MOLECULAR DETECTION OF ZOONOTIC TICK-BORNE PATHOGENS IN LIVESTOCK IN DIFFERENT PROVINCES OF SOUTH AFRICA

By

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DECLARATION

I the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

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DEDICATION

‘To my father, for the enduring spiritual upliftment’

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ABBREVIATIONS

µL	Microliter
µm	Micrometer
A.	*Anaplasma*
A.	*Amblyomma*
ank	Progressive ankylosis gene encoding for multipass transmembrane protein expressed in joints and other tissues.
ATBF	African Tick Bite Fever
B.	*Borrelia*
B.	*Babesia*
C.	*Coxiella*
CA	Cross Adsorption
CCI	Cell Culture Isolation
CDC	Center for Disease Control and Prevention
CME	Canine Monocytic Ehrlichiosis
CNS	Central Nervous System
CZC	Research Center for Zoonosis Control
DNA	deoxyribo-nucleic-acid
E.	*Ehrlichia*
EC	Eastern Cape Province
ECM	*erythema chronicum migrans*
EDTA	Ethylenediaminetetraacetic acid

EIA	Enzyme immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
FS	Free State Province
g	Grams
gltA	Citrate synthase encoding gene
H.	*Haemaphysalis*
H.	*Hyalomma*
HGA	Human Granulocytic Anaplasmosis
HGE	Human Granulocytic Ehrlichiosis
HME	Human Monocytic Ehrlichiosis
I.	*Ixodes*
IFA	Indirect Immunoflourenscent assay
IFAT	Indirect Immunoflourenscent assay technique
KZN	KwaZulu-Natal Province
LB	Lyme Borreliosis
LCVs	Large Cell Variants
LD	Lyme disease
min	Minutes
mL	Milliliter
MLVA	Multiple-Locus Variable number tandem repeat Analysis
MP	Mpumalanga Province
MSFG	Mediterranean Spotted Fever Group
msp2	Major surface protein 2
NaTHNaC	National Travel Health Network and Center

NW	North West Province
OIE	Office International des Epizootics
ORFs	Open Reading Frames
OVI	Onderstepoort Veterinary Institute
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEP	Postexposure Prophylaxis
PRP	Parasitology Research Program
PV	Parasitophorous Vacuole
R.	*Rhipicephalus*
R.	*Rickettsia*
RFLP	Restriction Fragment Length Polymorphism
RMSF	Rocky Mountain spotted fever
RNA	Ribonucleic acid
rOmpA	One of three major high-molecular-mass rickettsial outer membrane protein
rRNA	ribosomal RNA
s.l.	*sensu lato*
s.s.	*sensu stricto*
SARS	Severe Acute Respiratory Syndrome Coronavirus (sometimes shortened SARS CoV)
SCVs	Small Cell Variants
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sec/s	seconds
SFG	Spotted Fever Group
T.	*Theileria*

TBD(s)	Tick-Borne Disease(s)
TBE	Tris-Borate EDTA
TBF	Tick Bite Fever
WB	Western Blotting
x *g*	relative centrifugal force

RESEARCH OUTPUTS

Publications

1. K. Mtshali, M.S. Mtshali, J.S. Nkhebenyane, O.M.M. Thekisoe. (2012). Detection of *Salmonella*, *Clostridium perfringens* and *Escherichia coli* from fecal samples of captive animals at the National Zoological Gardens of South Africa, *African Journal of Microbiology Research*, 6(15), pp. 3662-3666.

2. Mtshali, K., Thekisoe M.M.O., Sugimoto C. (2013). Molecular detection of *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Ehrlichia canis* and *Rickettsia* species from ticks collected from dogs in South Africa (under review, Ticks and Tick-borne Diseases Journal).

Publication of results in conference proceedings

Mtshali K., Thekisoe M.M.O., Sugimoto C. Molecular detection of tick-borne zoonotic pathogens in livestock from in the different Provinces of South Africa., 41st Parasitological Society of Southern Africa Conference, Bloemfontein, 1-3 October, 2012.

ABSTRACT

Ticks and tick-borne diseases are a burden in the livestock industry, decreasing productivity and compromising food security, leading to high socioeconomic impacts on agro-exporting nations. Apart from being agricultural pests they can transmit pathogens of zoonotic significance. The aim of the study was to therefore detect and determine with PCR the prevalence of tick-borne zoonotic pathogens i.e. *Coxiella burnetii*, *Ehrlichia* spp., *Rickettsia* spp*., Anaplasma phagocytophilum*, *Borrelia burgdorferi sensu lato* from ticks collected from livestock. The sampling areas included both commercial and communal farms as well as domestic animals from KwaZulu-Natal, Free State, Eastern Cape, North West and Mpumalanga Provinces.

As a result a total of 1947 tick samples were collected which were then identified and further processed for PCR amplification. Tick species collected included *Rhipicephalus* spp. (n = 570), *R. sanguineus* (n = 275), *R. evertsi evertsi* (n = 650), *R. decoloratus* (n = 228)*, R. appendiculatus* (n = 10), *Amblyomma hebraeum* (n = 171), *Hyalomma marginatum rufipes* (n = 4), and *Haemaphysalis elliptica* (n = 38). The overall prevalence of infection with *B. burgdorferi* and *A. phagocytophilum* was 8±1.4% and 9±1.2% respectively, this was an unexpected finding since only one positive PCR confirmation of *A. phagocytophilum* has been reported in the country, since then no other studies have been successful in detecting this pathogen. There have been anecdotal cases of *B. burgdorferi* but the pathogen has, to the best my knowledge, not been detected and characterized by molecular methods. Both pathogens have not been isolated from ticks in South Africa previously. The tick vectors for these pathogens are not known to occur in the country, however this study managed to isolate *A. phagocytophilum* from *R. sanguineus, R. appendiculatus, R. evertsi evertsi* and *H. elliptica* and isolated *B. burgdorferi* from *Rhipicephalus* spp., *R. evertsi evertsi*, *R. decoloratus* and *A. hebraeum* which may act as main vectors and reservoir for livestock infections. I am however uncertain about their transmissibility neither to human hosts nor of their vectorial capacity, nevertheless tick species of *Amblyomma* readily bite

humans and may be able to transmit both pathogens and could therefore pose a serious threat to the public.

C. burnetii incidence was 16±1.6% amongst the ticks, this was also a first detection from ticks in the country but the findings seem to be consistent with previous serological studies, also all the tick species in the collection were found harboring the pathogen.

The prevalence of *E. ruminantium*, *E. canis* and *Ehrlichia*/*Anaplasma* was determined to be 29±2.2%, 20±3.6% and 18±3.8% respectively. No significant *Ehrlichia*/*Anaplasma* species were characterized except for *A. phagocytophilum* as reported above.

Rickettsia species that were isolated and characterized in the current study were *R. africae* and *R. conorii* as expected and the prevalence was 26±1.7%. All in all the target pathogens were successfully isolated, characterized and validated through sequencing reactions, however there still remains a task of determining the vectorial capacity of the ticks and evaluation of factors that could lead to their transmission to the public. In conclusion, these pathogens should be considered as part of routine screening in patients presenting with fevers of unknown origin especially amongst tourists where pathogens like *Rickettsia* seem to have become problematic in South Africa.

Chapter 1: Preamble

1.1. Background

Ticks are excellent vectors for disease transmission; they are second only to mosquitoes as vectors of human disease, both infectious and toxic (Andreotti *et al.*, 2011). They can carry and transmit a remarkable array of pathogens, including bacteria, spirochetes, rickettsiae, protozoa, viruses, nematodes, and toxins (Matjila *et al*., 2008; Berrada and Telford, 2009; Crowder *et al*., 2010).

From a livestock perspective, tick infestations and tick-borne diseases (TBDs) are important conditions affecting livestock health and productivity worldwide, examples of major TBDs in South Africa are anaplasmosis, babesiosis, ehrlichiosis and theileriosis (Ndlhovu *et al*., 2009). Not picky in their eating habits, they take their requisite blood meal from mammals, reptiles, amphibians and birds (Jongejan and Uilenburg, 2004). Their feeding habits can have detrimental effects on the host as some ticks secrete a cementing material to fasten themselves to the host. In addition, *Ixodes* ticks secrete anticoagulant, immunosuppressive, and anti-inflammatory substances into the area of the tick bite. These substances presumably help the tick to obtain a blood meal without the host noticing. These same substances also help any freeloading pathogens to establish a foothold in the host leading to secondary microbial infections (Berrada and Telford, 2009, Ndhlovu *et al*., 2009).

Apart from being agricultural pests, ticks can also carry pathogens which are transmissible to humans upon tick bite or by being directly in contact with infected animals causing diseases known as zoonoses for example, Lyme Disease (LD) and Human Granuloctic Anaplasmosis (HGA) caused by *Borrelia burgdorferi sensu lato* and *Anaplasma phagocytophilum* respectively. An individual tick often is infected with more than one pathogenic organism. Multiple infections can have medical significance, because co-infection can increase severity of symptoms in humans and animals (Ginsberg, 2008).

Zoonoses can become a serious limitation on exportation of animal products and thus international trade. They compromise food security causing a high socio-economic

impact on agro-exporting nations each year (Rojas, 2011). Moreover, the impact of these diseases is compounded in poor households where zoonoses affect both people and animals, because poor people keep fewer animals, they will suffer disproportionally from the illness or death of their animals. Livestock are often central to survival strategies in poor households as they may be sold to meet emergency expenditures such as school fees, treatment and hospitalization of family members or food in times of shortage (Epstein and Price, 2009).

Diseases that start as zoonoses may in the long run turn out to become communicable diseases of man. Although not necessarily the case for all of them, there are a few examples that have been proven to have originated as zoonoses, for example AIDS and measles. The conclusion from this is that zoonoses must be considered seriously as possible future human communicable diseases, and that ignoring them will pose a threat to public health (Cripps, 2000). In many countries the impact of zoonotic disease has hardly been investigated at all so it is difficult to estimate their contribution to human illness. Many veterinarians are less aware of the importance of zoonoses than is desirable and medical clinicians who encounter zoonoses in human patients may either fail to recognize them or concentrate on treating the individual patient rather than disease control (Cripps, 2000).

Endemic zoonoses usually do not get much attention as compared to newly emerging ones that attract the attention of the developed world. This is, in part, a consequence of under-reporting, resulting in underestimation of their global burden, which in turn as stated by Maudlin *et al*., (2009) artificially reduce their importance in the eyes of administrators and funding agencies.

1.2. Statement of the problem

In South Africa the role of ticks in disease transmission especially among livestock has been widely reported. Diseases include amongst others babesiosis, ehrlichiosis, theileriosis and anaplasmosis. In the agricultural sector research on vaccine candidate determination, eradication and control strategies are eminent. However their role as

vectors of human diseases has been under-reported all over the country. Gummow, (2003) reported that 63.6% veterinarians in South Africa suffer from a zoonotic disease. That approximately 46% of South Africans still live in rural areas where they are exposed to zoonosis as they regularly come into contact with farm animals. Zoonotic diseases of interest have been reported to include Rift Valley Fever, Rabies, Crimean-Congo Haemorrhagic Fever and African Tick Bite Fever however there are little or no reports of illnesses or deaths caused by these pathogens among locals. On the other hand there are several cases of illnesses reported from tourists returning from safari tours around the country; all the same though, these findings and associated publications are not those of our own scientists. Most if not all the tourists were diagnosed in their own countries, examples include tourists from Taiwan, Netherlands, France and Switzerland who contracted *Rickettsia africae* at one or more of our (South Africa's) many nature reserves (Raoult *et al*., 2001; Delfos *et al*., 2004; Tsai *et al*., 2004; Roch *et al*., 2008; Althausk *et al*., 2010), thus indicating lack of awareness, research and interest in this field from South African scientists.

A survey on the medical curricula at universities in South Africa showed that there are deficiencies at undergraduate training level in recognizing zoonotic conditions. This coupled with limited laboratory facilities and lack of funds to carry out comprehensive diagnostic procedures gives evidence that a large number of zoonotic conditions are currently misdiagnosed or go undiagnosed in the country (Gummow, 2003). The qualitative and quantitative effects of zoonosis in the country thus remain unavailable. This could negatively impact on our tourism industry and associated sectors as zoonoses can act as agents of bioterrorism, it is thus indicative that there is still room for development and improvement in this area.

In an effort to recover the prevalence of tick-borne zoonotic pathogens a retrospective survey needed to be conducted. Randomly selected study areas included communal and cormmercial farms, domestic households and a veterinary clinic from KwaZulu-Natal, Eastern Cape, North West, Mpumalanga and the Free State Provinces of South Africa. Ticks were collected from goats, cattle, sheep, dogs and incidentally from cats and horses and also from the vegetation. DNA from these samples was screened for

the target zoonotic pathogens. Results thereafter analyzed using statistical methods to determine the prevalence of infection of ticks.

Data on the prevalence and distribution of these parasites can be used for future research on development of drugs and other remedies to protect animals from ticks and TBDs in the country. Multidisciplinary approach to investigation, combination of new molecular and cellular biology diagnostic tools to distinguish these diseases by the unique bacterial pathogens may be the solution to keeping zoonoses in check and raising awareness about such diseases in the medical and tourism sectors.

1.3. Tick-borne pathogenic diseases and their vectors

1.3.1. Tick-borne diseases (TBDs)

Tick infestations and tick-borne diseases (TBDs) are important conditions affecting livestock health and productivity worldwide. Ticks are major vectors of arthropod-borne infections and can transmit a wide variety of pathogens, such as rickettsias, viruses, and protozoans, but may also carry more than one infectious agent and thus can transmit one or more infections at the same time (Matjila *et al*., 2008; Sparagano *et al*., 1999). These pathogens may cause diseases with varying severity depending on the species infected (Epstein and Price, 2009). Examples of major TBDs among livestock in South Africa are anaplasmosis, babesiosis, ehrlichiosis/ cowdriosis and theileriosis (Ndlhovu *et al*., 2009). In general, tick-borne protozoan diseases (e.g. theileriosis and babesiosis) and rickettsial diseases (e.g. anaplasmosis and heartwater) are pre-eminent health and management problems of cattle and small ruminants, as well as buffalo, affecting the livelihood of farming communities in Africa, Asia and Latin America. TBDs lead to great economic losses in terms of mortality and morbidity of livestock (Jongejan and Uilenberg, 2004; Sparagano *et al*., 1999). They have a significant impact on meat and milk production and consequently on livestock management (Ndhlovu *et al*., 2009).

Recently, TBDs were ranked high in terms of their impact on the livelihood of resource poor farming communities in developing countries (Jongejan and Uilenberg, 2004).

Apart from being agricultural pests TBDs pose a major threat to human health, manifested in the form of zoonoses. According to Stephen and Baum, (2008) zoonoses are infections that are spread from animals to humans either directly or through an arthropod vector. Most often, humans are “dead-end” hosts, meaning that there is no subsequent human-to-human transmission. There are hundreds of zoonoses, categorized by the organism causing the disease, by the animal reservoir and by the manner in which the disease is contracted, i.e., through arthropod bite, direct contact or ingestion. The animals may be wildlife, livestock, zoo or laboratory animals (Epstein and Price, 2009). Endemic zoonoses are found throughout the developing world, wherever people live in close proximity to their animals, affecting not only the health of poor people but often also their livelihoods through the health of their livestock (Maudlin *et al*., 2009). Therefore, zoonoses represent infections that can never be eliminated and must be considered as permanent and recurrent factors to be dealt with in protecting human health (Dennis and Piesman, 2005; Stephen and Baum, 2008). Zoonoses can become a serious limitation on exportation of animal products and thus international trade. They compromise food security causing a high socioeconomic impact on agroexporting nations each year (Rojas, 2011). Moreover, the impact of these diseases is compounded in poor households where zoonoses affect both people and animals; because poor people keep fewer animals, they will suffer disproportionally from the illness or death of their livestock. Livestock are often central to survival strategies in poor households as they may be sold to meet emergency expenditures such as school fees, treatment and hospitalization of family members or food in times of shortage (Maudlin *et al*., 2009). Unlike newly emerging zoonoses that attract the attention of the developed world, endemic zoonoses are by comparison neglected. This is, in part, a consequence of under-reporting, resulting in underestimation of their global burden, which in turn artificially reduce their importance in the eyes of administrators and funding agencies (Maudlin *et al*., 2009).

The ability of infectious agents to cross the species barrier explains why numerous zoonotic and vector-borne agents affect humans. Vorou *et al*., (2007) reports that, of the increasing number of pathogens of man, 1415 (61%) are zoonotic. Among emerging infectious diseases, 75% are zoonotic, originating principally from wildlife. The latter is a reservoir of microorganisms that, once transferred to humans, may emerge as public health threats. They further state that factors contributing to emergence of new diseases and their spread coincide with those of their vectors as will be discussed in the following chapter, i.e. increasing proximity of human and animal populations caused by growth of the human population, their mobility for recreational, cultural and socioeconomic purposes, and the efforts to keep well nourished. Other than tick transmitted zoonoses, air transportation and air travel may facilitate the global spread of emerging infectious diseases as occurred with the SARS epidemic (Cripps, 2000, Vorou *et al*., 2007).

With the increasing trend of treating animals as part of the family the risk of contraction of such diseases is exacerbated and this poses a threat to human health. Therefore this calls for a detailed epidemiological study of the pathogens between their hosts, vectors and reservoirs (Berrada and Telford, 2009). Reporting the incidence of bacterial pathogens and detecting the rise in incidence of a specific disease they cause remains the cornerstone of containment of emerging communicable threats (Vorou *et al*., 2007).

1. 3.2. Bacterial and rickettsial diseases

Tick-borne bacterial pathogens constitute a large domain of prokaryotic microorganisms, typically a few micrometres in length, with a wide range of shapes, from spheres to rods and spirals. These are recognizable in the agricultural sector and several, pathogenic to man. Known zoonotic tick-borne bacterial diseases include diseases that are caused by pathogens in the orders Spirochaetales (*Borrelia burgdorferi s.l.,* a complex of about ten species, three of which are pathogenic to man), Legionellales (*Coxiella burnetii*) and Rickettsiales (*Rickettsia africae*, *Anaplasma phagocytophilum*, *Ehrlichia* spp.*)* to mention just a few.

On the other hand rickettsial diseases are zoonoses caused by obligate intracellular bacteria grouped in the order Rickettsiales. Bacteria in this order were first described as short, gram-negative rods that retained basic fuschin when stained by the method of Gimenez, but over the last decade their classification has been changed and drastically reorganized due to technological advances in molecular genetics. The classification within Rickettsiales down to the species level continues to be modified as more data becomes available (Parola *et al*., 2005, Parola, 2006). An example can be made by *Coxiella burnetii* which has been removed from Rickettsiales and placed into Legionellales based on similarities of their genetic composition (Fournier *et al*., 1998; Heinzen *et al*., 1999). To date there are three diseases that are still commonly classified as rickettsial diseases, they include: (i) rickettsiosis caused by bacteria of the *Rickettsia* genus (spotted fever group and typhus group rickettsiosis), (ii) ehrlichiosis and anaplasmosis in the Anaplasmataceae family that has been re-organised and, (iii) scrub typhus caused by *Orienta tsutsugamushi* prevalent in the Asia-pacific region (Parola, 2006). For the purpose of this study and for the sake of clarity the diseases caused by *E. canis*; *E. ruminantium*; *A. phagocytophilum; R. africae*; *C. burnetii;* and *B. burgdorferi s.l.* shall be referred to by the names: canine monocytic ehrlichiosis (CME), heartwater, Human Granulocytic Anaplasmosis (HGA), African Tick Bite Fever (ATBF), coxiellosis (Q-fever) and Lyme borreliosis/Lyme Disease (LB/LD) respectively. This will serve to avoid confusion as most of these, at some point in history qualified to be classified as rickettsiosis/ rickettsial diseases and in many circumstances the term 'rickettsial disease' is used interchangeably with 'bacterial disease'. I shall discuss these pathogens further on in the sections to follow, for details on the diseases they cause, their vectors and reservoir hosts refer to Table 1. 3.

1.3.3. Mode of infection of tick host with bacterial/rickettsial pathogens

Ticks may become infected with bacteria by feeding on bacteremic animals or by transstadial and transovarial transmission. Transstadially the microbial parasite such as a virus or rickettsia is passed, from one developmental stage (stadium) of the host to its subsequent stage or stages, whereas in transovarial transmission the parasite is

passed from the maternal body to eggs within the ovaries hence the larvae become infective (Parola *et al*., 2005). All forms of transmission may occur for some bacteria; for example, the spotted fever group rickettsiae can be transmitted via all routes. Three points are essential to understanding the ways in which bacteria are transmitted by ticks and the consequences for tick-borne bacterial diseases. Firstly, rickettsiae multiply in almost all organs and fluids of ticks, in particular the salivary glands and ovaries, which enables transmission of organisms during feeding and transovarially, respectively. Secondly, each stage of ixodid tick feeds only once, and bacteria acquired by a tick during feeding can then be transmitted to another host only when the tick has molted to its next developmental stage. Finally, if bacteria such as the rickettsiae are transmitted both transstadially and transovarially in a tick species, this tick will also be the reservoir of the bacteria, and the distribution of the disease caused by the bacteria will be identical to that of its tick host stage. It is very rare, but ticks may become infected with bacteria by co-feeding, that is, several ticks feeding close to one another on the same host leading to direct spread of bacteria from an infected tick to an uninfected one. Sexual transmission from infected male to female has been reported in only some rickettsiae and some species of relapsing fever borreliae. Little is known about effects of bacteria on tick hosts themselves (Parola and Raoult, 2001).

1.4. Objectives of the study

This study aims at recovering the current status of TBDs in livestock around South Africa, the main focus being to search for zoonotic *Ehrlichia*, *Anaplasma, Coxiella, Rickettsia* and *Borrelia* from their tick hosts and, to evaluate the degree of co-infection of ticks with multiple pathogens both zoonotic and non-zoonotic. Additionally, this study aimed to collect and identify ticks currently infesting livestock within the sampled Provinces. Objectives of this study were fulfilled using polymerase chain reaction (PCR) based techniques.

1.4.1. General objectives

1.4.1.1. Identification of tick species currently infesting dogs, cattle, sheep and goats in different provinces of South Africa (Free State, KwaZulu-Natal, North West, Eastern Cape and Mpumalanga Provinces).

1.4.1.2. PCR detection of target and non-target species of pathogens from tick hosts, using established PCR assays.

1.4.1.3. Determination of microbial co-infection in ticks collected from dogs, sheep, goats and cattle in the designated study sites.

1.4.1.4. Sequencing of PCR positive results to validate the findings.

1.4.2. Specific objectives

1.4.2.1. PCR detection of *Coxiella burnetii* from ticks collected from dogs, sheep, goats, cattle and vegetation.

1.4.2.2. PCR detection of *Ehrlichia canis* from ticks collected from dogs.

1.4.2.3. PCR detection of *Ehrlichia ruminantium* from ticks collected from sheep, goats and cattle.

1.4.2.4. PCR detection of *Anaplasma phagocytophilum* from ticks collected from dogs, sheep, goats, cattle and vegetation.

