *A. hebraeum* tick that has *R. africae*. People need to watch out for those signs especially those who associate with cattle and other domestic animals or are performing many outdoor activities. If one sees such signs, that person should go to see the doctor or go to a clinic.

Although many cases are reported in foreign travellers, there have been cases where locals were reported to become sick with ATBF.

What you can do to prevent getting bitten by ticks?

Several studies have reported detection of *R. africae* in some other ticks besides *Amblyomma* ticks which are the known vector so people should avoid tick bites by wearing protective clothing and use of insecticides when going to the bushes for different activities.