1.4.2.5. PCR detection of *Borrelia burgdorferi* from ticks collected from dogs, sheep, goats, cattle and vegetation.

1.4.2.6. PCR detection of *Rickettsia* species from dog, sheep, goats, cattle and vegetation ticks.

Table 1.3.Tick-borne bacterial diseases, their vectors and hosts

Disease	**Causative agents**	**Primary tick vectors**	**Reservior host**	**Reference**
Canine Monocytic Ehrlichiosis	*Ehrlichia canis*	*Rhipicephalus sanguineus*	Canines: dogs, jackals, foxes	Amyx & Huxsoll, (1973); Price &Karstad, (1980), McBride *et al*., (1996)
Heartwater	*Ehrlichia ruminantium*	*Amblyomma hebraeum, A. variegatum A, lepidum*	Cattle, sheep, goats Wild ruminants	Allsopp *et al.*, (2003); (2004)
African Tick Bite Fever	*Rickettsia africae*	*A. hebraeum, A. variegatum*	Variety of ticks	Azad & Beard (1998); Kelly *et al*., (2010)
Meditteranean spotted fever Boutonneuse fever	*Rickettsia conorii*	*R. sanguineus*	Dogs, rodents	Azad & Beard (1998); Levin *et al*., (2012);
Coxielliosis (Query fever)	*Coxiella burnetii*	Broad, 64 species of ticks	Sheep, goats,cattle, dogs, pet rodents, reptiles, small mammals	Azad & Beard (1998); Heinzen *et al*., (1999)
Lyme Borreliosis / Lyme Disease	*Borrelia burgdorferi sensu lato Borrelia duttonii Borrelia* spp.	*Ixodes persulcatus* group*, Ornithodoros moubata Ornithodoros* spp.	Birds, rodents, ticks, mammals, lizards birds , shrews, voles, mice, hares, humans	Johnson *et al*., (1984) Olsen *et al*., (1995), Fivaz *et al*., (1990)
Human Granulocytic Anaplasmosis and Canine Granulocytic Anaplasmosis	*Anaplasma phagocytophilum*	*I. persulcatus group I. scalpularis I. ricinus I. pacificus*	White-tailed deer, mice, small rodents, Iberian dear, cattle, dogs, European wild boar, ticks & birds	de La Fuente *et al.*, (2005); Ginsberg, (2008), Ghafar & Amer, (2012)

Chapter 2: Collection and identification of ticks infesting livestock and companion animals in five Provinces of South Africa

2.1. Introduction

2.1.1. General overview of ticks

Ticks are obligate hematophagous arthropods that parasitize every class of vertebrates except for fish in almost every region of the world. There are two major tick families: the Ixodidae, or “hard ticks,” so called because of their sclerotized dorsal plate, which makes them to be the most important family in numerical terms and in medical importance, and the Argasidae, or “soft ticks,” so called because of their flexible cuticle, the third family, the Nuttalliellidae, is represented by only a single species *Nuttelliella namaqua*, that is confined to southern Africa (Parola & Raoult, 2001). *Nuttelliella namaqua* has morphological characteristics of both soft and hard ticks, characterized by having a pseudoscutum which resembles a scutum but not as smooth as that of the hard ticks (Sirigireddy, 2008). Slightly more than 650 species of ixodid ticks and 170 species of argasid ticks have been recorded worldwide, In Africa, ten genera of ticks commonly infest domestic animals: seven are ixodids consisting of more than 80 species that occur in South Africa, the remaining three genera are argasids (Horak *et al*., 2002; Sirigireddy, 2008). Two genera of the argasids (*Argas* and *Ornithodoros*) only attach to their hosts for short periods; they are more commonly found within the nest or housing of their hosts. The other argasid genus, *Otobius*, attaches to its hosts only as larvae and nymphs and only within the ear canal. All genera of ixodids feed slowly and attach to their hosts for long periods, depending upon the stage (Walker *et al*., 2007). Each of these ticks has its preferred hosts, geographic distribution and seasonal occurrence and many are vectors of disease to humans or animals (Horak *et al*., 2002).

2.1.2. Life cycle of ticks

There are four stages in the life cycle of an ixodid tick, namely egg, larva, nymph and adult, and each of the post-embryonic stages requires a blood-meal. Ixodid ticks have only one nymphal stage, whereas argasid ticks have two or more nymphal stages (Fig.2.1-2.3). The nymphs and adults of some species also use vegetation from which to quest for hosts. The larvae of several species ascend grass stems and leaves on which they can be seen in dense clusters while they await hosts. It is thus not uncommon for a person to be bitten by several larvae at more or less the same time (Horak *et al*., 2002). Mating generally occurs on the host. Pheromones play an important role in the behavior of ticks and facilitate tick's finding their hosts and their mates. They include assembly pheromones, which bring ticks together, and sex pheromones, which attract males to females and stimulate mounting. The life cycle of ixodid ticks is usually completed in 2–3 years, but it may take from 6 months to 6 years, depending on environmental conditions, including temperature, relative humidity, and photoperiod (Parola & Raoult, 2001).

Lifecycles of ticks are either adapted to one-, two-, or three-hosts (Horak *et al*., 2002). Ticks feeding on only one host throughout all three viable life stages are called one host ticks (Fig.2.1.). This type of tick remains on one host during the larval and nymphal stages, until they become adults, and females drop off the host after feeding to lay their batch of eggs. Other ticks feed on two hosts during their lives and are called two host ticks (Fig.2.2.). This type of tick feeds and remains on the first host during the larval and nymphal life stages, and then drops off and attaches to a different host as an adult for its final blood meal. The adult female then drops off after feeding to lay eggs. Finally, many ticks feed on three hosts, one during each life stage, and are appropriately named three host ticks (Fig.2.3.). These ticks drop off and reattach to a new host during each life stage, until finally the adult females lay their batch of eggs, from 400 to 120,000 depending on the species. In each case, the fed adult stage is terminal, that is, after laying one batch of eggs the female dies, and after the male has copulated, he dies as well (Jongejan and Uilenberg, 2004; Walker et al., 2007; http://www.alabamavms.org/.htm).

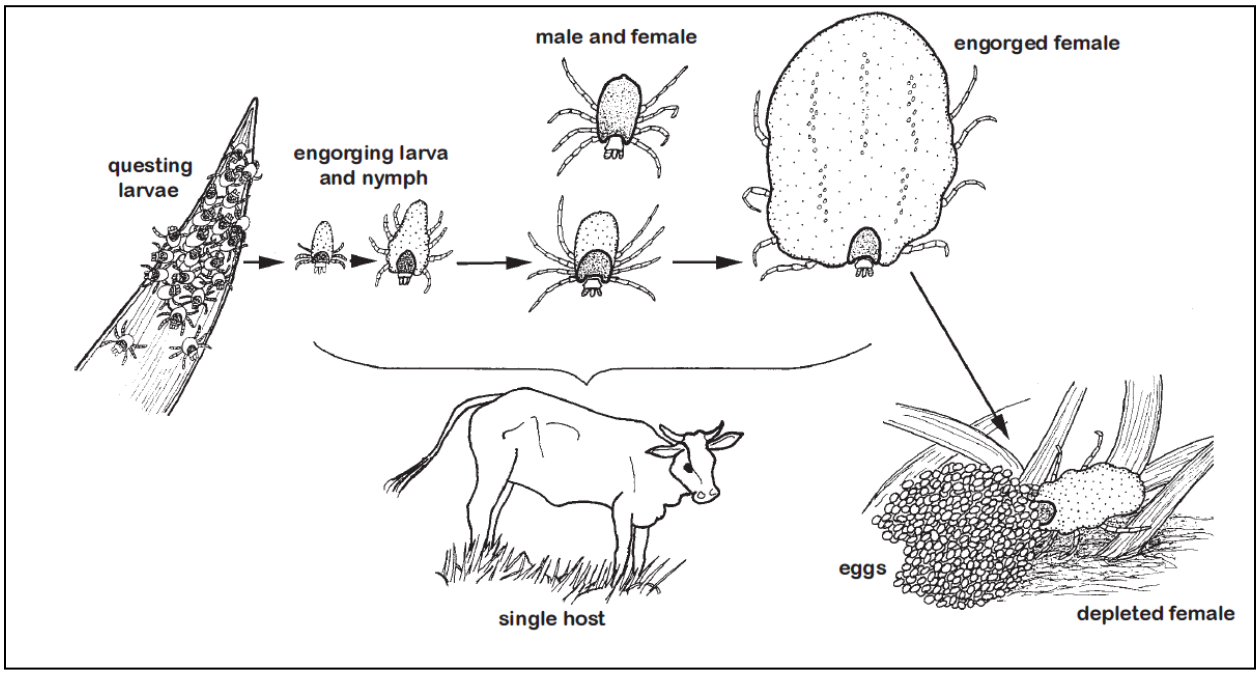

Figure 2.1. One host tick life-cycle (e.g. *Rhipicephalus decoloratus*), Source: Walker *et al*., (2007).

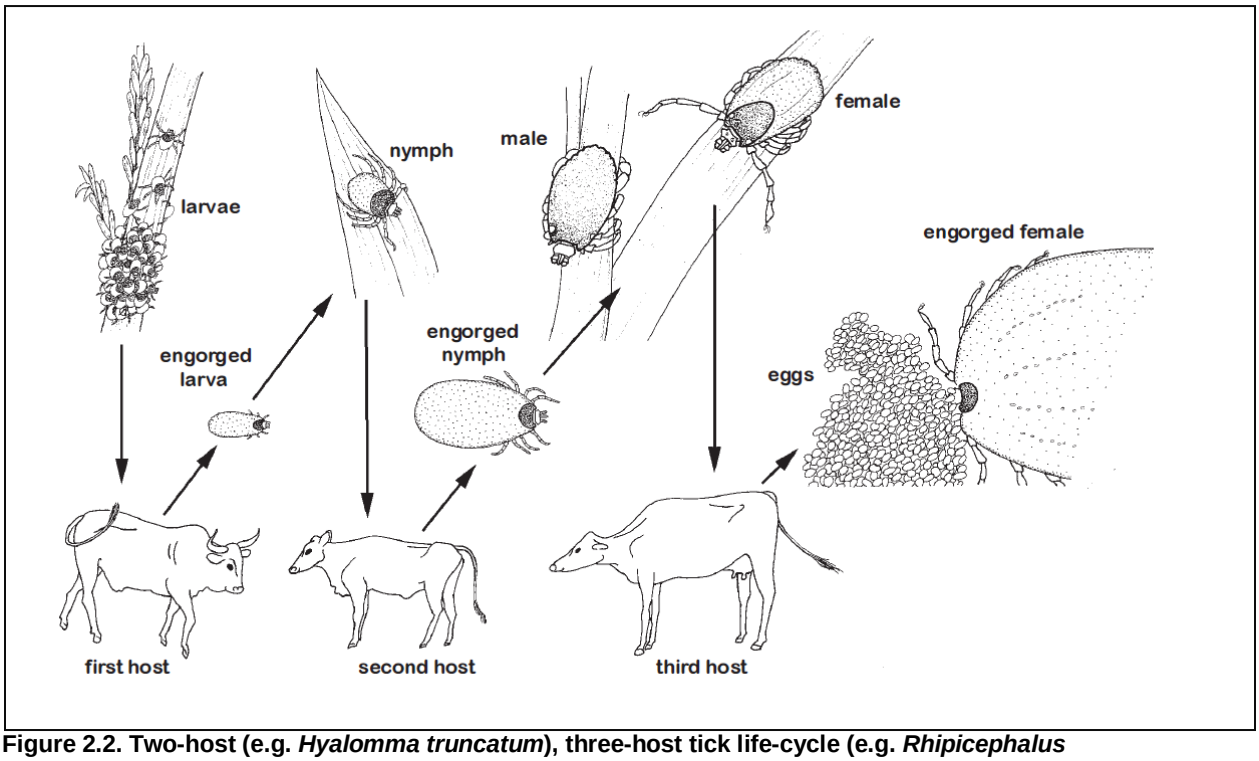

Figure 2.2. Two-host (e.g. *Hyalomma truncatum*), three-host tick life-cycle (e.g. *Rhipicephalus appendiculatus*), Source: Walker *et al*., (2007)

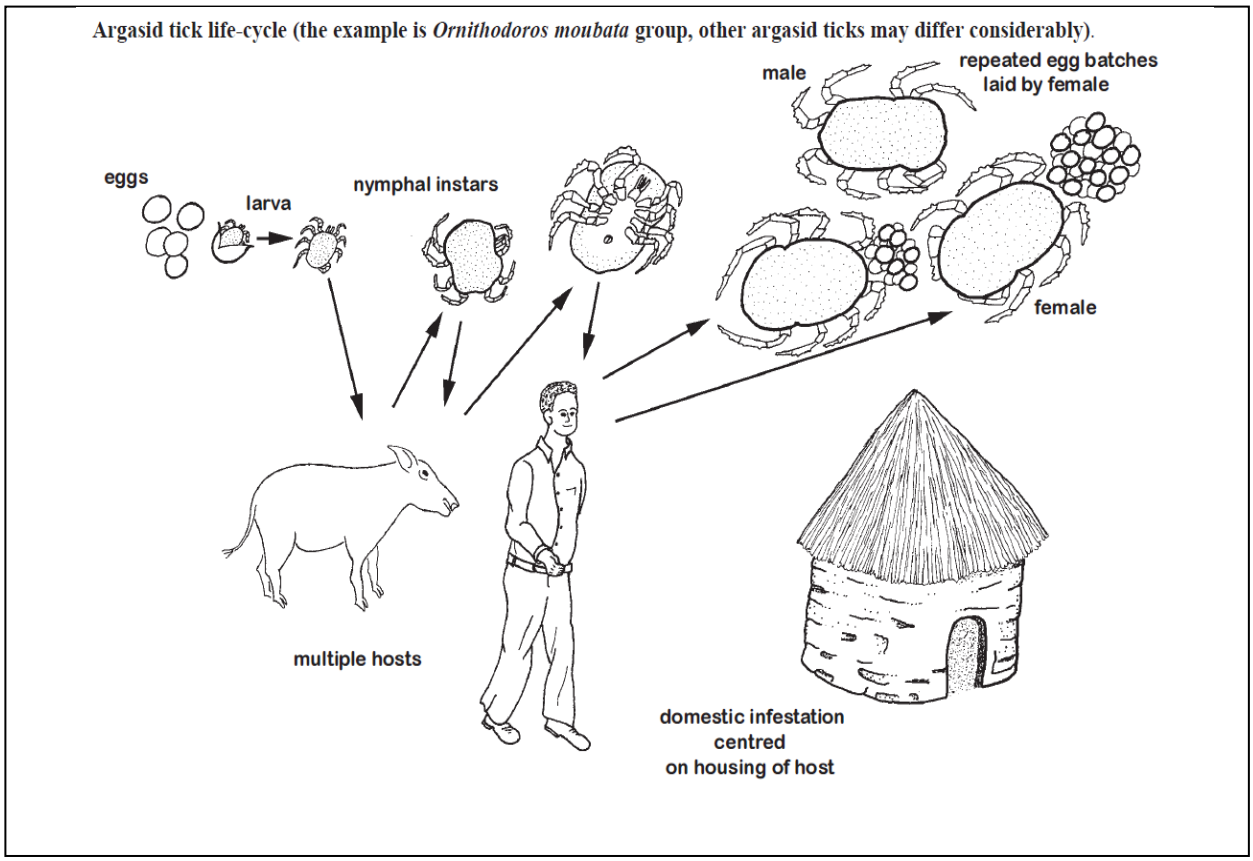

Figure 2.3. Argasid tick lif cycle,e.g. *Ornithodoros* (source: Walker *et al*., 2007)

2.1.3. Host seeking strategies

Ixodid ticks spend 90% of their life unattached from the host, and most of them are exophilic: they live in open environments, meadows, or forests. They are usually seasonally active, seeking their hosts when environmental conditions are most suitable. They are highly responsive to stimuli that indicate the presence of hosts. These include: chemical stimuli (such as CO_2, NH_3) phenols, humidity, and aromatic chemicals, and airborne vibrations and body temperatures associated with warm-blooded animals. For example, ticks are attracted by feet hitting the ground or by the CO_2 emitted by a car stopped in the bush. Two typical host-seeking behavior patterns occur among exophilic ticks. In the ambush strategy, ticks climb up vegetation and wait for passing hosts, with their front legs held out in the same manner as are insect antenna e.g., *Rhipicephalus sanguineus,* the brown dog tick, and *Ixodes ricinus* adults in Europe; *I. scapularis* and

Demarcentor variabilis in the United States. In the hunter strategy, ticks attack hosts. They emerge from their habitat and run toward their hosts when these animals appear nearby e.g. adult and nymph *Amblyomma hebraeum* and *A.variegatum* in Africa. Some species for example, the lone star tick, *A. americanum,* use both strategies (Parola and Raoult, 2001).

2.1.4. The role of ticks as hosts and vectors of disease causing pathogens

Ticks are considered to be second only to mosquitoes as worldwide vectors of human diseases, but they are regarded as the most relevant vectors of disease causing pathogens in domestic and wild animals (Andreotti *et al*., 2011). Animals are natural hosts of ixodid ticks but humans are usually only bitten when they intrude upon the tick's habitat, particularly when it is questing for a host either from the vegetation or from the ground (Horak *et al.,* 2002). Literature on ticks feeding on humans worldwide indicates that 24 ixodid species have frequently been collected from humans, while a further 48 species have been reported more rarely and according to Horak *et al.,* (2002) in South Africa *A. hebraeum, A. marmoreum, Haemaphysalis elliptica, H. silacea, Hyalomma marginatum rufipes, H. truncatum* and other *Hyalomma* sp., *Ixodes pilosus* group, *I. rubicundus, R. decoloratus, R. appendiculatus* , *R. evertsi evertsi, R. follies, R. gertrudae, R. glabroscutatum, R. maculates, R. muehlensi , R. sanguineus, R. simus, R. warburton*, *R. zambeziensis*, as well as *R. pulchellus* have been reported to occasionally feed on humans. Upon a bite, ticks can transmit a wide variety of pathogens, including protozoal parasites e.g. *Babesia*, bacteria e.g. rickettsiae, spirochetes or *Francisella* spp. and viruses e.g. nairovirus, coltivirus or flavivirus to both man and animals and may carry more than one infectious organism at a time (Matjila *et al*., 2008; Berrada and Telford, 2009; Crowder *et al*., 2010).The vectorial capacity of ticks is influenced by amongst others, factors such as their persistent bloodsucking habits, longevity, high reproductive potential, relative freedom from natural enemies, and highly sclerotized bodies that protect them from environmental stresses (Sparagano *et al*., 1999)

2.1.5. Effects of tick infestations on animals and humans

Ticks are responsible for direct damage to livestock through their feeding habits and their attachment can cause various kinds of dermatoses or skin disorders such as inflammation, pain and swelling. The damage is manifested as hide damage, damage to udders, teats and scrotum, tick worry, blood loss, injection of toxins, myiasis due to infestation of damaged sites by maggots and secondary microbial infections. Certain ticks can cause flaccid, ascending and sometimes fatal paralysis known as tick paralysis. Individuals bitten may have allergic or even anaphylactic reactions (Sparagano *et al*., 1999; Hlatshwayo, 2000; Ndhlovu *et al*., 2009).

There is also productivity losses associated with the various tick species that can occur depending on the pathogen they are able to harbor and transmit. These effects are usually more apparent at commercial level farming than in communal or smallholder farms where they are minimal (Hlatshwayo, 2000; Ndhlovu *et al*., 2009). Concepts such as economic damage threshold and economic threshold are inevitable when estimating damage caused by ticks and tick-borne diseases, the former measured as the minimum average weekly standard female tick burden sufficient to cause damage, equal in dollar value to the cost of applying tick control, the latter as the lowest pest population that causes a reduction in profit, or equivalently the pest population where the benefits of control equal the costs of eliminating the pest. Estimates in one survey in Zimbabwe put the cost at US$ 5.6 million per annum, and another report estimated annual costs for Angola, Botswana, Malawi, Mozambique, South Africa, Swaziland, Tanzania, and Zambia to a total of US$ 44.7 million due to heartwater disease caused by *Ehrlichia ruminantium* transmitted exclusively by *A. hebraeum* and *A. variegatum* in Southern Africa (Allsopp and McBride, 2009).

2.1.6. Control of ticks

Strategies into efficient control of ticks include habitat modifications such as vegetation management by cutting, burning and herbicide treatment, and drainage of wet areas. Effects of these control strategies are often short-lived and can cause severe ecological damage. Other alternatives such as host exclusion or depopulation may result in reduction in density of ticks, but this is mostly impractical and is also not ecologically sound. Use of chemical compounds such as organophosphates and pyrethroids which may be used in combination with pheromones to control ticks may also lead to environmental contamination and toxicity for animals and humans. Acaricides can be directly applied to wild animals or domestic hosts to kill attached ticks and disrupt tick feeding. Biological control methods include the introduction of natural predators (e.g. beetles spiders and ants), parasites (e.g. insects, mites, and nematodes), and bacterial pathogens of ticks, mass realease of sterilized males and immunization of hosts against ticks (Parola and Raoult 2001). Immunization can be accomplished by using blood vaccines containing virulent or attenuated organisms or using infected tick stabilates, recombinant antigens produced in transformed cells and by chemically synthesized antigens (Norval and Horak, 1994). The best solution at present is the integrated pest management which incorporates certain control measures together with environmental management (Parola and Raoult, 2001).

2.2 Aims of the study

This chapter aims to record tick species currently infesting livestock in the five sampled provinces of South Africa. The aim was achieved by collecting and identifying ticks currently infesting dogs, cattle, sheep, and goats in KwaZulu-Natal, Free State, Mpumalanga, Eastern Cape and North West Provinces of South Africa.

2.3. Materials and methods

2.3.1. Study area and study animals

Ticks were collected from dogs, cattle, goats, sheep and incidentally four horses and four cats and also from the vegetation from farms in the Free State (FS), KwaZulu-Natal (KZN), Eastern Cape (EC), North West (NW) and Mpumalanga (MP) Provinces. In KZN samples were collected from Wesselsneck [S 28° 20' 0.52" E 030° 02' 49.1"], Gcinalishona/Mjindini [S 28° 39' 00.5" E 030° 06' 56.3"], eTholeni [S 28° 25' 39.9" E 30° 13' 04.3"] and uMsinga Mountainview dip site [S 28° 41'43.1" E 030° 16'14.6"]; in FS, Hooningkloof [S 28° 30. 666' E 028° 42.701'], a farm situated at a livestock-wildlife interface, Sekoto farm [S 28° 36.094' E 028° 49.013'], Seotlong Hotel and Agricultural School [S 28° 35' E 28° 50'] and in Kestell [S 28° 20' E 28° 38']; in EC, at Amathole District Municipality [S 32°48' 30" E 27° 01' 49"]; in NW at a private Veterinary Clinic in Mafikeng [S 25° 51′ 0″ E 25° 38′ 0″] and in MP at Kameelpoort-KwaMhlanga [S 25° 46′ 6.3″ E 29° 28′ 42″].

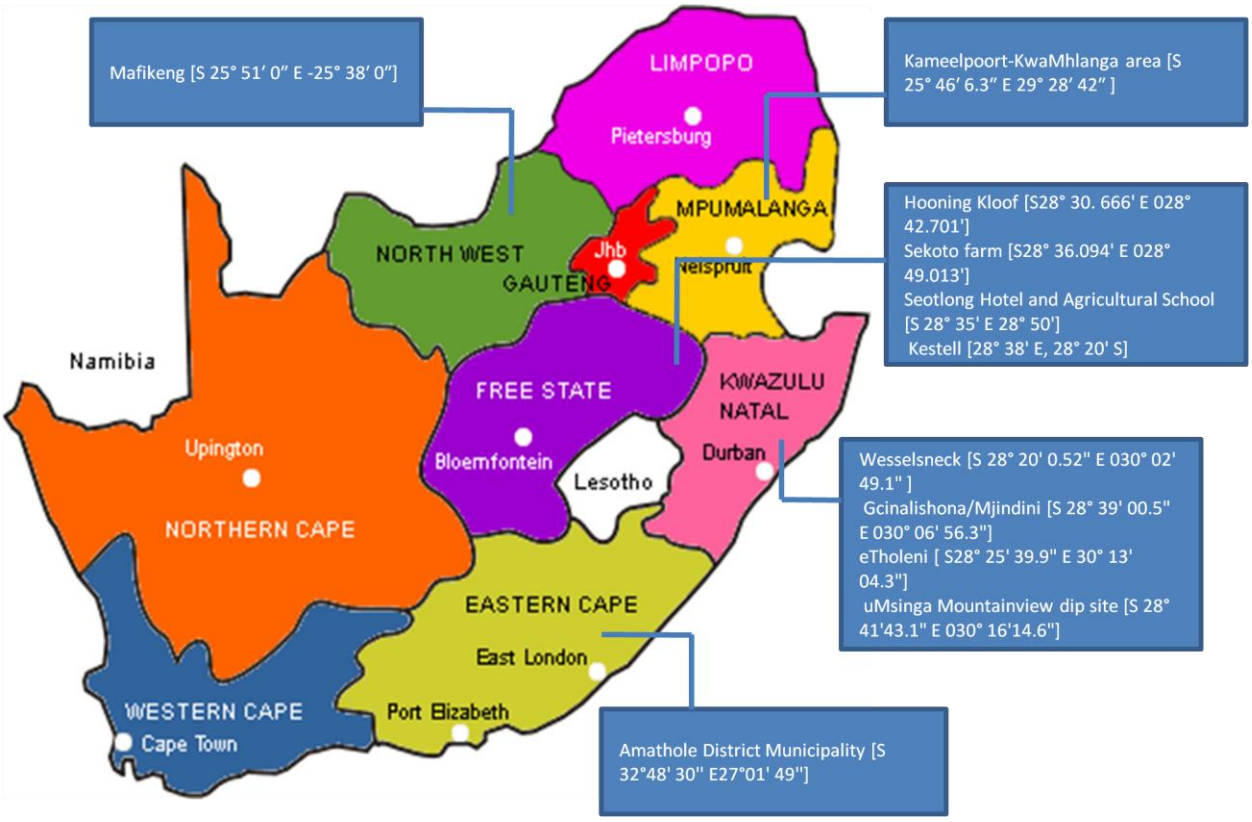

Figure 2.4. Map of South Africa, showing different provinces and sampling site co-orditates. Source: www.proseo.co.za.

2.3.2. Tick collection and identification

Ticks were collected from different body parts depending on the area of abundance and their visibility; these included the head, neck, perineum and the abdominal areas. Some ticks were collected from the vegetation by the flagging method as described by Eremeeva *et al*., (2006), this was only done in the FS province. Using sterile fine tipped forceps ticks were transferred into perforated collection vials. The ticks were identified to species level using tick identification guides freely available on the internet, the main descriptions used were those of Walker *et al*., (2007) from their guide of ticks occurring in Africa as well as Norval and Horak, (1994) description of ticks infesting livestock of southern Africa summarized in Table. 2.1. Thereafter, the ticks were surface sterilized twice with 75% ethanol and once with phosphate buffered saline (PBS) before they

were dissected and gutted (the engorged) or crushed whole (the males) using ethanol flamed scissors and later preserved in PBS and stored at -34°C until further processing.

Table 2.1. Brief morphological descriptions used for identification of tick species

Tick Species	**Tick Description**
***Rhipicephalus* species**	Basis capitulum hexagonal,hypostome and palps short (or medium), Most species in-ornate but 4 species ornate, eyes flat to orbited, eleven festoons, anal grove posterior to anus, adanal plates present in males legs are uniformly coloured one specis has banded legs (*R.e. mimeticus*)
Rhipicephalus decoloratus	Small inconspicuous ticks, short mouthparts and slender legs, males considerably smaller than females, brownish yellow in color and darker colored intestines visible through the lightly sclerotized scuta, males found usually paired with female, 3 + 3 columns of teeth on the hypostome.
Rhipicephalus evertsi evertsi	Medium sized, dark brown color of their heavily punctuate scuta, beady eyes, leg color ranging from orange to red, coxae 1 internal spurs visible.
Rhipicephalus appendiculatus	Medium sized brown ticks with short mouthparts. Leg size of males increase gradually from the first to the fourth pair, caudal appendage is broad in engorged males, coxa 1 dorsally visible, have broader cervical fields with sharply raised margins, fewer punctations, slightly convex eyes and the posterior grooves are not deeply sunken.
Rhipicephalus sanguineus	Dull yellow-mid brown color, basis capituli sharp, palp pedicel short, eyes slightly convex, caudal appendage broad in fed males, interstitial punctuation size is small to medium and sparsely distributed, coxae 1 not dorsaly visble.
Amblyomma hebraeum	Large, conspicuous, long mouthparts, brightly ornamented scuta of both male and females colors ranging from pink to orange, flat eyes, brown and white banded legs, males with

	yellow colored festoons, coxae 1 external spur medium, and internal short .
Hyalomma marginatum rufipes	Large, dark-brown bodies, large mouthparts, ornate conscutum in females, scutum heavily punctuated, beady eyes and long, red and white banded legs, males more circular rather than elongate body shape of *H. trancatum*.
Haemaphysalis elliptica	Small, yellow-brown, mouthparts form a distinctive conical shape, conspicuous lateral extensions to palp articles 2, coxae 4 of males have only medium-sized spurs.

Desciptions were reproduced from Norval and Horak, (1994), Coetzer *et al*. (1994), and Walker *et al*., (2007)

2.4. Results

A total of N = 1947 ticks were collected from the designated study areas. The species of ticks collected included *Rhipicephalus* spp. (n = 570), *R. sanguineus* (n = 275), *R. evertsi evertsi* (n = 650), *R. decoloratus* (n = 228), *R. appendiculatus* (n = 10), *Amblyomma hebraeum* (n = 171), *Hyalomma marginatum rufipes* (n = 4), and *Haemaphysalis elliptica* (n = 38). The morphological differences between the collected tick species are shown on Plate I-IX. Tick species identified as *Rhipicephalus* species included among others, *R. decoloratus*, *R. simus*, *R. e. evertsi*, and *R. appendiculatus*. The number of ticks collected from the livestock and the provinces in which the ticks were collected are summarised in Table 2.2.

The ticks collected from KwaZulu-Natal (n = 529) included *R. sanguineus* (44%), *A. hebraeum* (15.3%) and *Rhipicephalus* spp (40%). *Rhipicephalus* spp. included *R. e. evertsi* and *R. decoloratus*. In the sampled areas, regardless of the kraal the animal came from, co-infestation with *A. hebraeum* and *Rhipicephalus* spp. by visual inspection was more or less the same. The areas mostly infested on the cattle were the anal area, the udders and the rump and no ticks were visible on the face and ears as well as other

body parts. *R. appendiculatus* and *H. elliptica* were absent from the collections in this area.

In the Free State Province (n = 998), *Rhipicephalus* spp. (20.4%), *R. sanguineus* (0.2%), *R. decoloratus* (12.9%), *R. e. evertsi* (62.1%), *H. elliptica* (3.2%), and *H. m. rufipes* (0.1%) were found infesting the animals and the only tick species found questing on the vegetation was *R. appendiculatus* (1.0%). *R. sanguineus* and *H. elliptica* were only found infesting dogs. *R. e. evertsi* seemed to be exclusively the only tick to infest sheep and goats and only found attached to the anal area. *R. appendiculatus* was found in cattle attached in and around the ears and the head, whereas *R. decoloratus* was found in different stages throughout the whole body, more especially around the thurl, stifle and the heart girth. Predilection sites of other *Rhipicephalus* species such as *R. simus* were not clearly recorded as they were initially misidentified.

The North West Province collection (n = 39) comprised of ticks collected from pet dogs brought in at a private veterinary clinic in Mafikeng. The dominant tick species identified was mainly *R. sanguineus* (94.9%) and only one (5.0%) *H. elliptica* tick was collected.

In Mpumalanga Province (n = 21) tick samples were collected from numerous goats, a few cattle and cats and only one dog. The species collected from goats included *H. m. rufipes* (14.3%), *R. e. evertsi* (33%) and *Rhipicephalus* spp. (9.5%); from cattle, *R. decoloratus* (4.7%) and *A. hebraeum* (14.3%); *R. sanguineus* (9.5%) from a dog and; *H. elliptica* (19%) as well as a nymph of *A. hebraeum* from the cats.

Eastern Cape (n = 360) samples were collected from goats and cattle only, the most abundant tick species being *Rhipicephalus* spp.; 40% (which included *R. appendiculatus* and *R. simus*) and *R. decoloratus* (27.5%); followed by *A. hebraeum* (24%); and a few species of *R. e. evertsi* (6.3%).

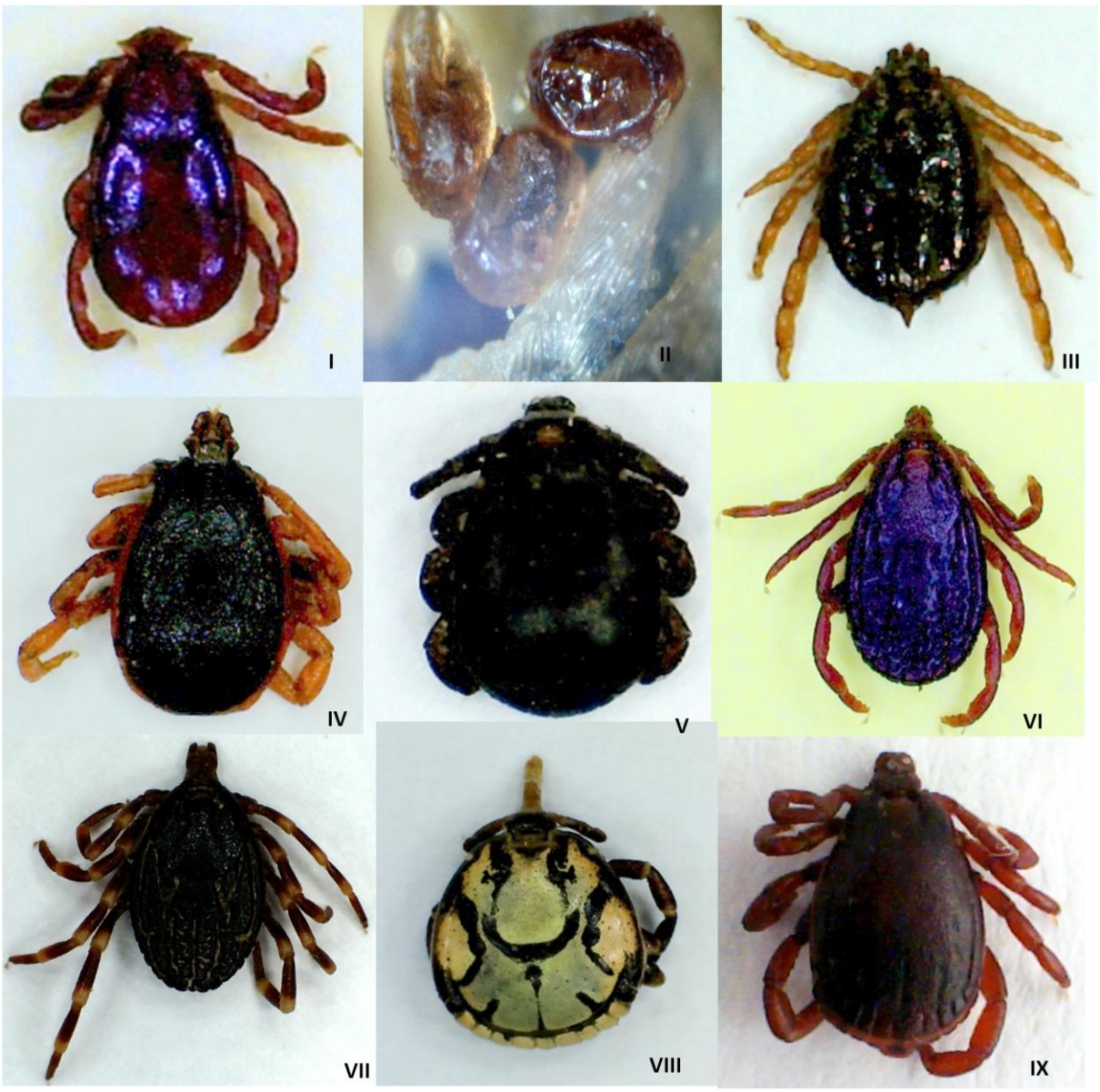

Plate I-IX.Morphological differences between the tick species collected from livestock across five Provinces of South Africa. *Haemaphysalis elliptica* male, I; *Rhipicephalus* eggs, II; *R. decoloratus* male,III; *R. evertsi eversti* male, IV; *R. sanguineus* male ,V; *R. appendiculatus* female, VI; *H. marginatum rufipes* female, VII; *Amblyomma hebraeum* male, VIII; and *R. simus* male, IX. All pictures were taken by the author, using a Discovery USB Deluxe 20x400x magnification microscope (Veho, UK).

Table 2.2. Tick species collected and the number of ticks collected per Province

Tick Species	**Number of species of ticks per province**					**Total number of ticks**
	Kwa Zulu- Natal	**Free State**	**Eastern Cape**	**Northwest**	**Mpumalanga**	
Amblyomma hebraeum	81	0	87	0	3	**171**
Haemaphysalis elliptica	0	32	0	2	4	**38**
Hyalomma marginatum rufipes	0	1	0	0	3	**4**
***Rhipicephalus* species**	213	204	151	0	2	**570**
R. appendiculatus	0	10	0	0	0	**10**
R. decoloratus	-	129	99	0	1	**229**
R. evertsi evertsi	-	620	23	0	7	**650**
R. sanguineus	235	2	0	37	1	**275**
Total number per Province	**529**	**998**	**360**	**39**	**21**	**1947**

2.5. Discussion

Baker *et al.,* (1989) reported *R. decoloratus*, *R. appendiculatus* and *R. e. evertsi* to be the most prevalent species on cattle raised on commercial farms in KwaZulu-Natal. *R appendiculatus* was absent from my collection and this may be due the seasonality factor, nevertheless my findings are consistent with Baker's.

In the Eastern Free State the principal ticks infesting cattle belonging to resource poor farmers are *R. decoloratus* (53.1%), *R. e. evertsi* (44.7%), *R. follis* (1.0%), *R. gertrudae* (0.7%) and *R. warburtoni* (0.4%) (Hlatshwayo *et al.*, 2002; Mbati *et al.*, 2004). This is similar to my findings, in contrast though, in the current study I was unable to find the latter three species of ticks however I also report finding *H. m. rufipes*, absent from the previous authors's findings. In the south west region of the FS, Fourie and Horak (1991) recorded that *A. marmoreum*, *H. m. rufipes* and *H. truncatum* were the predominant species and later on Fourie *et al.* (1996) found *I. rubicundus* and *H. m. rufipes* as the most prevalent tick species in the same region.

R. sanguineus and *H. elliptica* were the only ticks species found from the North West Province since collections were done only on dogs, however, Bryson *et al.* (2002a) noted that the adults of *A. hebraeum*, *R. appendiculatus* and *R. e. evertsi* were the most numerous tick species in the Province amongst livestock.

In a survey conducted in the Eastern Cape Province, *R. decoloratus*, *A. hebraeum*, *R. appendiculatus* and *R. e. evertsi* were found to be the most common tick species infesting cattle (Rechav, 1982), this is consistent with the findings of the current study. In addition to that a number of *R. simus* ticks were found in the current study.

R. sanguineus and *H. elliptica* were only found infesting dogs, this is appropriate as they are commonly known to feed on dogs as their preferred hosts in South Africa and most parts of the world (Keirans, 1992). The kennel or brown dog tick, *R. sanguineus*, is

practically an exclusive parasite of domestic dogs and its life cycle is adapted to living in kennels or human dwellings with its dog host. It is thus possible for people to be bitten by ticks in the supposed safety of their own homes (Horak *et al*., 2002). Only a few species of *Haemaphysalis* have adapted to domestic livestock. In South Africa *H. elliptica* has been reported infesting wild and domesticated cats and dogs only (Norval and Horak, 1994; Walker *et al*., 2007). *R. sanguineus* transmits *Ehrlichia canis* and *Rickettsia conorii*, whereas *H. elliptica* mainly transmits *Babesia* species (McBride *et al*., 1996; Matjila *et al*., 2008).

Of the 129 species of *Amblyomma* ticks described to date, only *A. hebraeum* was found in this study mainly among cattle and goats. It is the main vector of *E. ruminantium* as well as *R. africae* in sub-Saharan Africa, the causative agents of heartwater and ATBF respectively. It is therefore regarded as one of the most important vector of disease among livestock, second to *A. variegatum*. They parasitize a wide variety of mammalian hosts and also reptiles and amphibians, with hosts weighing more than 100 kg in mass being regularly infested, while hosts below this mass are not usually infested (Ndhlovu *et al*., 2009). Immature stages of *A. hebraeum* have been reported to infest small mammals and birds, which are said to play an important role in dispersing the ticks (Jongejan and Uilenberg, 2004). This factor has become apparent in this study where a nymph of *A. hebraeum* was found attached to a cat in Mpumalanga Province, this is also backed by similar findings reported by Horak *et al*., (2010) in the Gauteng and Eastern Cape Provinces whereby a total of 16 nymphs were recovered from cat hosts.

Rhipicephalus comprises 70 species. Most *Rhipicephalus* spp. are found on mammals on the African continent. *R. appendiculatus*, the brown ear tick, is the most important rhipicephalid tick in eastern and southern Africa where it occurs on a wide variety of domestic and wild ruminants. This tick prefers to feed on the ears of its hosts in the adult stage of its three-host life cycle (Jongejan and Uilenberg, 2004). *R. appendiculatus* has been listed as one of the rhipicephalid ticks that frequently feed on humans with 18 records reported in the year 2000 (Horak *et al*., 2002). This tick is able to tolerate a wide range of climatic conditions and some strains can cause paralysis in

sheep (Ndhlovu *et al*., 2009). *R. conorii* can be transmitted by this tick (Walker *et al*., 2007). Furthermore *Theileria parva* and *A. bovis* can be transmitted by *R. appendiculatus* (Walker *et al*., 2007).

R. decoloratus preferably feeds on cattle where they complete all their life stages. These one-host ticks, may become very numerous on cattle herds, particularly those with a low degree of resistance, and cause considerable direct damage as the preferred feeding sites are often of good leather potential. This is in accordance with the findings in the current study. They feed readily on a large variety of wild ruminants as well (Jongejan and Uilenberg, 2004). Due to a lack of suitable hosts they tend to shift their host preferences which may be the reason why they were found infesting goats in the Eastern Cape Province. *R. decoloratus* transmits *Borrelia theileri*, protozoans such as *Babesia* spp. and bacterial pathogens like *A. marginale* (Walker *et al*., 2007).

R. evertsi evertsi preferably parasitize cattle, sheep, donkeys and horses. The adults were found on the hairless area around the anus as well as on the outer ear canal, no immature stages were collected, this might be because immature stages are known to attach on the deeper parts of the inner surface of the ear. This tick is known to transmit *B. caballi*, *T. equi* and *A. marginale*. The large numbers of over-wintered nymphs that emerge to adults in spring engorge on new-borne lambs and can lead to paralysis (Walker *et al*., 2007).

Unlike most other ixodid ticks, which wait on the vegetation for a host to pass, adult *Hyalomma* actively run out from their resting sites when a host approaches (Jongejan and Uilenberg, 2004). These ticks, in particular the species of *H. truncatum* are known to carry and transmit *Borrelia* spirochetes, the causative agent of LD and tick relapsing fever (Norval and Horak 1994). Both *H. truncatum* and *H. m. rufipes* can transmit *R. conorii* (Walker *et al*., 2007).

2.6. Conclusions

Factors such as changes in environment which favor ticks and increase their range, density, and likelihood of human interaction; furthermore evolving socio-economic factors and behaviors (urbanization, increasing outdoor recreation, reforestation, changing agricultural practices and pet ownership) can increase the risk that humans can come into contact with ticks and the disease agents they carry (Dennis & Piesman, 2005). Urban and peri-urban areas are often also heavily infested with ticks, particularly where pets and livestock are allowed to roam freely and infestation can be acquired in either of these localities. Many tourists visit, and also hike in national, provincial and privately owned nature reserves and on farms in South Africa, while a considerably larger number of people live and work there. Free-living ticks are often plentiful in these localities and humans thus unwittingly become infested (Horak *et al*., 2002).

The abundance of these ticks in the sampled Provinces increases the chances of detection of the pathogens that they carry and their potential of harboring pathogens that are transmissible to humans. A key challenge for future research in tick-borne diseases is the manipulation of molecular diagnostic methods to detect diseases that we know and to characterize new potential diseases, while keeping focus on transmission of those we already know to be important (Dennis & Piesmann, 2005). Education and awareness could play a major role in the control and prevention of tick bites amongst humans.

Chapter 3: Molecular detection of *Anaplasma phagocytophilum*, *Ehrlichia canis* and *E. ruminantium* from ticks collected from livestock and companion animals in five Provinces of South Africa

3.1.1. Introduction to Ehrlichiosis

Ehrlichioses are tick-borne diseases that cause significant morbidity and mortality in dogs and people worldwide (Dumler *et al*., 2005). In 2005 the Office International des Epizootics (OIE) undertook a great task of compiling an updated report of ehrlichiosis worldwide and according to this organization ehrlichiosis is a group of diseases, usually named according to the host species and the type of white blood cell most often infected. According to this report; canine monocytic ehrlichiosis (CME) is caused by *Ehrlichia canis* and, occasionally, *E. chaffeensis.* Canine granulocytic ehrlichiosis and equine granulocytic ehrlichiosis are caused by *Anaplasma phagocytophilum* and *Ehrlichia ewingii.* Human granulocytic anaplasmosis (HGA) and tick-borne fever (a disease of ruminants) are also caused by *A. phagocytophilum* and that human monocytic ehrlichiosis (HME) is caused by *Ehrlichia chaffeensis* and *E. ewingii.* Heartwater also known as cowdriosis is caused by *E. riminantium*. In 2001, the taxonomy of the ehrlichiae was changed. Formerly, they belonged to the genus *Ehrlichia*, tribe Ehrlichieae and family Rickettsiaceae. Some species of *Ehrlichia* have now been reclassified into the genera *Anaplasma* or *Neorickettsia*, and all were placed in the family Anaplasmataceae. *A. phagocytophilum* contains the organisms formerly known as *E. equi* and *E. phagocytophila*, which were previously thought to have been different species of *Ehrlichia*. Many members of Anaplasmataceae have adapted to existence within arthropods, some are transmitted by tick bites and cause human infections such as ehrlichiosis and anaplasmosis (OIE, 2005). The genera *Ehrlichia* and *Anaplasma* possess all pathogens in the family that are transmissible by ticks and that generally infect peripheral blood cellular elements, including leukocytes, platelets, and erythrocytes (Dumler *et al*. 2005).

In South Africa, canine ehrlichiosis is encountered commonly in veterinary practice and there is evidence of human ehrlichiosis (Inokuma *et al*., 2005). Inokuma *et al*., (2005) further elaborate that there are currently eight species in the family Anaplasmataceae that are pathogenic in dogs i.e. *E. canis*, *E. chaffeensis*, *E. ewingii, E. ruminantium*, *A. platys*, *A. phagocytophilum*, *N. helminthoeca*, and *N. risticii*. Thus far *E. canis*, *E. ewingii*, *E. ruminantium*, *E. chaffeensis*, *A. phagocytophilum* and *N. risticii* have been found to cause illnesses in humans (OIE, 2005; Kawahara *et al*., 2005; Inokuma *et al*. 2005; Vorou *et al*., 2007).

In the same study Inokuma *et al*., (2005) reported the first *Anaplasma* species that was closely related to *A. phagocytophilum* in South Africa from blood specimen collected from dogs in Bloemfontein which were found to be carriers and also detected similar strains of *Anaplasma* species in sheep that had signs of heartwater and was concurrently infected with *E. ruminantium*. The *E. ruminantium* DNA (the 16S rRNA gene) in 2001 was detected by PCR in a dog suffering from atypical canine ehrlichiosis in South Africa and an identical sequence was later isolated in serum from the owner who had died 3 weeks after the death of her pet dog from biliary fever (Allsopp and McBride, 2009). *A. phagocytophilum* has also been isolated from lambs in U.S (Berrada and Telford, 2009). It was associated with tick-borne fever in cattle, sheep, and goats in India and Europe for several decades (OIE, 2005; Vorou *et al*., 2007). Ehrlichiae are distributed throughout the world. Although *A. phagocytophilum* is found worldwide, tick-borne fever (or a similar syndrome) has been reported only in ruminants in United States, Europe, India and South Africa (Vorou *et al*., 2007).

Ehrlichiae are transmitted by ticks in the family Ixodidae. *Ehrlichia canis* is transmitted by the brown dog-tick *Rhipicephalus sanguineus*. Recently it was also shown to be experimentally transmitted by *Dermacentor variabilis*, the American dog tick. *E. chaffeensis* is mainly transmitted by *Amblyomma americanum*, the Lone Star tick. *A. americanum* is also thought to be the primary vector for *E.ewingii.* In addition, evidence of infection has been found in *D. variabilis* and *R. sanguineus*. The bacterium *E. ruminantium* is transmitted by *Amblyomma* species in Africa and the Carribean Islands,

A. variegatum and *A. hebraeum* being important vectors in sub-Saharan Africa (Allsopp *et al*., 2003, Robinson *et al*., 2009). *A. phagocytophilum* is transmitted by species of *Ixodes*. In the U.S., the vectors include *I. scapularis* (the black-legged tick) and *I. pacificus*. *I*. *ricinus* transmits this organism in Europe. Transovarial transmission is not known to occur; the ticks seem to first become infected as larvae or nymphs. Transstadial transmission has been proven for some species, including *E. canis* in *R. sanguineus* and *E. chaffeensis* in *A. americanum*. Infections can also be transmitted by blood transfusions, and mechanical transmission by biting insects has been suggested as a possible means of spread (OIE, 2005).

3.1.2. Aims of the study

This study was aimed mainly at determining the prevalence of infection of ticks collected from livestock (cattle, dogs, sheep and goats) with *Anaplasma phagocytophilum* in Eastern Cape, Free State, KwaZulu-Natal, Mpumalanga and North West Provinces of South Africa. By the by to determine the prevalence of *E. ruminantium* in ruminants (cattle, goats and sheep), *E. canis* as well as potentially zoonotic *Ehrlichial/Anaplasma* species e.g. *E. chafeensis* amongst ticks collected from dogs in these Provinces.

3.2. *Anaplasma phagocytophilum*

Human granulocytic anaplasmosis (HGA) is a tickborne rickettsial infection of neutrophils caused by *A. phagocytophilum* (formerly *Ehrlichia equi*, *E. phagocytophila*). The human disease was first identified in 1990 in a patient from Wisconsin, despite the fact that the pathogen was defined as a veterinary agent in 1932. The patient died with a severe febrile illness 2 weeks after a tick bite. During the terminal phases of the infection, clusters of small bacteria were noted within neutrophils in the peripheral blood, assumed to be phagocytosed gram-positive cocci. A careful review of the blood smear suggested the possibility of human ehrlichiosis, an emerging infection with similar bacterial clusters in peripheral blood monocytes among infected patients in the southeast and south-central United States. Since 1990, U.S. cases have markedly

increased, and infections are now recognized in Europe (Dumler *et al*. 2005). The CDC reports that cases have gone up from 348 cases in year 2000 to 1006 cases in year 2008. The incidence (the number of cases for every million persons) of anaplasmosis has also increased, from 1.4 cases per million persons in 2000 to 4.2 cases per million persons in 2008. The case fatality rate has remained low, at less than 1%. A high international seroprevalence suggests infection is widespread but unrecognized (Dumler *et al*. 2005).

A. phagocytophilum, an emerging human pathogen of public health importance, is transmitted to humans most commonly by tick bites. The agent has been detected in various species of *Ixodes* ticks around the world including *I. scapularis* in the eastern United States, *I*. *pacificus* in the western United States, *I*. *ricinus* in Europe, and *I*. *persulcatus* in the Asiatic region of Russia adjacent to China as well as in *Dermacentor silvarum* ticks in northeastern People's Republic of China, where *A. phagocytophilum* strains were isolated from wild and domestic animals. HGA was reported in the southern area of the Russian Far East that borders China (Prendki *et al*., 2011). In South Africa the bacterium was isolated from whole blood obtained from a dog which tested positive for morulae during microscopic observation and subsequently from three other blood specimens obtained from dogs in the same area (Inokuma *et al*., 2005, Matjila *et al*., 2008). The vector for the bacterium remains unknown as *I. persulcatus* group is absent in the country. Tick infection is established after an infectious blood meal, and the bacterium is transstadially but not transovarially passed. Transmission and propagation of *A. phagocytophilum* occurs in large mammals such as horses, deer, cattle, goats, sheep and cats in Europe. Small mammals and not ticks are said to be reservoirs of infection of anaplasmoses in this region (Vorou *et al*., 2008). The major mammalian reservoir for *A*. *phagocytophilum* in the eastern United States is the white-footed mouse, *Peromyscus leucopus*, although other small mammals and white-tailed deer (*Odocoileus virginianus*) can also be infected. White-footed mice have transient (1 - 4 weeks) bacteremia; deer are persistently and subclinically infected.

Human infection occurs when humans impinge on tick-small mammal habitats (Dumler *et al*. 2005).

3.2.1. Aetiology and pathogenesis

Anaplasma species are small (0.2 - 1.0 µm in diameter) obligate intracellular bacteria with a gram-negative cell wall, but lack lipopolysaccharide biosynthetic machinery. The bacteria reside in an early endosome, where they obtain nutrients for binary fission and grow into a cluster called a morula. *A*. *phagocytophilum* prefers to grow in myeloid or granulocytic cells and has been propagated in human HL-60 and KG-1 promyelocytic leukemia cells, THP-1 myelomonocytic cells, endothelial cell cultures, and in two tick cell lines (IDE8 and ISE6) that were derived originally from embryos of *Ixodes scapularis* (Dumler *et al*., 2005; de la Fuente *et al*., 2005). Although *A. phagocytophilum* has a broad geographic distribution, all the strains identified thus far appear to have considerable serological cross-reactivity and minor degree of variation in the nucleotide sequences of the 16S rRNA, *groESL*, *gltA*, *ank* and *msp2* genes, with the exception of some *ank* sequences from the infected German ticks that are different from other *ank* sequences of human and animal strains (de la Fuente *et al*., 2005).

3.2.2. Clinical signs

The first symptoms of anaplasmosis typically begin within 1 - 2 weeks after the bite of an infected tick. A tick bite is usually painless (CDC, 2012). Aside from the bacterial clusters, common features among patients include fever, headache, myalgia, malaise, absence of skin rash, leukopenia, thrombocytopenia, and mild injury to the liver (Skerget *et al*., 2003; Dumler *et al*., 2005). Common clinical signs of canine anaplasmosis mimic those seen with Lyme disease and include high fever, depression, anorexia, lethargy, and inflammation of multiple joints (polyarthritis), Esneault-Muller, (2006). *A. phagocytophilum* may cause disease in dogs; tick-borne fever in horses, domestic ruminants, white-tailed deer, and humans, as well as several species of small rodents (Granick *et al*., 2009).

3.2.3. Diagnosis

During the first week of illness a microscopic examination of peripheral blood smear may reveal morulae (microcolonies of *Anaplasma*) in the cytoplasm of white blood cells in up to 20% of patients (Butler *et al*., 2008). However, this method is insensitive and requires special expertise. Culture of HGE is possible, but it is very labor intensive and has not been validated as far as sensitivity is concerned (Schouls *et al*., 1999). Serological tests based on enzyme immunoassay (EIA) technology are available from some commercial laboratories, but indirect immunofluorescence assay (IFA) using *A. phagocytophilum* antigen is the main method of diagnosis. During the acute phase of illness, a sample of whole blood can be tested by polymerase chain reaction (PCR) assay to determine if a patient has anaplasmosis (CDC). Same applies for the infected animals (Butler *et al*., 2008). The sensitivity and specificity of PCR for the detection of *A. phagocytophilum* probably exceeds those of the other methods described to date and several PCR assays for its detection have been developed (Schouls *et al*., 1999).

3.2.4. Treatment

Doxycycline is the first line of treatment for adults and children of all ages. Other antibiotics, including broad spectrum antibiotics are not considered highly effective against *A. phagocytophilum*, and the use of sulfa drugs during acute illness may worsen the severity of infection. Treatment with doxycycline twice daily or tetracycline in animals results in favourable prognosis (CDC (2012): http://www.cdc.gov/anaplasmosis/symptoms/index.html; Lester *et al*., 2005).

3.3. *Ehrlichia canis*

E. canis is the aetiological agent of Canine Monocytic Ehrlichiosis (CME) (Mc Bride *et al*., 1996). First discovered by Donatien and Lestoquard in Algeria in 1935, it has since then become an important canine disease worldwide (Skotarczak, 2003; Shaw and Day, 2005; Allsopp and McBride, 2009). It was later reported in southern India and other parts of Africa in the 1940s. Subsequently, *E. canis* was relatively unrecognized until it

was associated with outbreaks of canine tropical pancytopenia in Singapore and Malaysia from 1963 to 1968 and was identified as being the cause of an epizootic of canine tropical pancytopenia in U.S. military dogs stationed in Vietnam in late 1968 (Shaw and Day, 2005). *E. canis* infections have since been well documented in the United States, Israel, Brazil, and Vietnam, and serologic and/or molecular evidence of infection in temperate regions where *Rhipicephalus sanguineus* is commonly found, including Central and South America, the Caribbean, southern Europe, southeast Asia and India, has also been reported (Mc Bride *et al*., 1996; Zhang *et al*., 2008). Shaw and Day, (2005) state that *E. canis* is transmitted transtadially but not transovarially by *R. sanguineus* (the Brown dog tick) and experimentally by *Dermacentor variabilis* (The American dog tick). The prevalence of *E. canis* is dependent on the distribution of the vector ticks which occur mainly in tropical and subtropical regions. CME is a potentially fatal disease in dogs that requires rapid and accurate diagnosis in order to initiate appropriate therapy leading to a favorable prognosis. Initially HME was blamed on *E. canis* but based on the 16S rRNA sequence the bacteria was differentiated and found to be a new species named *E. chaffeensis*, now known as the cause of the disease in humans. *E. canis* causes a febrile systemic disease that is often severe and can be fatal in dogs, like *E. chaffeensis* in humans. However, in experimentally infected dogs, *E. chaffeensis* causes only a mild febrile response with no hematological disorders and *vice versa* an *E. canis*-like agent (a new strain of *E. canis*) inflicts a chronic asymptomatic infection in humans (Skotarczak, 2003). This organism was isolated from a man in Venezuela in 1996 and subsequently from six patients in the same region suggesting that the zoonotic potential of *E. canis* is not yet fully elucidated (Perez *et al*., 1996; Harrus and Waner, 2005; Allsopp and McBride, 2009).

3.3.1. Aetiology and pathogenesis

E. canis is a small Gram-negative, pleomorphic, coccoid, obligate intracellular bacterium of the *E. canis* genogroup. The bacteria vary in size from small (0.4 µm), medium (0.7 µm), large (1 µm), and occasionally very large (≤2 µm). They stain dark blue to purple with use of Romanovsky's stains, including Wright's and Giemsa stains (Parola and Raoult, 2001; Shaw and Day, 2005; Allsopp and Mc Bride, 2009). *E canis* parasitizes

circulating monocytes intracytoplasmically in clusters of organisms called morulae and it infects dogs and other members of the Canidae family. The incubation period of CME is 8 - 20 days, the ehrlichial organisms enter the bloodstream and the lymphatics and localize in macrophages, mainly in the spleen and liver where they replicate by binary fission. From the macrophages, disseminate the infection to the other organs (McBride *et al*., 1996; Shaw and Day, 2005). Ehrlichiaea survive by inhibiting the phagosome-lysosome fusion thus increasing their chances of survival (Harrus and Waner, 2005).

3.3.2. Clinical signs

The first, acute stage, beginning after 8 – 20 days following transmission by infected tick, lasts 2 – 4 weeks. The acute phase may be manifested by fever, depression, dyspnoea, anorexia, lethargy and slight weight loss. In laboratory findings: thrombocytopenia, leucopoenia, mild anaemia, and hypergammaglobulinemia**.** The second phase is subclinical and follows the acute phase and may last 40 – 120 days or even years, in which dogs can remain persistently infected for years without clinical signs but with mild thrombocytopenia. Ultimately this stage is chronic, characterized by haemorrhages, epitasis and edema, with clinical signs; the results of laboratory study resemble the first phase of the disease (Skotarczak, 2003; Harrus and Waner, 2005; Allsopp and McBride, 2009). *E. canis* infection should be suspected in patients with fever, headache, malaise, myalgia, gastrointestinal symptoms, relative bradycardia, leukopenia, thrombocytopenia, and a recent exposure to either dogs or ticks (Conrad, 1989).

3.3.3 Diagnosis

Back in 1996 definitive diagnosis of canine ehrlichiosis was based on hematologic, biochemical, and serologic findings. However, the most clinically reliable method for diagnosis of canine ehrlichiosis was by serologic techniques such as immunofluorescent antibody test (IFA) and plate latex agglutination that detect *E. canis* serum antibodies. Nevertheless, serologic tests have several disadvantages. The use of

cell culture isolation (CCI) to detect *E. canis* in blood from infected dogs was reported to be both sensitive and specific (McBride *et al*., 1996) but PCR-based assays for *E. canis* detection are even more sensitive as compared with that of CCI and are the most commonly used methods now. Diagnosis is based on compatible history, clinical presentations and clinical pathological findings in combination with serology, PCR and *in vitro* culturing of the orgarnism (Harrus and Waner, 2005).

3.3.4. Treatment

Dogs infected with *E. canis* remain infected for their entire lives, even if they received antibiotic treatment with doxycycline. Recovery has been observed in humans without treatment, prompt therapy with tetracycline is advised (Conrad, 1989). Control of infection is based solely on the optimal application of acaricides and tick removal (Harrus and Waner, 2005).

3.4. *Ehrlichia ruminantium*

The aetiological agent of heartwater, *E*. *ruminantium* (formerly *Cowdria ruminantium*) is a tick-borne disease of cattle, goats, sheep and some wild ruminants (Allsopp *et al*., 2004). It is one of the most important tick-borne pathogens infecting wild and domestic ruminants throughout sub-Saharan Africa. Heartwater is present on some Caribbean islands and it poses a threat to mainland America (Allsopp *et al*., 2003). In South Africa it was first described in the 1800s in Limpopo by the Voortrekker pioneer Louis Trichardt after a stock of sheep died following a massive tick infestation. It is widely known as one of the major causes of stock losses in the sub-Saharan region, where it is endemic in most parts and is responsible for 17.5 million head of livestock. Goats in rural endemic parts are especially threatened with an undisclosed amount of losses per year (Allsopp *et al*., 2004; Allsopp and Mc Bride, 2009). The mortality rate in susceptible livestock ranges from 6% to 90%. Breeds of domesticated ruminants vary in their susceptibility, with higher morbidity and mortality rates among non-native than indigenous breeds. Up to 80% of merino sheep may die, but the mortality rate can be only 6% in Persian or

Afrikaner sheep (OIE, 2007). It is transmitted by ticks of the genus *Amblyomma*. In South Africa the only vector is *A. hebraeum* (Allsopp *et al*., 2004). At least twelve species of *Amblyomma* can transmit *E. ruminantium*. In addition to *A. variegatum* and *A. hebraeum*, *A. lepidum* (in East Africa and the Sudan); *A. astrion*; *A. pomposum*; *A. sparsum*; *A. gemma*; *A. cohaerans*; *A. marmoreum*;and *A. tholloni* (the elephant tick) are capable of transmitting experimental infections. Two North American species, *A. maculatum* (the Gulf Coast tick) and *A. cajennense*, can transmit *E. ruminantium* in the laboratory, but neither has been implicated in natural infections. *E. ruminantium* gene segments have been found, by PCR, in the ticks *Rhipicephalus evertsi*, *Hyalomma truncatum* and *H. marginatum*; however, the organism was not isolated (OIE, 2007).

Economic impact of heartwater, although difficult to quantify due to under-reporting and occurrence factors such as the use of acaricides, antibiotic prophylaxis, immunization by infection and treatment, resistance of breeds and endemic stability; the estimated cost due to the disease totals about US$ 30 million in 10 per cent of stock losses per annum in South Africa, despite the US$1 and 5 million spent on prophylaxis and vaccination respectively (Allsopp *et al*., 2004).

3.4.1. Aetiology and pathogenesis

E. ruminantium is a pleomorphic rickettsia with colonies containing one to several thousands in an individual organism within the endothelial cells. Colonies consist of coccoid organisms that vary in size from small (0.4 µm), medium (0.76 µm) to large (1.04 µm). It is closely related to *E. canis* and *E. chaffeensis* based on the 16S gene sequence analysis (Allsopp *et al*., 2004, Allsopp 2009). The Gram negative bacterium stains purplish-blue with Giemsa. In common with all species in the family Anaplasmataceae growth takes place in an intracellular vacuole bounded by a lipid bilayer membrane derived from the eukaryotic host cell membrane. *E. ruminantium* replicates mainly by binary fission of reticulated bodies while the elementary bodies represent the infective stage. Sequential development of the organism has been described in both vertebrate and invertebrate host cells. Strains of *E. ruminantium* are very diverse, some strains are highly virulent while others appear to be non-pathogenic.

E. ruminantium has a high level of genomic plasticity. Several different genotypes can co-exist in a geographic area, and may recombine to form new strains (OIE, 2007).

3.4.2. Clinical signs

Typically heartwater is characterized by high fever, nervous signs and hydropericardium where the sac surrounding the heart becomes filled with an excessive amount of fluid hence the name heartwater. Fluid is also observed in the chest (hydrothorax), lungs (oedema), and the brain and eventually may result in death (Inokuma *et al*., 2005).

3.4.3. Diagnosis

Observation of brain smears by microscopy is used during post mortem to identify the pathogen. Serological tests include IFA where the infected peritoneal macrophages are used as an antigen (Martinez *et al*., 1990; OIE, 2005). Serological tests for *E. ruminantium* are criticized for lacking specificity, whilst PCR amplification and probing for a section of the organism genome referred to as the pCS20 region is being commended as the most reliable and sensitive test for the organism (Allsopp *et al*., 2005).

3.4.4. Treatment

There is no reliable vaccine and infected animals frequently die before treatment with tetracyclines can be administered (Allsopp *et al*., 2005). There are also inactivated vaccines as well as nucleic acid vaccines such as OVI-DNA vaccine which is a cocktail of four *E. ruminantium* ORFs (Open Reading Frames) derived from the 1H12 clone from a genetic locus involved in nutrient transport. Ruminants infected with *E. ruminantium* are treated with doxycycline. Currently the only suggested way of treating this in human patients is with oral doxycycline which, unfortunately, is not always well tolerated. The most commonly used vaccine in South Africa is Ball 3 *E. ruminantium* but is sometimes ineffective and has to be used in combination with other antibiotics (Tshikudo, 2009).

3.5. Materials and methods

3.5.1. Study area

Ticks were collected from dogs, cattle, goats, sheep and incidentally four horses and four cats from farms in the Free State (FS), KwaZulu-Natal (KZN), Eastern Cape (EC), North West (NW) and Mpumalanga (MP) Provinces. In KZN samples were collected from Wesselsneck [S 28° 20' 0.52" E 030° 02' 49.1"], Gcinalishona/Mjindini [S 28° 39' 00.5" E 030° 06' 56.3"], eTholeni [S 28° 25' 39.9" E 30° 13' 04.3"] and uMsinga Mountainview dip site [S 28° 41'43.1" E 030° 16'14.6"]; in FS, Hooningkloof [S28° 30. 666' E 028° 42.701'], a farm situated at a livestock-wildlife interface, Sekoto farm [S28° 36.094' E 028° 49.013'], Seotlong Hotel and Agricultural School [S 28° 35' E 28° 50'] and in Kestell [S 28° 20' E 28° 38']; in EC, at Amathole District Municipality [S 32°48' 30" E 27° 01' 49"]; in NW at a private Veterinary Clinic in Mafikeng [S 25° 51′ 0″ E 25° 38′ 0″] and in MP at Kameelpoort-KwaMhlanga area [S 25° 46′ 6.3″ E 29° 28′ 42″], refer to Figure 2.4. for the Map of South Africa.

3.5.2. DNA extraction from ticks

The ticks were surface sterilized twice with 75% ethanol then washed once in phosphate buffered saline (PBS) solution before they were dissected and gutted (the engorged) or crushed whole (the males) in individual sterile Eppendorf tubes (Hamburg, Gemany) then later preserved in PBS and then stored at -34°C until further use. Ticks of the same species collected from one animal were grouped as one sample for DNA extraction. Some ticks laid eggs within the collection vials, these were also washed in PBS, spun down at full speed (16 000 xg) in the microcentrifuge then crushed and stored as described above. DNA was extracted from tick extracts and eggs using the salting out method as described by Miller *et al*., (1988). DNA was dissolved in 50 - 200 µl (depending on the size of the pellet) of double distilled water and extracts were stored at -34 °C.

3.5.3. Polymerase chain reaction

The experimental procedures were carried out in the laboratories of the Parasitology Research Program of the University of the Free State (UFS) - Qwaqwa Campus, South Africa and the Research Center for Zoonosis Control (CZC) of Hokkaido University, Japan. The extracted DNA (n = 721) from whole ticks together with an additional 55 DNA samples (obtained from dog ticks in the Free State, Phuthaditjhaba area) from a previous study (Leodi, 2010) were subjected to PCR amplification using oligonucleotide sequences listed in Table 3.1. to screen for the presence of *A. phagocytophilum*, *Ehrlichia/Anaplasma, E. canis* and *E. ruminamtium.* Thereafter electrophoresed on 1.5% agarose gel stained with GelRed and/or ethidium bromide and size fractionated using a 50/100 bp DNA ladder (Figure 3.1 - 3.4). PCR was performed using AmpliTaq Gold® 360 Master Mix (Applied Biosytems, U.S.A.) with the following cycling conditions: Initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55 - 60°C for 30 s and extension at 72°C for 60 s/kb followed by final extension at 72°C for 7 min and held at 4°C ∞, using CZC's Veriti®Thermocycler (Applied Biosystems, U.S.A.) and the PRP's Multigene OptiMAx Thermal Cycler (Life Technologies Corporation, U.S.A.).

3.5.4. Purification and sequencing

For experiments conducted at CZC, the positive PCR products were purified using USB ExoSAP-IT Enzymatic PCR Product cleanup (Affymetrix, Japan) by incubating at 37 °C followed by 80 °C. Cycle sequencing reactions were performed using an ABI Prism Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, U.S.A.) on an ABI 3130 DNA Sequencer. For experiments conducted at PRP the PCR products were sent to Inqaba Biotechnological Industries (Pty) Ltd (R.S.A.) for sequencing. The sequence data of the PCR products were analyzed using the BLAST 2.0 program (National Center for Biotechnology Information, Bethesda, Maryland, U.S.A.; http://www.ncbi.nlm.nih.gov/blast/) for homology searching. Sequences were analyzed and aligned using BioEdit Sequence Alignment Editor © 1997-2004 (Tom Hall Isis Pharmaceuticals, Inc), see Figure 3.5., sequences used in the alignment were obtained from NIH's GenBank Database http://www.ncbi.nlm.nih.gov/genbank/).

Table 3.1. Oligonucleotide sequences used to amplify the target pathogens

Pathogen	Primer sequences (5' - 3')	Expected fragment size (bp)	Annealing temp	Reference
Anaplasma phagocytophilum	EHR-521 TGT AGG CGG TTC GGT AAG TTA AAG EHR-747 GCA CTC ATC GTT TAC AGG GTG	250	60 °C	Welc-Faleciak *et al*., (2009)
***Ehrlichia/Anaplasma* spp.**	Ehr- F: GGA ATT CAG AGT TGG ATC MTG GYT CAG Ehr- R biotin: CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT	352-460	55°C	Matjila *et al*. , 2008
Ehrlichia canis	E.c 16S fwd: TCGCTATTAGATGAGCCTA CGT E.c 16S rev:GAGTCTGGACCGTATCTCAGT	154	55°C	Peleg *et al*., 2010
Ehrlichia ruminantium	pCS20 F3- CTT GAT GGA GGA TTA AAA GCA pCS20 B3- GTA ATG TTT CAT GTG AAT TGA TCC	279	57°C	Nakao *et al*., (2010)

3.6. Results

3.6.1. *Ehrlichial Anaplasma* species detection

Using broad range primer set Ehr-F and Ehr-R an overall 18% *Ehrlichia/Anaplasma* DNA (n=122) was detected by PCR across the four sampled Provinces from the dog- and cat-tick samples (Fig 3.1.). NW samples tested negative for these pathogens, 22% infection in KZN was observed followed by FS with 18% and the dog-tick sample from MP was also positive for *Ehrlichia/Anaplasma* DNA, the cat-tick samples were all negative.

3.6.2. *Ehrlichia canis* detection

To confirm the presence of *E. canis,* primer set E.c 16S fwd and E.c 16S rev were used to screen the same samples mentioned above (n = 122) and all but three that had been positive for *Ehrlichia/Anaplasma* DNA also tested positive for *E. canis* DNA and

additionally five more samples that had tested negative for *Ehrlichia/Anaplasma* DNA were positive for *E.canis* giving an overall infection of 20% with *E. canis*. Only 2 of the crushed-egg DNA samples (n = 14) were positive for *E. canis*. The size fractionated amplified PCR products are shown in Figure 3.2., depicting an amplicon size of 154 bp. The sequences generated were 100% homologous with other published sequences of *E. canis* (GenBank Accession no: CP000107.1, DQ228514.1, HQ844983.1).

3.6.3. *Anaplasma phagocytophilum* detection

Using PCR primers specific for *A. phagocytophilum,* primer set EHR-747 and EHR-521 to screen 107 dog and cat-tick DNA samples that were also tested with *Ehrlichia/Anaplasma* broad range primer set Ehr-F and Ehr-R, 20 (19%) of these samples tested positive for *A. phagocytophilum* and gave a corresponding band of 250 bp during electrophoresis (Figure 3.3.). Only three of the 20 (15%) *A. phagocytophilum* positive samples had tested positive for the presence of *Ehrlichia/Anaplasma* DNA. The prevalence of *A. phagocytophilum* was 63% in NW (n = 16) and 21% in FS (n = 28), the four cat-tick DNA samples from MP were all positive, the dog-tick sample negative and none of the dog-tick samples from KZN (n = 59) tested positive for the bacterium. In the dog and cat population combined *A. phagocytophilum* was isolated from 15% *Haemaphysalis elliptica*, 60 % *Rhipicephalus sanguineus* and 25 % of unidentified ticks and it was absent from *A. hebraeum* ticks.

The overall infection rate amongst cattle-, goat- and sheep-ticks was 6%, 17% and 1.25% respectively for all Provinces combined. An infection rate of 1.25% (n = 80) *A. phagocytophilum* was obtained among the FS sheep-tick samples. Thirty two percent (n = 28), 15% (n = 13) and 0 % (n = 20) infection rates for EC, MP and FS respectively were obtained amongst the goat-tick population. The four horse-tick DNA samples from FS were negative for *A. phagocytophilum*. An overall 5% was obtained for both EC (n = 95) and KZN (n = 84), and 7.3% for FS (n = 123) when the cattle-tick DNA samples were subjected to PCR screening.

All in all the overall prevalence of infection of ticks with *A. phagocytophilum* was 9% (50/562) for all the screened tick DNA samples combined across the five sampled Provinces, for all groups of animal from which they were collected. Prevalence of infection with *A. phagocytophilum* in the FS was 6% (n = 263), 3% (n=143) in KZN, 63% (n = 16) in NW, 11% (n = 123) in EC and lastly 35% (n = 17) in MP. Of the positive tick samples collected from ruminants the bacterium was detected from 50.0% *Rhipicephalus* spp. 23.0% *R. e. evertsi,* 19.2% *R. decoloratus* and 7.7% *A. hebraeum*. The crushed-egg DNA samples from all animal groups combined (n = 17) were all negative for *A. phagocytophilum* DNA by PCR. Tick DNA samples collected from vegetation (n = 12) also tested negative. The sequences were 96% - 100% identical to published sequences of *A. phagocytophilum* (GenBank accession no: DQ648489.1) obtained from ticks of cattle in India. The alignment of the sequences obtained with similar previously published sequences is shown in Figure 3.5.

3.6.4. *Ehrlichia ruminantium* detection

The overall rate of infection with *E. ruminantium* in the sampled Provinces was 37%, 26%, 18% and 0% for the FS, KZN, EC and MP respectively for all tick samples combined. Ruminant tick samples were not collected from the NW Province. Amongst the goat, sheep and cattle tick samples the overall prevalence of infection with *E. ruminantium* was 69%, 34% and 19% respectively for all Provinces combined.

Of the 91 screened sheep-tick DNA samples from the FS, 34% infection was obtained. All (100%) the goat-tick samples (n = 22) from the FS were positive for the bacterium, in EC (n = 29) infection was 76% and MP (n = 13) goat ticks were all negative for *E. ruminantium* DNA by PCR. The four horse-tick DNA samples from FS tested negative for *E. ruminantium* DNA. Amongst cattle ticks 26% (n = 82) and 27% (n = 124) were obtained in KZN and FS respectively and none (0%) of the 95 DNA samples from EC tested positive for this pathogen.

The number of positive DNA samples totaled 130/460 (28%) and of those that were positive, *R. evertsi evertsi* had the highest infection rate of 55.4%, followed by

Rhipicephalus spp. (34.6%), *A. hebraeum* (6.2%) and lastly *R. decoloratus* (3.8%). The size fractionated amplified PCR products are shown in Figure 3.4., showing an amplicon size of 279 bp. The sequences generated were 100% identical to pCS20 ribonuclease region of the *E. ruminantium* Welgevonden strains AY236058.1, CR767821.1, the type species which was obtained from an *A. hebraeum* tick collected in the former north eastern Transvaal in South Africa.

The summary of the overall infection rates of ticks collected from livestock and from the vegetation with *A. phagocytophilum*, *Ehrlichia/Anaplasma*, *E.canis*, *E. ruminantium* in the five sampled Provinces of South Africa is given on Table 3.2 below.

3.7. Discussion

The overall 20% prevalence of infection with *E. canis* was detected solely from *R. sanguineus* ticks (of the identified ticks). None of the *H. elliptica* or *A. hebraeum* ticks carried *E. canis* this is probably because of its high affinity for *R. sanguineus* as a carrier (Harrus and Waner, 2005). Being a chronic disease in canines, it would be expected for the prevalence to be much higher yet it is surprisingly low in this case, however the actual incidence of *E. canis* worldwide may be over-estimated due to its diagnosis being reliant on biochemical and serological tests known to detect latent infections (Mc Bride *et al*., 1996). Its absence from the NW ticks could be as a result of frequent administering of doxycyclines of well cared for dogs that are brought in at the veterinary clinic. Other than *E. canis* in South Africa, another potential threat to canine health is *E. ruminantium* (the agent of heartwater in ruminants). Lately dogs with symptoms suggestive of CME but showed no morulae during microscopic diagnosis and tested negative with *E. canis* specific primers were subject to screening with *E. ruminantium* primers which target the PCS20 gene and from that 36 of 50 dogs were positive for this pathogen (Allsopp and Allsopp, 2001).

In addition to the previous detection of *A. phagocytophilum* from whole blood specimens of three dogs in Bloemfontein (Inokuma *et al*., 2005; Matjila *et al*., 2008), this is to my knowledge a second report of the bacterium in the country and the first detection of the pathogen in ticks. Egypt reported 13.7% and 5.3% *A. phagocytophilum* infection rates of ticks collected from dogs, sheep and goats (Ghafar and Amer, 2012), making the current study's findings slightly higher. Tick infection with *A. phagocytophilum* is established after an infectious blood meal, and the bacterium is transstadially but not transovarially passed (Dumler *et al*., 2005), thus eliminating the theory that the ticks do carry the pathogen but are unable to pass it on to their host and also clarifies how the ticks in this study acquired the bacterium. This is a clear indication that *A. phagocytophilum* exists amongst South Africa's livestock population, but is probably maintained within the cycle between livestock and ticks as *Ixodes persulcatus* group (the vectors of HGA) is absent in the country. The absence of the bacterium from the crushed-egg DNA samples is in accordance with the finding that, *A. phagocytophilum* is transstadially and not transovarially transmitted. In the absence of *I. persulcatus* group, *R. sanguineus*; *Rhipicephalus* spp.; *R. e. evertsi; R. decoloratus; H. elliptica;* and *A. hebraeum* should therefore be considered as possible vectors/reservoirs of the pathogen amongst livestock populations. The true incidence of *A. phagocytophilum* however, remains unknown in South Africa.

Farmers in pan Africa battle with *E. ruminantium* infections amongst livestock as the disease they cause accounts for annual cost losses totaling US$ 44.7 million, in Angola, Botswana, Malawi, Mozambique, South Africa, Swaziland, Tanzania, and Zambia combined. When livestock from heartwater-free areas are introduced to boost stock production, they succumb to the disease, this is to such an extent that heartwater losses are regarded as just another factor to be endured in the endemic areas of Africa, and so presumptive diagnoses are usually not confirmed and the real economic cost is therefore difficult to quantify (Allsopp and McBride, 2009).

Amblyomma hebraeum, the main vector of *E. ruminantium* in South Africa occurs mainly in Limpopo, KwaZulu-Natal and in the Eastern Cape Provinces (Pfitzer *et al*., 2004). In this study it was also collected in Mpumalanga, it was absent in the Free State

tick collection. Seeing that all the *Rhipicephalus* tick species carried *E. ruminantium* it may be because it was already present in the blood meal. However its gene segments, but not the entire organism, have been isolated in *Rhipicephalus* spp. in previous studies, and their vectorial capacity is yet to be demonstrated (OIE, 2007). In the Eastern Cape, an area neighboring Cwaru village (the sampling site), Magwiji, and another north-east of it, Cala, were found to be serologically negative for *E. ruminantium* amongst the cattle population (Marufu, 2008), the negative result was justified by the absence of *A. hebraeum*, this is in agreement with the findings in the current study, only in this study the vector was ample among the collected samples. Surprisingly the bacterium was isolated from 76% of the ticks collected from goats in the same area. This could be an indication of endemic instability of *E. ruminantium* in the studied area or it could be that the animals had recently been treated. Previous studies have reported *E. ruminantium* amongst ticks to be as low as 4.7% and 11.3% in communal grazing land in Rietgat, 5.7% and 25% at Kruger National Park and Songimvelo Game Reserve respectively (Peter *et al*., 1999, Bryson *et al*., 2002b).

Although humans are believed to be resistant to *E. ruminantium* infection, they can act as transport hosts of *Amblyomma* spp. ticks, including *A. variegatum* according to the Centers for Epidemiology and Animal Health (2008). On the contrary *E. ruminantium* has been reported to cause illness in humans, an example of a case is one in South Africa where the strain was detected in serum from a woman who had reportedly died of biliary fever after the death of her pet dog, which suffered from atypical canine ehrlichiosis (Inokuma *et al*., 2005). Allsopp *et al*., (2005) reported three cases of suspected fatal ehrlichiosis (in Gauteng area), the first being the previously mentioned case. The second, a six-year-old child who died a week after hospital admission with a clinical picture of encephalitis together with complaints of severe headache, sleepiness, and an unsteady gait. Post-mortem examination revealed severe vasculitis affecting the midbrain and pons regions, as well as prominent pulmonary edema. The third case was also a child who died after a short illness. The clinical features resembled those of the second case. Both children were accustomed to playing outdoors, one on an agricultural smallholding and the other on the borders of a game reserve, but only one

child had definite evidence of tick bite. At that time there was no information concerning evidence of tick bites, the unique *E. ruminantium* variant detected in the woman was assumed to have been possibly acquired from her sick dog. A follow up study by Louw *et al*., (2005) later that year confirmed that the suspected fatal cases mentioned above and an additional case of a child from Cape Town were indeed caused by *E. ruminantium*. This could signal the emergence of *E. ruminantium* as a zoonotic agent in heartwater-endemic areas of Africa and it has been suggested by Louw *et al.*, (2005) and Allsopp and McBride (2009) that children living in endemic areas presenting with a clinical picture of encephalitis of unknown origin should be treated with doxycyclines due to the rapid course of the disease.

3.8. Conclusions

As more proof that demonstrates the zoonotic potential of *E. ruminantium* becomes available, one cannot help but wonder about the risk associated with the pathogen as it is transmitted by *Amblyomma* species which are known to actively hunt for their hosts and will readily bite humans. Thus zoonotic strains should be isolated and studied to differentiate them from non-zoonotic ones. Early diagnosis and proper vaccination remains a feasible method of keeping heartwater disease in check as there is no reliable method of treating the disease and animals often die before they can be treated.

Further studies on *A. phagocytophilum* in South Africa should be conducted to shed light on the epidemiology of the pathogen and why there are infrequent if any clinical cases of infection with it amongst human populations. The current study revealed the essence of using species specific-primers in the detection of *A. phagocytophilum* as *Ehrlichia/Anaplasma* primers failed to pick up a significant (20%) presence of *A. phagocytophilum* from the screened samples and showed a bias towards *Ehrlichia*, hence for this reason it is possible that the actual presence of the bacterium may be under-estimated. *E. canis*, *E. ruminantium* and *A. phagocytophilum* should become part of routine screening when presented with patients showing clinical signs similar to those of HGA and HME. Moreover medical practitioners should become acquainted with newer findings in micropathobiology as more often than not they are not aware of these,

consequently misdiagnosing patients, which in some unfortunate circumstances may result in death.

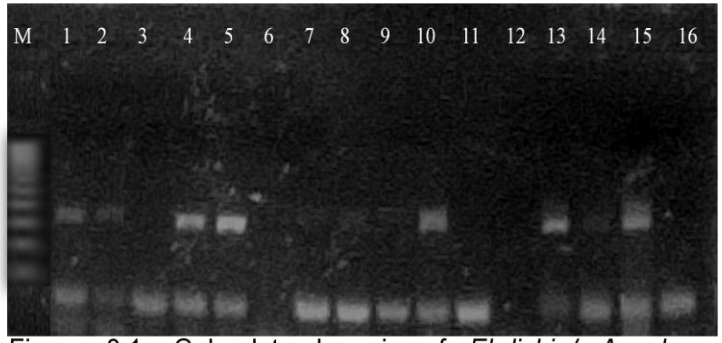

Figure 3.1. Gel eletrophoresis of *Ehrlichia/ Anaplasma* amplified PCR product with amplicon size of 352-460bp, M: DNA ladder (100bp), 1;2;4;5;10;13;15: positive samples, 3;6-9;11;12;14: negative sample, 15:*E. canis* positive control, 16: negative control (DDH_2O).

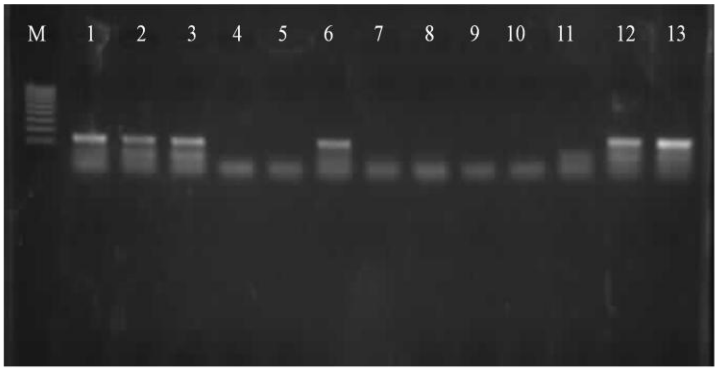

Figure 3.2. Gel eletrophoresis of *E. canis* amplified PCR product with amplicon size of 154 bp, M: DNA ladder (100bp), 1-3; 6;12: positive samples, 4;5; 7-11: negative samples, 13: *E. canis* positive control.

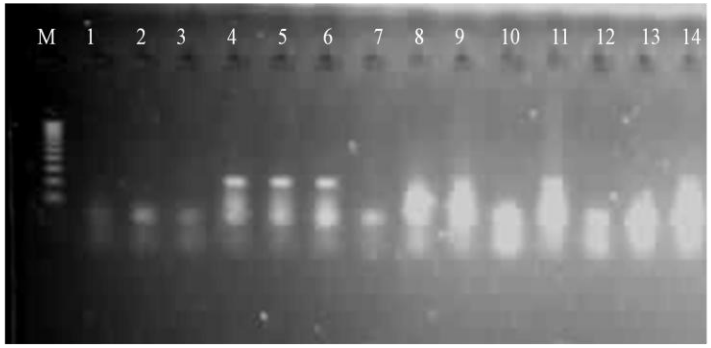

Figure 3.3. Gel eletrophoresis of *A. phagocytophilum* amplified PCR product with amplicon size of 250 bp, M: DNA ladder (100bp), 4-6: positive sample, 1-3;7;10;12: negative samples, 8;9;11;13; 14: inconclusive smears.

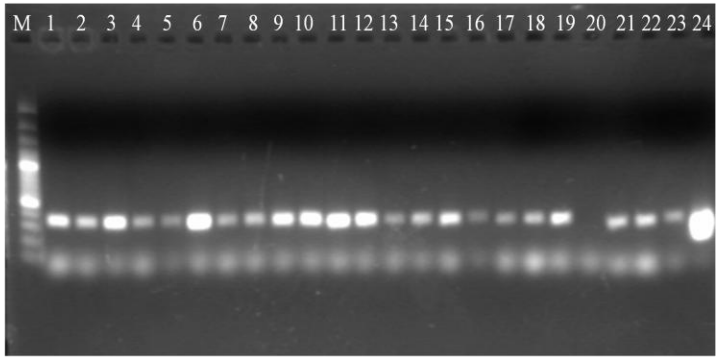

Figure 3.4. Gel eletrophoresis of *E. ruminantium* amplified PCR product with amplicon size of 279 bp , M: DNA ladder (50 bp), 1-19; 21-23: positive samples, 20: negative sample, 24: *E. ruminantium* positive control.

Table 3.2. Summary of the overall prevalence infection of ticks with *A. phagocytophilum*, *E. canis* and *E. ruminantium* across the five sampled Provinces of South Africa

Province	Study group	*E. canis*			*E. ruminantium*			*A. phagocytophilum*		
		+ve	Total screened	Overall %	+ve	Total screened	Overall %	+ve	Total screened	Overall %
Free State	Dogs	13	50	26	-	-	-	6	28	21
	Cattle	-	-	-	34	124	27.4	9	123	7.3
	Goats	-	-	-	22	22	100	0	20	0
	Sheep				31	91	34	1	80	1.25
	Flagged	-	-	-	-	-	-	0	12	0
North West	Dogs	0	16	0	-	-	-	10	16	63
KZN	Dogs	10	55	19	-	-	-	0	59	0
	Cattle	-	-	-	21	82	26	4	84	5
EC	Cattle	-	-	-	0	95	0	5	95	5
	Goats	-	-	-	22	29	76	9	28	32
MP	Dogs	1	1	#	-	-	-	1	1	#
	Cats	0	4	#	-	-	-	3	3	#
	Goats	-	-	-	0	13	0	2	13	15

Overall infection rate of ticks across five sampled Provinces

Study group	*E. canis*			*E. ruminantium*			*A. phagocytophilum*		
	+ve	Total screened	Overall %	+ve	Total screened	Overall %	+ve	Total screened	Overall %
Goats	-	-	-	44	64	69	11	61	18
Cattle	-	-	-	55	301	18	18	302	6
Dogs	24	122	20	-	-	-	20	107	19
Flagged	-	-	-	-	-	-	0	12	0
Sheep	-	-	-	31	91	34	1	80	1.25

Percentage negligible, sample size too small

Overall incidence of infection of ticks in individual Provinces

***E. canis*:** FS =26%, NW= 0%, KZN=19%; ***E. ruminantium*:** FS=37%, KZN=26%, EC=18%, MP=0%;

***A. phagocytophilum*:** FS=5.8%, NW= 63%, KZN=3.5%, EC =11%, MP=29%

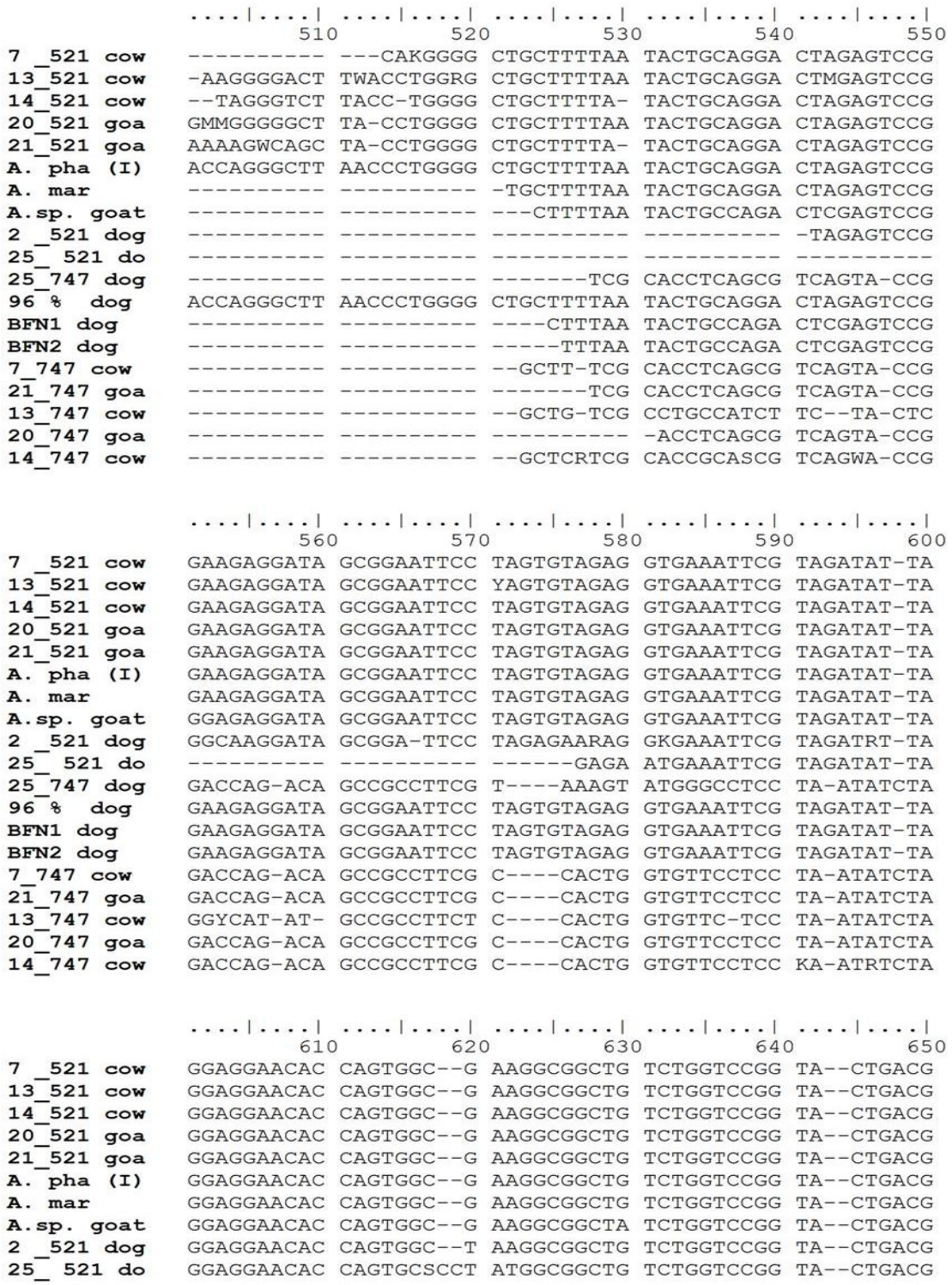

Figure 3.5. Alignment of *A. phagocytophilum* sequences obtained vs published sequences generated from BioEdit, BFN1 and 2 represent *A. phagocytophilum* sequences isolated from blood samples of dogs from Bloemfontein, A. pha (I) represents *A. phagocytophilum* sequence obtained in cattle ticks from India, A sp. Goat represents *Anaplasma* species similar to *A. phagocytophilum* obtained from goats in South Africa,2; 7; 13; 14;20; 21;25_ 521 and & _747 are sequences generated using forward (EHR_521) and reverse (EHR_747) primers on goat, cow and dog-tick samples, A. mar represents *Anaplasma marginale* sequence used for the sake of comparison.

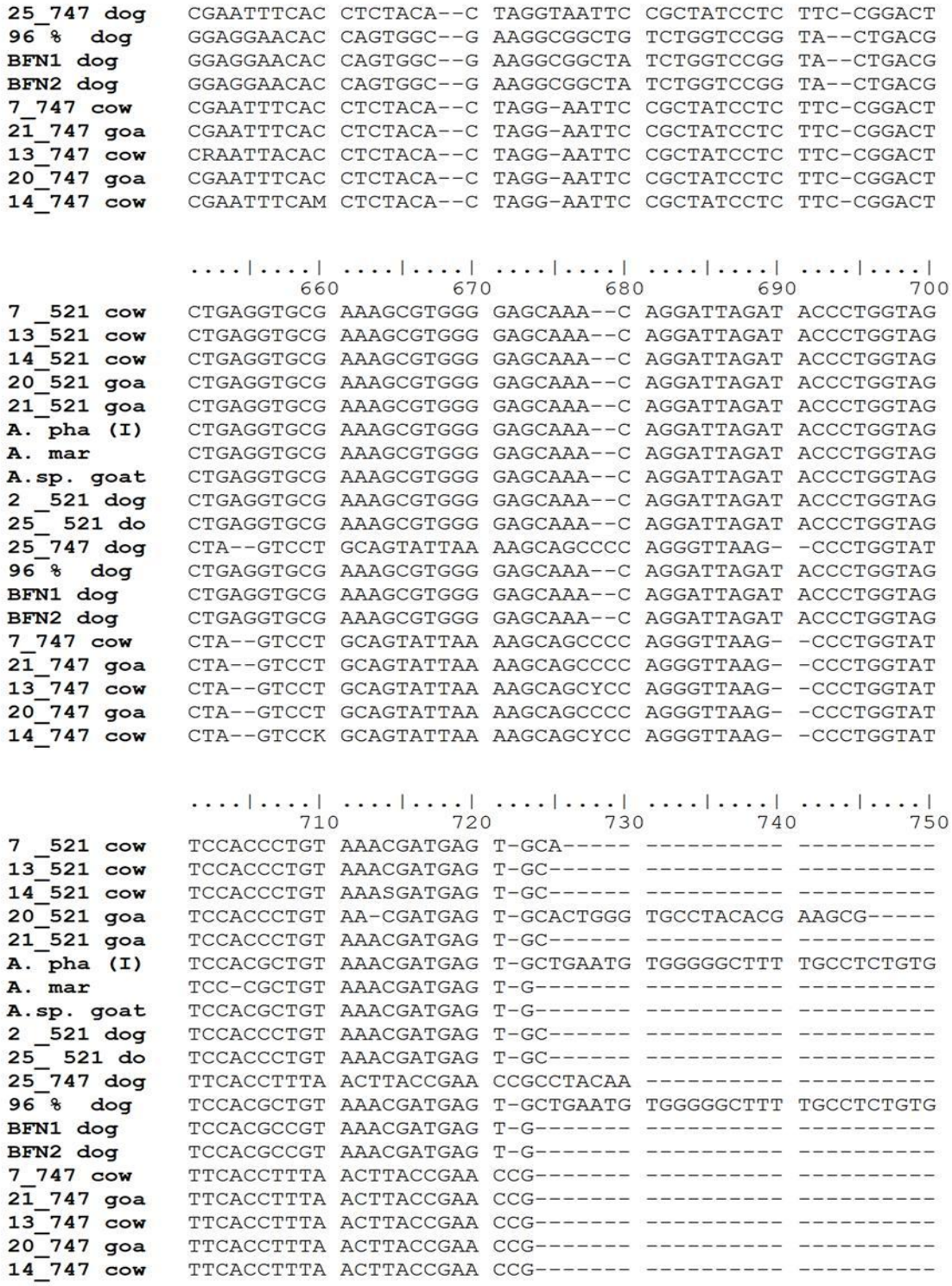

Figure 3.5. Alignment of *A. phagocytophilum* sequences obtained vs published sequences generated from BioEdit, BFN1 and 2 represent *A. phagocytophilum* sequences isolated from blood samples of dogs from Bloemfontein, A. pha (I) represents *A. phagocytophilum* sequence obtained in cattle ticks from India, A sp. Goat represents *Anaplasma* species similar to *A. phagocytophilum* obtained from goats in South Africa,2; 7; 13; 14;20; 21;25_ 521 and & _747 are sequences generated using forward (EHR_521) and reverse (EHR_747) primers on goat, cow and dog-tick samples, A. mar represents *Anaplasma marginale* sequence used for the sake of comparison.

Chapter 4: Molecular detection of *Coxiella burnetii* from ticks collected from livestock and companion animals in five Provinces of South Africa.

4.1. Introduction

Query fever (Q-fever) is a worldwide zoonotic disease caused by *Coxiella burnetii*, a member of the family Coxiellaceae (Mediannikov *et al*., 2010; Matthewman *et al.*, 1997). It was first reported in abattoir workers in Queensland, Australia in 1937 by E.H. Derrick. Subsequently, F.M. Burnet and M. Freeman isolated a fastidious intracellular bacterium from guinea pigs that had been injected with blood or urine from Derrick's patients and named it *Rickettsia burnetii* in 1939. This bacterium was morphologically and biochemically similar to other gram-negative bacteria. Later on in 1948, on the basis of cultural and biochemical characteristics, C.B. Philip classified *R. burnetii* in a new genus, *Coxiella*, naming it after H.R. Cox, who first isolated this microorganism in the United States (Schutte *et al*., 1976; Heinzen *et al*., 1999; Fournier *et al*., 1998, Guatteo *et al*., 2011). It has long been considered a *Rickettsia,* this was proven by Burnet and Freeman in 1939, although with advancement in diagnostic tools, it was however found to be phylogenetically distant to other Rickettsias and more closely related to bacteria such as *Legionella* spp.*, Rickettsiella grylli*, and *Francisella* spp. (Schutte *et al*., 1976; Fournier *et al*., 1998; Heinzen *et al*., 1999). It is prevalent and endemic in most places in the world except New Zealand (Raoult *et al*., 2005).

The true reservoir is wide and includes mammals, birds and arthropods, mainly ticks. Cattle, sheep and goats are most commonly identified as sources of human infection and the disease is prevalent mostly in rural areas worldwide. Other animals, however, including common pets such as cats, rabbits, pigeons and dogs may also serve as sources. Q-fever is usually transmitted by aerosol inhalation (Berri *et al*., 200). Hard and soft ticks infected during feeding, may transmit *C. burnetii* transovarially and transstadially and excrete it via feces, saliva and coxal fluid (Skerget *et al*., 2003; Mediannikov *et al*., 2010). About 32 species of Ixodid ticks and 32 species of Argasid ticks have been reported to harbor the pathogen in the former USSR alone

(Mediannikov *et al*., 2010). Q-fever has been reported all over the African continent, with generally higher serological indices in the countries West of Africa isolated from *Amblyomma variegatum*, *Hyalomma truncatum* and *Rhipicephalus senegalensis* (Mediannikov *et al*., 2010).

The first cases of *C. burnetii* in South Africa were reported round about the 1950's from a boy, with fever and atypical pneumonia, an adult male with fever and delirium and, from an adult male with intermittent fever of unknown origin and serological evidence of the infection. Later in 1953 in a survey conducted by South African Institute for Medical Research, it was reported that serologically the disease was the most common rickettsial disease of man at the time in the country (Schutte *et al*., 1976). It is further reported that the disease is common in South Africa and seroprevalence of *C. burnetii* infected people is high in the neighboring country, Zimbabwe (Matthewman *et al*., 1997). In Japan, a country previously thought not to have Q-fever, indicated seropositivity rates of 20%, which strongly suggests that infections with *C. burnetii* should be considered as a re-emerging infectious disease (Heinzen *et al*., 1999).

Public health and animal health issues are both closely related to Q-fever, this makes it an important disease to public policy makers and the food industry (Guatteo *et al.*, 2011). *C. burnetii* is classified as a category B bioterrorism agent by the Centers for Disease Control and Prevention and the National Center for Allergy and Infectious Diseases (Moodie *et al*., 2008). A bioterror event from such an organism can have immense public, political and economic impact (Massung *et al*., 2012) therefore public health agencies are obligated to prepare for such a scenario.

4.1.1. Aetiology and pathogenesis

C. burnetii is a small, gram-negative, nonmotile, pleomorphic, oval to rod-like, obligate intracellular bacterial pathogen. Its dimensions vary from 0.4 - 1.0 µm in length and from 0.2 - 0.4 µm in diameter. *C. burnetii* is biphasic, it has metabolically dormant cells

called small cell variants (SCVs) which are initially phagocytosed by eukryotic host cells and sequestered in a phagolysosome. SCVs are then triggered to develop into more metabolically active large cell variants (LCVs). SCVs and LCVs are morphologically distinct and can be separated on the basis of their different buoyant densities (Heinzen *et al*., 1999). The pathogen resides in the gamma subdivision of the Proteobacteria (Schutte *et al*., 1976), within a parasitophorous vacuole (PV) that has characteristics of a secondary lysosome. *In vitro*, it enters passively (viable and nonviable organisms are equally internalized) and replicates within a wide variety of epithelial, fibroblast and macrophage-like cell lines. *In vivo*, the initial target is the alveolar macrophage, although the organisms can subsequently dissemate to replicate within a wide variety of tissues (Heinzen *et al*., 1999). *C. burnetii* completes its developmental lifecycle in the phagolysosome of the eukaryotic cell (Kelly *et al*., 1993), the bacterium is acidophilic and it grows and proliferates in acidic media as low as 4 in pH (Rolain *et al*., 2005).

4.1.2. Clinical signs

Presentation of the disease is extremely variable and infection may lead to asymptomatic seroconversion, acute disease (ranging from a flu-like syndrome to severe pneumonia requiring intensive care), or chronic infection (manifesting mainly as endocarditis) (Matthewman *et al*., 1997). *C. burnetii* is a zoonotic disease of man which manifests as a febrile illness, self-limiting fever, and influenza-like symptoms in acute phase, with the chronic phase being characterized by endocarditis, granulomous hepatitis and osteomyelitis (Willems *et al*., 1993), meningioencephalitis may also occur (Matthewman *et al*., 1997), abortion, premature birth (33%), low weight in newborns and stillbirths have been reported in pregnant women (39%) (Benenson and Tigertt, 1956; Skerget *et al*., 2003; Guatteo *et al*., 2011). Man is the only host which has been reported to show clinical symptoms to infection with *C. burnetii* (Kelly *et al*., 1993) nevertheless 60% of the infected population may remain asymptomatic (Skerget *et al*., 2003). Although it is inapparent in wild and domestic animals, bronchopneumonia in sheep and perinatal disease in sheep, goats and cattle has been ascribed to it (Schutte *et al*., 1976). *C. burnetii* has, however, recently also been reported to lead mainly to reproduction disorders such as abortion and stillbirths, weak calf delivery, metritis and

infertility with associated economic impact for the herd (Guatteo *et al*., 2011). Variability in the clinical expression may be caused by host factors, the extent of exposure, or bacterial virulence factors (Raoult *et al*., 2005).

4.1.3. Diagnosis

Immunodiagnostic procedures that detect anti-*C. burnetii* antibodies in serum are the usual means of diagnosis more especially in diagnosis of human infections. IFAT is the preferred method for both acute and chronic forms of the disease (Engelkirk & Duben-Engelkirk, 2008). PCR has fast become a method of choice when it comes to diagnosis of this pathogen and several nucleic acid based assays have been developed to date, these include Restriction Fragment Length Polymorphism (RFLP) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), targeting the 16S/ 23S gene, *ComI* gene, *icd* gene etc., some more expensive than others such as MLVA and Micro-array typing techniques (Massung *et al*., 2012).

4.1.4 Treatment

Vaccination may be necessary for people at high risk, such as laboratory workers, abattoir workers, veterinarians, individuals with special conditions such as cardiac valve prosthesis and immunocompromised individuals. Earlier vaccines were rather crude and consisted of formalin-killed and ether-extracted *C. burnetii* containing 10% yolk sac, although with time newer more effective vaccines were developed. The most thoroughly tested Q-fever vaccine in use today is “Q-Vax” (Waag, 2007).

C. burnetii is reportedly sensitive to the tetracyclines. Doxycycline is possibly the most active tetracycline against C. *burnetii.* Current Q-fever postexposure prophylaxis (PEP) guidelines for the general population are 100 mg of doxycycline (or 500 mg tetracycline 2×/day for 5 days), started 8 - 12 days postexposure (Moodie *et al*., 2008).Treatment with this drug should continue for at least three days after remission of fever. There have been reports of the successful treatment of Q-fever with cotrimoxazole and erythromycin (Aitken *et al*., 1987). Rifampin and sulfamethoxazole-trimethroprim are recommended for treatment of endocarditis (Engelkirk & Duben-Engelkirk, 2008).

4.2. Aims of the study

In this chapter the aim was to determine by PCR the rate of infection with *Coxiella burnetii* amongst ticks collected from livestock (cattle, dogs, sheep and goats) as well as from the vegetation in the Eastern Cape, Free State, KwaZulu-Natal, Mpumalanga and North West Provinces of South Africa.

4.3. Materials and methods

4.3.1. Study area

Ticks were collected from dogs, cattle, goats, sheep and incidentally four horses and four cats from farms in the Free State (FS), KwaZulu-Natal (KZN), Eastern Cape (EC), North West (NW) and Mpumalanga (MP) Provinces of South Africa. In KZN samples were collected from Wesselsneck [S 28° 20' 0.52" E 030° 02' 49.1"], Gcinalishona/Mjindini [S 28° 39' 00.5" E 030° 06' 56.3"], eTholeni [S 28° 25' 39.9" E 30° 13' 04.3"] and uMsinga Mountainview dip site [S 28° 41'43.1" E 030° 16'14.6"]; in FS, Hooningkloof [S28° 30. 666' E 028° 42.701'], a farm situated at a livestock-wildlife interface, Sekoto farm [S28° 36.094' E 028° 49.013'], Seotlong Hotel and Agricultural School [S 28° 35' E 28° 50'] and in Kestell [S 28° 20' E 28° 38']; in EC, at Amathole District Municipality [S 32°48' 30" E 27° 01' 49"]; in NW at a private Veterinary Clinic in Mafikeng [S 25° 51′ 0″ E 25° 38′ 0″] and in MP at Kameelpoort-KwaMhlanga [S 25° 46′ 6.3″ E 29° 28′ 42″], refer to Figure 2.4. for the Map of South Africa.

4.3.2. DNA extraction from ticks

The ticks were surface sterilized twice with 75% ethanol then washed once in Phosphate Buffered Saline (PBS) solution before they were dissected and gutted (the engorged) or crushed whole (the males) in individual sterile Eppendorf tubes

(Hamburg, Gemany) then later preserved in PBS and then stored at -34 °C until further use. Ticks of the same species collected from one animal were grouped as one sample for DNA extraction. Some ticks laid eggs within the collection vials, these were also washed in PBS, spun down at full speed (16 000 xg) in the microcentrifuge then crushed and stored as described above. DNA was extracted from tick extracts and eggs using the salting out method as described by Miller *et al*., (1988). DNA was dissolved in 50 - 200 µl (depending on the size of the pellet) of double distilled water and extracts were stored at -34 °C.

4.3.3. Polymerase chain reaction

The experimental procedures were carried out in the laboratories of the Parasitology Research Program of the University of the Free State (UFS) - Qwaqwa Campus, South Africa and the Research Center for Zoonosis Control (CZC) of Hokkaido University, Japan. The extracted DNA from whole ticks together with an additional 55 DNA samples (obtained from dog ticks in the Free State, Phuthaditjhaba area) from a previous study (Leodi, 2010) were subjected to PCR amplification using oligonucleotide sequences; CB-1: 59 ACT CAA CGC ACT GGA ACC GC and CB-2: 59 TAG CTG AAG CCA ATT CGC C (Parola and Raoult, 2001), targeting the superoxide dismutase enzyme gene of *C. burnetii* bacterium with an expected product size of 257 bp. Thereafter electrophoresed on 1.5% agarose gel stained with GelRed and/or ethidium bromide and size fractionated using a 50/100 bp DNA ladder and then photographed under UV transillumination (Figure 4.1.). PCR was performed using AmpliTaq Gold® 360 Master Mix (Applied Biosytems, U.S.A.) with the following cycling conditions: Initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 57±5°C for 30 s and extension at 72 °C for 60 s/kb followed by final extension at 72°C for 7 min and held at 4°C ∞, using CZC's Veriti®Thermocycler (Applied Biosystems, U.S.A.) and the PRP's Multigene OptiMAx Thermal Cycler (Life Technologies Corporation, U.S.A.).

4.3.4. Purification and sequencing

For experiments conducted at CZC, the positive PCR products were purified using USB ExoSAP-IT Enzymatic PCR Product cleanup (Affymetrix, Japan) by incubating at 37°C followed by 80°C. Cycle sequencing reactions were performed using an ABI Prism Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, U.S.A.) on an ABI 3130 Automated Sequencer. For experiments conducted at PRP the PCR products were sent to Inqaba Biotechnological Industries (Pty) Ltd (R.S.A.) for sequencing. The sequence data of the PCR products were analyzed using the BLAST 2.0 program (National Center for Biotechnology Information, Bethesda, Maryland, U.S.A.; http://www.ncbi.nlm.nih.gov/blast/) for homology searching. Sequences were analyzed and aligned using BioEdit Sequence Alignment Editor © 1997 - 2004 (Tom Hall Isis Pharmaceuticals, Inc).

4.4. Results

A total of 776 DNA samples were obtained from whole ticks when combined with 55 DNA samples (obtained from dog ticks in the Free State, Phuthaditjhaba area) from a previous study (Leodi, 2010). From the obtained DNA samples a total of 527 samples were screened for the presence of *C. burnetii* bacterium. The results of the gel electrophoresis with a product size of 257 bp are shown in Figure 4.1. The overall infection rate with *C. burnetii* was 16% (83/527) for all the screened samples from the five sampled provinces. The summary of the infection rates of ticks with *C. burnetii* for the five sampled Provinces is given on Table 4.1.

The overall prevalence of infection with *C. burnetii* in individual Provinces was obtained to be 13% for KZN (n = 164), 27% for FS (n = 207), 31% for NW (n = 16), and 6% for MP (n = 17) when all tick samples of all groups of animals as well as samples collected from the vegetation were combined. In the EC Province all the screened DNA samples (n = 123) were negative for *C. burnetii* DNA.

Amongst the dog- and cat-tick DNA, 41% overall infection with *C. burnetii* (n = 128) was observed across the four sampled Provinces with highest infection rate of 55% in FS, KZN with 32%, NW with 31% and lastly the MP dog-tick sample being the only one that tested positive in that area.

The only goat-tick samples positive for the bacterium were those from the FS (20%; n = 20) whereas EC (n = 29) and MP (n = 13) DNA samples tested negative. Thirty-two percent (n = 34) sheep-ticks carried the pathogen. Prevalence of infection of cattle-ticks with *C. burnetii* was, 3% for KZN (n = 108), 13% for FS (n = 82) and 0% for EC (n = 94). Tick samples collected from the vegetation (n = 12), horses (n = 4) and cats (n = 4) were all negative when screened for the presence of *C. burnetii* DNA.

Of the total positive samples (83/527), the highest infection rate (35.4%) was obtained amongst the unidentified dog-tick samples, followed by 26% *Rhipicephalus sanguineus* and 23% *R. evertsi evertsi*. Whilst the rest of the positive samples were from 6% of *Rhipicephalus* spp., 4.9% *R. decoloratus*, 2.4% *Haemaphysalis elliptica*, 1.2% *A. hebraeum* and 1.2% *Rhipicephalus* spp. crushed-egg DNA.

The *C. burnetii* PCR positive samples were sequenced and they revealed a 96 - 99% maximum identity with *Coxiella burnetii*_CbUKQ154 complete genome (GenBank accession number: CP001020) isolated in the U.S. and *Coxiella burnetii* R.S.A. 331 complete genome (GenBank accession number: EU448153.1) when using NCBI, BLAST.

4.5. Discussion and conclusions

Many species of ticks are found infected with *C. burnetii* (about 40% natural infection) and a significant number of viable organisms are present in their feces (Kelly *et al*., 1993, Psaroulaki *et al*., 2006). Q-fever has become a public health problem with 2,357 human cases notified in 2009 alone in the Netherlands. Its incidence though is generally unknown and may be underestimated (Fournier *et al*., 1998). In South Africa *C. burnetii*

infections have been demonstrated only serologically in cattle, goats and sheep (Schutte *et al*, 1976; Kelly *et al*., 1993) and also in wild dogs at Kruger National Park (van Heerden *et al*., 1995), making the current study the first of its kind with an overall prevalence of 16% in all tick species obtained across the five sampled provinces. In a similar study in Cyprus it was detected from 11/141 (7.8%) *R. sanguineus* and *Hyalomma* ticks through PCR (Psaroulaki *et al*., 2006). In contrast to a similar study in rural Senegal (Mediannikov *et al*., 2010), ticks found on some groups of animals (cattle, dogs, goats, and sheep) and not in others (cats and horses) were infected with *C. burnetii* in the current study. Mediannikov and colleagues reported a rate of *C. burnetii* infection of ticks ranging between 10.2 - 37.6%, similarly in the current study the infection rates ranged between 1.2 - 35.4% amongst ticks. The distribution of *C. burnetii* in the country is reported to coincide with the yellow dog tick, *H. elliptica*, which is recognized as a carrier tick in South Africa and the blue tick, *R. decoloratus* (Gummow *et al*., 1987). In the current study however all the tick species identified carried the pathogen, suggesting that they may also act as reservoirs.

The seroprevalence in domestic dogs has been reported to be 15% in Zimbabwe, a neighboring country (Kelly *et al*., 1993), 21.8% in Queensland, Australia and (Cooper *et al*., 2011) and 5.5% in Iraq (Havas and Burkman, 2011). In the latter study Havas and Burkman, (2011) did a comparative survey in the period 2007 to 2008 on the incidence of infection of feral dogs versus Military Working Dogs (MWD) in Iraq and this revealed a seroprevalence of 5.5% in feral dogs and none of the MWDs seroconverted. In that study MWDs were less of a threat as sentinels for human populations whilst feral dogs although rarely come into contact with humans posed a risk for human infections. In this study however, a notably high infection rate (41%) amongst dog-ticks was observed thus exacerbating the risk of transmission to the pet owners as dogs usually have a much more intimate relationship with their owners when compared to the other groups of animals. It has been proposed that in some regions pets may actually be more commonly implicated than domestic ruminants in the transmission of this disease to humans (Cooper *et al*., 2011).

Seropravelence of *C. burnetii* amongst cats and cattle in South Africa is reported to be 2% and 8 - 26.5% respectively (Gummow *et al*., 1987; Frean and Blumberg, 2007). The prevalence of *C. burnetii* when examined from fetal and placental tissue impressions as well as from serum of cattle and sheep were 81% and 93% respectively in 12 South African farms A - L, representative of about four Provinces (Schutte *et al*., 1976). When compared to that of cattle in Zimbabwe (39%), the 5% prevalence obtained here is significantly low (Matthewman *et al*., 1997). In the country Q-fever is reported to be serologically common amongst human populations (Schutte *et al*., 1976). Other countries like Senegal and Zimbabwe have demonstrated seropositivity of 3.7% and 37% respectively amongst human populations (Matthewman *et al*., 1997; Frean and Blumberg, 2007; Mediannikov *et al*., 2010). The majority of the cases of Q-fever in the 50's were reported mainly among immigrants (Schutte *et al*., 1976), this could have been because of a high likeliness of the presence of an endemic situation. The disease was kept at levels below threshold amongst livestock populations more especially in cattle and because of this low incidence the threat of transmission to humans remained very minimal thus eliminating the need for vaccination (Gummow *et al*., 1987). It is my suspiscion that this is also the case with the current findings as they depict a very low overall infection rate of 16%. Gummow *et al*., (1987) further suggest that the incidence can be lowered even further by making farmers and all stakeholders aware that *C. burnetii* is widespread throughout the country and that management practices, such as proper handling and destruction of afterbirth, pasteurization of milk and control of ticks can help effect this.

Nevertheless, it is crucial to look at a disease like Q-fever from all angles e.g. from this study it can be directly inferred by determining the prevalence of *C. burnetii* from ticks, that the animals they infested would also be positive for the bacterium. From this it can be deduced that dogs, goats, cattle and sheep may transmit Q-fever to humans by other forms other than tick bites e.g. infected birth fluids and membranes, ingestion of raw milk, inhalation of airborne *C. burnetii* particles¸ feces and by urine as other studies have reported (Schutte *et al*., 1976; Rhode *et al*., 1993; Matthewman *et al*., 1997; Capuano *et al*., 2004).

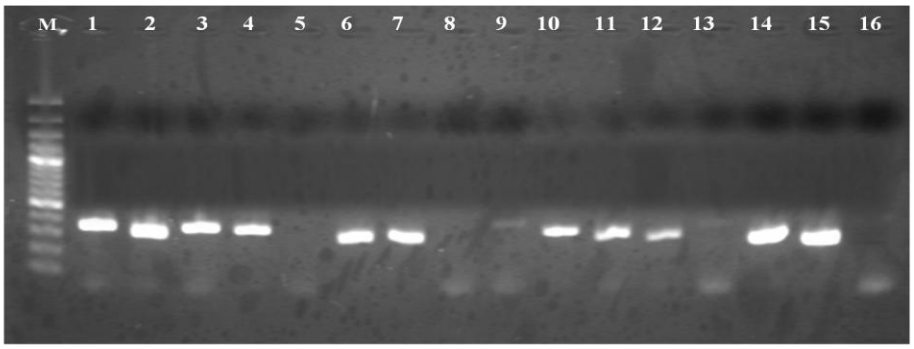

Figure 4.1. Gel eletrophoresis of *C. burnetii* amplified PCR product with amplicon size of 257 bp, M: DNA ladder (50 bp) ,1-4;6-7;10-12 & 14: positive samples, 5; 8-9; 13: negative samples,15: positive control,16: negative control.

Table 4.1. Prevalence of *Coxiella burnetii* in ticks across the five sampled Provinces

Study animals		KwaZulu-Natal	Free State	Eastern Cape	North West	Mpumalanga	Overall % (+) per animal population
Dogs							
	Tot tested	56	55	-	16	4*	**131**
	No. Pos	18	30	-	5	1	**54**
	%	32	55	-	31	25	**41**
Sheep							
	Tot tested	-	34	-	-	-	**34**
	No. Pos	-	11	-	-	-	**11**
	%	-	32	-	-	-	**32**
Goats							
	Tot tested	-	20	29	-	13	**62**
	No. Pos	-	4	0	-	0	**4**
	%	-	20	0	-	0	**6.5**
Cattle							
	Tot tested	108	82	94	-	-	**284**
	No. Pos	3	11	0	-	-	**14**
	%	3	13	0	-	-	**5**
Total Screened		164	207[υ]	123	16	17	**527[υ]**
Overall (+) samples		21	56	0	5	1	**83**
Overall % (+) per Province		**13**	**28**	**0**	**31**	**6**	**16**

*** number includes 1 dog and 3 cat tick-DNA samples, [υ] includes 12 tick-DNA samples collected from the vegetation from the FS Province and 4 horse-tick DNA, not shown on the table**

Chapter 5: Molecular detection of *Rickettsia* species from ticks collected from livestock and companion in five Provinces of South Africa.

5.1. Introduction

Tick-borne rickettsioses are caused by obligate intracellular bacteria belonging to the spotted fever group (SFG) of the genus *Rickettsia* within the family *Rickettsiaceae* (Raoult *et al*., 2001; Parola *et al*., 2005; Frean and Blumberg, 2007). They include typhus, spotted fever group (SFG) rickettsiosis, and scrub typhus (Raoult *et al*., 2001; Parola, 2006; Roch *et al*., 2008). Since its discovery in 1919 by Wolbach, *Rickettsia rickettsii* remained for approximately 90 years as the only conclusive tick-borne rickettsial pathogen inflicting disease in man in the Western Hemisphere (Parola *et al*., 2005). *R. conorii* infection was once considered the only tick-transmitted rickettsial infection in Europe and Africa, causing both Mediterranean spotted fever (MSF) and African tick-bite fever (ATBF) (Raoult *et al*., 2001). It follows that it was only in 1996 that a strain distinct from other previously described rickettsiae but similar to a bacterium previously isolated from *A. variegatum* by the likes of Angus Pijper in the 1930s was characterised. This strain was subsequently named *R. africae* and was confirmed to be the causative agent of ATBF in southern Africa and Guadeloupe (Kelly *et al*., 1996). SFG rickettsioses are important emerging tick-borne human infections worldwide and are the second most common cause of fever among travelers returning from the developing world after malaria but before dengue and typhoid fever. Among SFG rickettsias, ATBF is reportedly the most common rickettsioses in sub-Saharan Africa (Raoult *et al*., 2001; Portillo *et al*., 2007; Roch *et al.*, 2008).

The first human case of infection with ATBF was described in 1992 as occurring in Zimbabwe (Ndip *et al*., 2004; Portillo *et al*., 2007). In South Africa, there are three rickettsias associated with the rickettsiosis disease i.e., *R*. *africae*, *R. conorii* and

recently *R. aeschlimanii* has been reported (Pretorius and Birtles, 2004; Rutherford *et al.*, 2004). In Limpopo Province several years back, a case of a construction worker who had symptoms similar to those caused by *R*. *mongolitimonae* (encountered in Mongolia in 1991) which was later confirmed by sequencing of the r*OmpA* fragment of *Rickettsia* spp. that revealed 99% similarity with the corresponding r*OmpA* fragment of *R. mongolitimonae* was reported, although not many cases have come up since then (Pretorius and Birtles, 2004; Vorou *et al*., 2007).

The vector for *R. africae* is specific, *A. variegatum* is the tick transmitting *R. africae* in the sub-Saharan area and *A. hebraeum* is found more specifically in South Africa (Raoult *et al*., 2001; Parola, 2006; Portillo *et al*., 2007; Althaus *et al*., 2010). MSF is reportedly transmitted by *R. sanguineus* in southern Europe (Parola *et al.*, 2005). Some cases have also been described in the West Indies, probably transmitted by *Amblyomma* ticks, as *R. microplus* and *R*. *sanguineus* ticks did not show *R*. *africae* DNA when subjected to PCR in previous studies in Guadeloupe. *R*. *africae* is maintained transtadially and transovarially in *A*. *hebraeum* and the organism is transmitted at each feeding stage (Kelly, 2006), while there is no evidence of transovarial transmission of *R. conorii* in *R. sanguineus* ticks, nonetheless vertical transmission has been recently described (Socolovschi *et al*., 2009). Cattle and goats are the most important domestic hosts, although sheep, horses, donkeys, giraffes, buffaloes, antelopes and warthogs are also frequently attacked (Althaus *et al*.,2010). ATBF appears to be acquired after travel in the countryside and through contact with ticks that parasitize cattle or wild animals; especially *Amblyomma* ticks (Kelly *et al*., 1996; Fournier *et al*., 1999; Raoult *et al*., 2001). However, little attention has been given to the epidemiology of rickettsial diseases in areas where wildlife, livestock, and humans interact and this is quite frequent in Africa (Macaluso *et al*., 2003).

5.1.1. Aetiology and pathogenesis

SFG rickettsias are approximately 0.3 by 1.0 µm and have a cell wall typical of Gram-negative bacteria (Walker, 2004). Gimenez-stained cells infected with *R. africae* have rod-shaped intracellular organism 0.3 - 0.5 by 0.9 -1.6 µm. They appear to occur freely in the cytoplasm under electron microscopy, have an outer slime layer and a trilaminal

cell wall (Kelly *et al*., 2006). They invade the endothelial cells at the cutaneous portal of entry, where they multiply and proliferate and cause a focus of dermal and epidermal necrosis, an eschar. They are carried via the blood stream where they infect endothelials lining the blood vessels. They continue to multiply and burst to adjacent cells, thereafter activating the immune, inflammatory and coagulation system of the host. Progressive dissemation and injury of endothelial cells leads to presentation of clinical signs in the patient (Walker, 2004).

5.1.2. Clinical signs

The National Travel Health Network and Center (NaTHNaC, http://www.nathnac.org/travel/factsheets/pdfs/rickettsial.pdf) in 2004 reported that the spotted fever group of rickettsial diseases are clinically similar, after inoculation from a bite, a febrile illness develops following a short incubation period of a few days that may persist for up to two weeks if untreated. Common nonspecific laboratory abnormalities in rickettsioses include mild leukopenia, anemia, and thrombocytopenia although hyponatremia, hypoalbuminemia, and hepatic and renal abnormalities may also occur (Parola *et al*., 2005). The spectrum of rickettsial infections in Africa ranges from mild to severe. *R. conorii* causes a moderate to severe illness with a case-fatality rate of approximately 3%. Disease is most often characterized by a single eschar and a generalized maculopapular rash that may involve the palms and the soles (Rutherford *et al*., 2004). In ATBF, multiple eschars may be present, but the generalised rash is frequently absent. Lymphadenopathy in the region of the bite and oedema are more common symptoms (Brouqui *et al*., 1997). Livestock when infected with *R*. *africae*, show no clinical or laboratory signs of disease. They are, however, intermittently rickettsemic and may then be sources of infection for ticks (Caruso *et al*., 2002; Kelly, 2006). It is worth noting though, that rickettsiae were first identified in ticks before being recognized as human pathogens (Matsumoto *et al*., 2007).

5.1.3. Diagnosis

Serological tests are the most frequently used and widely available methods for diagnosis. The Weil-Felix agglutination test, the oldest assay, is based on the detection

of antibodies to various *Proteus* antigens that cross-react with rickettsiae. It is said to lack specificity and sensitivity however it continues to be used in many developing countries and in countries with higher level of technical development (Parola *et al*., 2005; Frean and Blumberg, 2007; Frean *et al*., 2008). Serology should be considered as an initial method to recognize and diagnose rickettsial diseases, but sole use of this method particularly if no rickettsiae have been previously isolated or detected in the considered area is highly discouraged (Parola *et al*., 2005) .

Serological tests such as cross-absorption (CA) techniques, western blotting (WB) and imnnunoflourescent assays (IFA) can be used in reference centers to help to differentiate rickettsial infections by antibody evaluation as they are highly specific (Fournier *et al*., 1999; Raoult *et al*., 2001, Parola *et al*., 2005). However, serologic cross-reactions are common when diagnosing serum samples using microimmunofluorescence assays (Fournier *et al*., 1999). Rickettsiosis can also be diagnosed from skin biopsies, cell or tissue culture, polymerase chain reaction (PCR) assays, and “suicide” PCR assays (Raoult *et al*., 2001; Ndip *et al*., 2004; Parola *et al*., 2005; Parola, 2006; Roch *et al*., 2008). In addition *R. africae* can be distinguished from other SFG rickettsioses by SDS-PAGE (Kelly *et al*., 1996) as well as other genomic methods such as RLFP analysis of PCR amplification products.

5.1.4. Treatment

TBF can be life-threatening in patients of any age group, treatment with the most effective agent, doxycycline, is the recommended therapy for all patients. Chloramphenicol and the 4-fluorinated quinolones show *in vitro* activity, but clinical data on efficacy is limited. It is the only available option in critically ill patients unable to tolerate oral medication, as parenteral tetracycline is unavailable in South Africa. Erythromycin has poor efficacy and there is insufficient clinical data to recommend the new macrolides such as clarithromycin and azithromycin, although they may have a place in supplementing initial doxycycline treatment (Frean and Blumberg, 2007; Frean *et al*., 2008).

5.2. Aims of the study

The main aim of this chapter was to detect/characterize the prevalence of *Rickettsia* species amongst ticks collected from livestock (cattle, dogs, sheep and goats) as well as from the vegetation in the Eastern Cape, Free State, KwaZulu-Natal, Mpumalanga and North West Provinces of South Africa.

5.3. Materials and methods

5.3.1. Study area

Ticks were collected from dogs, cattle, goats, sheep and incidentally four horses and four cats and a few from the vegetation from farms in the Free State (FS), KwaZulu-Natal (KZN), Eastern Cape (EC), North West (NW) and Mpumalanga (MP) Provinces. In KZN samples were collected from Wesselsneck [S 28° 20' 0.52" E 030° 02' 49.1"], Gcinalishona/Mjindini [S 28° 39' 00.5" E 030° 06' 56.3"], eTholeni [S 28° 25' 39.9" E 30° 13' 04.3"] and uMsinga Mountainview dip site [S 28° 41'43.1" E 030° 16'14.6"]; in FS, Hooningkloof [S28° 30. 666' E 028° 42.701'], a farm situated at a livestock-wildlife interface, Sekoto farm [S28° 36.094' E 028° 49.013'], Seotlong Hotel and Agricultural School [S 28° 35' E 28° 50'] and in Kestell [S 28° 20' E 28° 38']; in EC, at Amathole District Municipality [S 32°48' 30" E 27° 01' 49"]; in NW at a private Veterinary Clinic in Mafikeng [S 25° 51′ 0″ E 25° 38′ 0″] and in MP at Kameelpoort-KwaMhlanga [S 25° 46′ 6.3″ E 29° 28′ 42″], refer to Figure 2.4. for the Map of South Africa.

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use. Ticks of the same species collected from one animal were grouped as one sample for DNA extraction. Some ticks laid eggs within the collection vials, these were also washed in PBS, spun down at full speed (16 000 xg) in the microcentrifuge then crushed and stored as described above. DNA was extracted from tick extracts and eggs using the salting out method as described by Miller *et al*., (1988). DNA was dissolved in 50 - 200 µl (depending on the size of the pellet) of double distilled water and extracts were stored at -34 °C.

5.3.3. Polymerase chain reaction

The experimental procedures were carried out in the laboratories of the Parasitology Research Program of the University of the Free State (UFS) - Qwaqwa Campus, South Africa and the Research Center for Zoonosis Control (CZC) of Hokkaido University, Japan. The extracted DNA (n=721) from whole ticks together with an additional 55 DNA samples (obtained from dog ticks in the Free State, Phuthaditjhaba area) from a previous study (Leodi, 2010) were subjected to PCR amplification using CS-78: GCA AGT ATC GGT GAG GAT GTA and CS-323: GCT TCC TTA AAA TTC AAT AAA TC primers to amplify a 401 bp product of the *gltA* (citrate synthase) gene of *Rickettsia* (Ndip *et al*., 2004) as well as RpCS-877p: GGG GAC CTG CTC ACG GCG G and RpCS-1273r: CAT AAC CAG TGT AAA GCTG , amplifying the r*OmpA* and *gltA* genes of *Rickettsia* spp. (Inokuma *et al.,* 2008). Thereafter electrophoresis of the PCR produts on 1.5% agarose gel stained with GelRed and/or ethidium bromide and size fractionation using a 100 bp DNA ladder was conducted (Figure 5.1). PCR was performed using the AmpliTaq Gold® 360 Master Mix (Applied Biosytems, U.S.A.) with the following cycling conditions: Initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 60 s/kb followed by final extension at 72°C for 7 min and held at 4°C ∞, using CZC's Veriti®Thermocycler (Applied Biosystems, U.S.A.) and the PRP's Multigene OptiMAx Thermal Cycler (Life Technologies Corporation, U.S.A.).

5.3.4. Purification and sequencing

For experiments conducted at CZC, the positive PCR products were purified using USB ExoSAP-IT PCR Product cleanup (United States Biochemical Corporation, Cleveland, OH) by incubating at 37°C followed by 80°C, cycle sequencing reactions were performed using an ABI Prism Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI 3130 DNA Sequencer. For experiments conducted at PRP the PCR products were sent to Inqaba Biotechnological Industries (Pty) Ltd (R.S.A.) for sequencing. The sequence data of the PCR products were analyzed using the BLAST 2.0 program (National Center for Biotechnology Information, Bethesda, Maryland, U.S.A.; http://www.ncbi.nlm.nih.gov/blast/) for homology searching. Sequences were analyzed using BioEdit Sequence Alignment Editor © 1997 - 2004 (Tom Hall Isis Pharmaceuticals, Inc).

5.4. Results

Primer set RpCS-877 and RpCS-1273r were used to detect for *Rickettsia* spp. from all dog-, cat- and horse-tick DNA samples and also from a pool of cattle-tick DNA samples (FS and KZN). Sequencing of the partial *gltA* and r*OmpA* genes revealed mainly the presence of *R. africae* and *R. conorii*. Infection of ticks with *Rickettsia* spp. using these primers was 28% (n = 82) in KZN, 37% (n = 119) in FS, 38% (n = 16) in NW, and it was not detected in ticks collected from MP, whilst EC was excluded in the study. From the dog and cat samples the bacteria were detected in 52% (n = 54), 21% (n = 52) and 38% (n = 16) of the FS, KZN and NW tick DNA samples, but were absent in tick samples from MP (n = 4) giving an overall 37% prevalence of infection across all sampled Provinces for this group of animals. Among the identified tick species infesting dogs and cats the pathogens were present exclusively in *R. sanguineus*. The overall infection rate of the cattle-tick DNA pool with *Rickettsia* spp. was 27% with FS (n = 65) registering 22% and KZN (n = 30) 40% individually. One of the four horse-tick DNA samples screened positive for *Rickettsia* species DNA. All in all the overall prevalence of *Rickettsia* species for all sampled animals in all Provinces using this set of primers was 32% (71/221).

The prevalence of infection of ticks with *Rickettsia* spp. using primer set CS-78 and CS-323 for PCR screening was 23% (102/439) for all screened samples. The DNA samples screened with these primers included those of ticks collected from cattle, goats, sheep and vegetation only. In the sampled Provinces individually the incidence of *Rickettsia* spp. was 45%, 24%, 17% and 0% for KZN (n = 75), EC (n = 122), FS (n = 229) and MP (n = 13) respectively. The overall infection rates per sampled group of animals were as follows: 33%, 26% and 20% in ticks collected from sheep (n = 83), goats (n = 65) and cattle (n = 279) respectively. From ticks collected from the vegetation the infection with *Rickettsia* spp.was 16% (n = 12) however they tested negative when using the RpCS-877p and RpCS-1273r primers above. The prevalence of infection was 33% (n = 83) in FS when sheep ticks were screened for the pathogen, whilst in the other Provinces tick samples were not collected from sheep. Amongst the goats an incidence of 26% (n = 23) and 38% (n = 29) was recovered from the FS and EC provinces but the bacterium was not detected in MP samples (n = 13). KZN led with an infection rate of 45% (n = 75), followed by 19% (n = 93) for EC, then 4% (n = 111) for FS in ticks collected from cattle. Of the positive samples the bacterium was isolated from 36% *Rhipicephalus* spp., 35% *R. evertsi evertsi*, 20% *A. hebraeum*, 2.0% of both *R. decoloratus* and *R. appendiculatus* tick species, 5.0% from unidentified ticks and 0.9% from eggs of *Rhipicephalus* spp. ticks. Sequences generated had a 96 - 100% maximum identity with previously published sequences of *R. africae*, *R conorii*, *R. prowazekii* and *Rickettsia* spp.' *gltA* and r*OMP*A gene sequences (GenBank Accession no: HQ335125.1, HM538186.1, U59733.1, CP001612.1, AE006914.1, AF178035). The overall infection rate of ticks collected from the five Provinces with *Rickettsia* species for both sets of primers combined was obtained to be 26% (173/660). The summary of infection rate of ticks collected from livestock in the five Provinces with *Rickettsia* species is given on Table 5.1.

5.5. Discussion and conclusions

R. africae was present in ticks of all groups of animals tested and it was isolated in most tick species in the collection with varying rates of infection. It was detected from *A. hebraeum* ticks as expected, others such as *Rhipicephalus* species may have been acting as reservoirs as previous studies have suggested. As an example *R. africae* has been isolated from *R. decoloratus* in Botswana but it was assumed that the pathogen may have been acquired from the blood meal of the oryx from which it had been feeding, however this tick species has not been demonstrated as being able to transmit this pathogen (Portillo *et al*., 2007). While the role of *Rhipicephalus* ticks in the transmission of *R. africae* is not clear, their infection may be dependent on coincidental transmission by other species, such as *A. hebraeum* (Macaluso *et al*., 2003). *R. conorii* on the other hand was much more frequent amongst the dog ticks exclusively in *R. sanguineus*.

There is an obvious increase in reported cases of *Rickettsia* species and, although *R. africae* is widely distributed in Africa with serosurveys of up to 100% infection in humans, reports of ATBF in indigenous people are unexpectedly rare. According to Kelly, (2006) this finding could be because indigenous people acquire the infection at a generally young age, when the disease might be very mild or subclinical and medical attention is not sought. Furthermore because inoculation eschars are difficult to see in pigmented skin and the fact that definitive diagnosis of ATBF requires sophisticated diagnostic tests not available in developing countries, the infection is often missed. Another definitive reason to this is that, although *R. sanguineus* (the vector of *R. conorii*) lives in peridomestic environments shared with dogs (e.g., kennels, yards, and houses) it has a relatively low affinity for humans and low infection rates with *R. conorii* (10%) when compared to 75% *R. africae* infection rate in *Amblyomma* ticks (Fournier *et al*; 1999; Prabhu *et al*., 2011), therefore because of these circumstances, cases of MSF are sporadic and typically encountered in urban areas (Parola *et al*., 2005; Frean and Blumberg, 2007). An overall 37% infection rate of ticks of dogs with *Rickettsia* spp. obtained in this study is very high, when compared to the above stats, therefore the potential risk of contraction of the pathogen by the pet owners more especially is

increased, however *Ripicephalus* spp*.,* unlike *A. hebraeum* which actively hunt for their host, rarely ever bite humans (Fournier *et al*., 1999; Macaluso *et al*., 2003; Althaus *et al*., 2010).

Although *R*. *africae* has been detected in *Amblyomma* ticks and patients from >14 African countries, the reported case incidence rate of ATBF is only 60 - 80 per 100,000 patients each year in our neighboring country, Zimbabwe (Frean *et al*., 2008; Althaus *et al*., 2010). Evidence is on the other hand accumulating that tick-borne rickettsioses are underreported and underappreciated cause of illness in sub-Saharan Africa and most reported cases are as a result of an outbreak (Raoult *et al*., 2001; Rutherford *et al*., 2004). ATBF, however, is quite common in international travelers with up to 11% of visitors to disease-endemic areas having evidence of infection. Examples include among others, travelers from Japan, Taiwan, Netherlands, France and Switzerland who contracted the disease after being bitten by ticks in South Africa whilst on safari tours and at several of R.S.A.'s tourist attraction sites (Raoult *et al*., 2001; Delfos *et al*., 2004; Althaus *et al*., 2010; Roch *et al*., 2008; Fujisiwa *et al*., 2012). Not much work has been done in South Africa with regards to rickettsioses, however, ticks like *A. hebraeum* have the potential of transmitting rickettsias of medical importance. Consequently they could pose a serious threat to humans as such pathogens, some who's zoonotic potential not yet demonstrated, continue to be on the rise (Pretorius and Birtles, 2004) and could potentially damage the country's tourism industry if nothing is done about it.

Rutherford *et al*., (2004) explicitly report a fatal case of infection with SFG rickettsiosis in Kenya where a woman reportedly died of the infection by *Rickettsia* as a result of being initially misdiagnosed as having malaria. This highlights the fact that, although people in endemic areas may not frequently display clinical signs for the diseases, *Rickettsia* should be considered as part of routine screening in patients with fever and that it is important to determine the prevalence and distribution of rickettsiae in the region.

Although this study demonstrated *R. africae* and *R. conorii* infection in ticks by PCR and DNA sequence analysis, the best way to confirm the infection amongst populations is by

detecting the pathogen from blood or tissue samples of suspected patients; this remains to be achieved in South Africa. It could be however speculated from this study that sheep, cattle, horses, goats and dogs act as reservoirs of this infection in the country . Other issues that should be addressed are a full description of the clinical spectrum of these rickettsioses in South African patients and the determination of risk factors for severe illness. Therefore, infection especially with SFG rickettsias must be taken into account in the differential diagnosis of suspected patients returning from South Africa, having had a recent history of tick exposure. Additional surveillance is needed to increase physician and public awareness of the potential risk of disease transmission to humans from exposure to ticks.

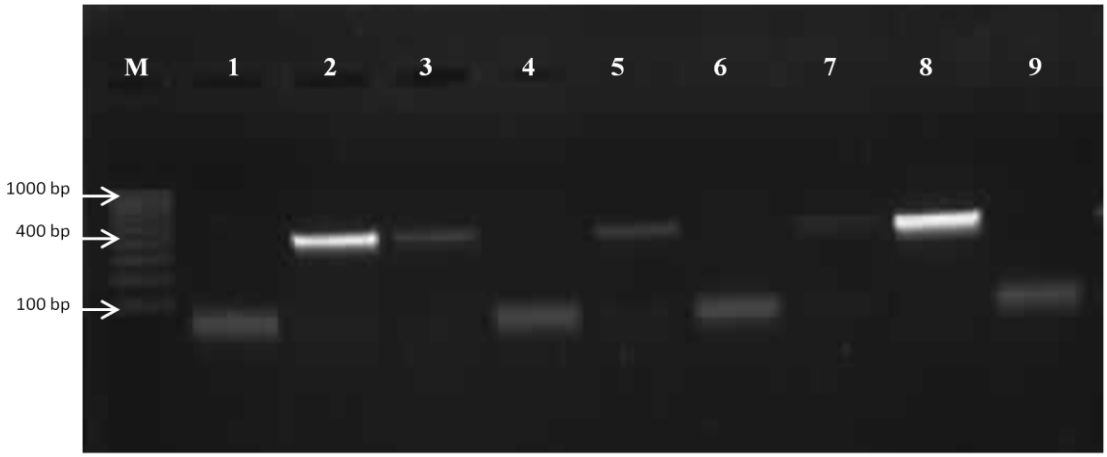

Figure 5.1. Gel eletrophoresis of *Rickettsia* spp.amplified PCR product with amplicon size of 401 bp, M:DNA ladder (100bp), 1;4;6: negative samples, 2;3;5;7: positive samples, 8: positive control (*R. helvetica*), 9: negative control (DDH_2O).

Table 5.1. Prevalence of *Rickettsia spp.* in ticks across the five sampled provinces

Sampling site and study animals		***Rickettsia* spp. (CS)**			***Rickettsia* spp. (RpCS)**		
Province	**Study group**	**+ve**	**Total screened**	**Overall %**	**+ve**	**Total screened**	**Overall %**
Free State	**Dogs**	-	-	-	28	54	*52*
	Cattle	4	111	*4*	14	65	*22*
	Goats	6	23	*26*	-	-	-
	Sheep	27	83	*33*	-	-	-
	Flagged	2	12	*17*	-	-	-
	Total	***39***	***229***	***17***	***42***	***119***	***35***
North West	**Dogs**	-	-	-	6	16	*38*
	Total	-	-	-	***6***	***16***	***38***
KZN	**Dogs**	-	-	-	11	52	*21*
	Cattle	34	75	*45*	12	30	*40*
	Total	***34***	***75***	***45***	***23***	***82***	***28***
EC	**Cattle**	18	93	*19*	-	-	-
	Goats	11	29	*38*	-	-	-
	Total	***29***	***122***	***24***	-	-	-
MP	**Dogs**	-	-	-	0	4	*0*
	Cats	-	-	-	-	-	-
	Goats	0	*13*	*0*	-	-	-
	Total	***0***	***13***	***0***	***0***	***4***	***0***
Overall prevalence		***102***	***439***	***23***	***71***	***221***	***32***

CS- PCR results of primer set CS-78 and CS-323; RpCS- PCR results of primer set RpCS-877 and RpCS-1273r

Chapter 6: Molecular detection of *Borrelia burgdorferi sensu lato* from ticks collected from livestock and companion animals in five Provinces of South Africa.

6.1. Introduction

Human Lyme borreliosis (LB) sometimes referred to as Lyme disease (LD) is the most prevalent arthropod-borne infection in temperate climate zones around the world but covers mainly the Northern Hemisphere and is caused by *Borrelia* spirochetes (Turčinavičienė *et al*., 2006). LD is a zoonosis which is maintained in nature in enzootic cycles involving various tick species and some of the hosts of these ticks (Gern and Falco, 2000). The disease was first reported in the USA by A.C. Steere in 1975, when there was a cluster of cases of juvenile arthritis with an unusually high incidence of erythematous rash in the town of Old Lyme, Connecticut (Parola and Raoult, 2001). Subsequently its clinical manifestations were described by a scholar in Yale University in 1977, and named LD in 1980. Willy Burgdorfer and his colleagues isolated a spirochete and confirmed it as the causative agent of LD. The spirochete was formally named *Borrelia burgdorferi* in 1984. Lyme disease is the most common vector-borne disease distributed in over 30 countries and regions of Asia, Europe, America, Africa and Oceania, in Europe with more than 0.3 million clinical cases per year. In the USA, it is particularly severe with approximately 20 - 100 cases per 100,000. The number of cases is increasing, and the disease was listed as a key target for prevention and control by WHO in 1992 (Niu *et al*, 2011).

The *B. burgdorferi sensu lato* complex comprises at least 13 species, *B. burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, *B. lusitaniae*, *B. valaisiana*, *B. bissettii*, *B. andersonii*, *B. japonica*, *B. tanukii*, *B. turdi*, *B. sinica*, *B. spielmanii* and *B. californiensis* (Gern and Falco, 2000; Niu *et al*., 2011). The genus *Borrelia* contains several major human and animal pathogens. Among these are the relapsing fever *Borrelia* species, LD *Borrelia* species, and the etiologic agent of avian spirochetosis, *Borrelia anserina*. Within the LD *Borrelia* group *Borrelia burgdorferi sensu lato*, three different human pathogenic species, *B. burgdorferi sensu stricto*, *Borrelia garinii*, and *Borrelia afzelii* are currently

recognized. A fourth species, *Borrelia japonica*, was recently described, but its relevance as a human pathogen is still unclear (Olsen *et al*., 1995). *Borrelia* spirochetes in ticks vary considerably between different geographical regions (Niu *et al*., 2011). Molecular analysis has indicated that these *B. burgdorferi* isolates are genetically divergent. A closely related cluster containing several tick-borne *Borrelia* species and genomic groups associated with LB has been defined (Turčinavičienė *et al*., 2006).

B. burgdorferi s. l. is transmitted mainly by ticks within the *Ixodes ricinus* group. The following five species of *B. burgdorferi* have been isolated from ticks: *B. burgdorferi s.s.* (*I. scapularis*, *I. dammini*, *I. ricinus* and *I. pacificus*), *B. garinii* (*I. ricinus* and *I. persulcatus*), *B. afzelii* (*I. ricinus* and *I. persulcatus*), *B. lusitaniae* (*I. ricinus*) and *B. valaisiana* isolated from *I. ricinus* and *I. columnae* (Niu *et al*., 2011). These tick species parasitize various mammals, birds, and lizards. In Europe, *B. burgdorferi s. l.* has been isolated from or detected in *I. ricinus*, *I. persulcatus*, *I. uriae*, *I. hexagonus*, birds , shrews, voles, mice, hares, and humans. In the United States, avian reservoirs of the LD pathogen have been suggested since *B. burgdorferi s.s.* has been isolated from passerine birds and from *I. scapularis* larvae carried by birds (Olsen *et al*., 1995). In North America, the infection is transmitted by deer ticks between small mammals or, inadvertently, to people (Bushmich, 1994). *B. burgdorferi* has however been isolated from *Rhipicephalus*, *Haemaphysalis*, *Boophilus* and *Dermacentor* species of ticks in China (Niu *et al*., 2011). No information regarding the tick vector of *B. burgdorferi* in South Africa is currently available. The pathogen can be transmitted both transstadially and transovarially once the ticks become successfully infected. Commonly it is found in the digestive tract of its tick vectors but it has also been found in the haemolymph and saliva (Fivaz and Petney, 1989).

6.1.1. Aetiology and pathogenesis

The spirochetes are flexible helical cells with dimensions of 0.18 - 0.25 by 4 - 30 µm. The organism is motile with both rotational and translational movements; the coiling of the cell is regular. On the average, seven periplasmic flagella are located at each cell

end, and these flagella overlap at the central region of the cell (Johnson *et al*., 1984; Fivaz and Petney, 1989). Among borreliae, *B. burgdorferi* is the longest and narrowest (0.2 - 0.3 µm by 20 - 30 µm) with a structure and biochemical characteristics similar to that of other members of the genus and has the least flagellae (Fivaz and Petney, 1989; Parola and Raoult, 2001). A multilayered outer envelope or membrane surrounds the protoplasmic cylinder, which consists of the peptidoglycan layer, cytoplasmic membrane, and the enclosed cytoplasmic contents. The diamino acid present in the peptidoglycan I is ornithine. Cytoplasmic tubules are absent. The cells are gram negative and stain well with Giemsa and Warthin-Starry stains and are microaerophilic. Unstained cells are not visible by bright-field microscopy but are visible by dark-field or phase-contrast microscopy. The optimal growth temperature is 34 to 37°C in a complex liquid medium called BSK II medium but also occurs on various solid media, and the organism has a generation time of 11 to 12 h at 35°C until they number 10^5 to 10^8/mL blood (Johnson *et al*., 1984; Parola and Raoult, 2001; Guerrant *et al*., 2010). Like all other borreliae the spirochete is obligate parasitic with no known free-living stages (Johnson *et al*., 1984). Rarely the disease is acquired through accidental inoculation of infected blood, contact of blood with abraded or lacerated skin, mucous membranes, or the conjunctiva, or transplacental or perinatal transmission from mother to fetus. Transmission to humans by aerosol, fomites, human saliva, urine, feces, or sexual contact has not been documented (Guerrant *et al*., 2010). Chance of transmission of the pathogen depends upon attachment time with maximum transmission occurring after about 72 h and the likelihood of contracting an infection seems to depend on the seasonal activity patterns of the ticks (Fivaz and Petney, 1989). From the blood the spirochetes may invade the central nervous system (CNS), eye, liver, and other organs (Guerrant *et al*., 2010).

6.1.2. Clinical signs

The onset of LB is often marked by the development of an *erythema chronicum migrans* (ECM) rash at the site of the tick bite. The skin leshion is followed by further clinical manifestations which include pyrexia, fatigue, arthralgia and arthritis and a variety of

neurological and cardiac symptoms (Fivaz *et al*., 1990). The infection can persist for years and may also result in a chronic cutaneous manifestation, *acrodermatitis chronica atrophicans* (Fivaz *et al*., 1990; Rijpkema *et al*., 1995; Gern and Falco, 2000). Other symptoms in humans may include profound fatigue, chronic muscle weakness, sleep disturbance, chronic encephalitis, photophobia, auditory hyperacusis, extreme irritability or emotional lability, word-finding problems, dyslexic errors when speaking or writing and spatial disorientation (Strijdom and Berk, 1996).

Animals such as cattle, sheep, horses, dogs and rats can be infected by the pathogen, and in most cases, play a role as reservoirs with varied clinical symptoms (Niu *et al*., 2011). Many domestic animals infected with *B. burgdorferi* seroconvert but do not show clinical signs. Those animals that do develop clinical signs exhibit primarily single or shifting limb lameness and swollen joints, with or without fever. Less commonly observed clinical signs reported include behavioral changes, seizure activity, encephalitis, renal dysfunction, cardiac arrythmia, and reproductive disorders. With the possible exception of cattle, domestic animals do not commonly demonstrate an erythematous skin lesion at the site of tick bite, although *B. burgdorferi* has been cultured from normal-appearing skin (Bushmich, 1994).

6.1.3. Diagnosis

Presumptive diagnosis of LD in domestic animals and patients relies primarily on clinical signs, exposure to an LD-endemic area, supportive serology (IFA, ELISA or WB) and response to therapy, other causes of lameness and joint swelling must be ruled out (Bushmich, 1994; Strijdom and Berk, 1996). Because of the large number of asymptomatically infected animals in endemic regions, serological evidence alone is insufficient to support a diagnosis of LD in domestic species (Bushmich, 1994). Recently, PCR has been used to amplify *B. burgdorferi* DNA (*osp*A and 16S rRNA genes) in blood, cerebrospinal fluid, skin, synovial fluid, and urine samples from infected patients and several other assays have been developed for detection of the spirochetes in ticks and other animals (Parola and Raoult, 2001).

6.1.4. Treatment

Treatment regimens have been largely extrapolated from laboratory animal studies and human patient experience. Antibiotics from the penicillin and tetracycline families are typically employed. Exact drugs used vary by species (Rijpkema *et al*., 1995). In the early stages of LD, doxycycline (200 mg/day, given for 20 - 30 days) is the recommended treatment. *B. burgdorferi* is, however, also susceptible to β-lactams, including ceftriaxone, which is used to treat neurological forms of the disease (Guerrant *et al*., 2010; Parola and Raoult, 2001). The vaccine, derived from a lipidated outer surface protein of *B. burgdorferi* which works inside the tick itself (antibodies ingested during the blood meal by the tick can neutralize the bacteria in the tick gut), thus preventing transmission of the spirochete is also available (Parola and Raoult, 2001).

6.2. Aims of the study

The aim of this chapter was to detect and characterize the prevalence of *B. burgdorferi* amongst ticks collected from livestock (cattle, dogs, sheep and goats) as well as from the vegetation in the Eastern Cape, Free State, KwaZulu-Natal, Mpumalanga and North West Provinces of South Africa.

6.3. Materials and methods

6.3.1 Study area

Ticks were collected from dogs, cattle, goats, sheep and incidentally four horses and four cats and also from the vegetation from farms in the Free State (FS), KwaZulu-Natal (KZN), Eastern Cape (EC), North West (NW) and Mpumalanga (MP) Provinces. In KZN samples were collected from Wesselsneck [S 28° 20' 0.52" E 030° 02' 49.1"], Gcinalishona/Mjindini [S 28° 39' 00.5" E 030° 06' 56.3"], eTholeni [S 28° 25' 39.9" E 30° 13' 04.3"] and uMsinga Mountainview dip site [S 28° 41'43.1" E 030° 16'14.6"]; in FS,

Hooningkloof [S28° 30. 666' E 028° 42.701'], a farm situated at a livestock-wildlife interface, Sekoto farm [S28° 36.094' E 028° 49.013'], Seotlong Hotel and Agricultural School [S 28° 35' E 28° 50'] and in Kestell [S 28° 20' E 28° 38']; in EC, at Amathole District Municipality [S 32°48' 30" E 27° 01' 49"]; in NW at a private Veterinary Clinic in Mafikeng [S 25° 51′ 0″ E 25° 38′ 0″] and in MP at Kameelpoort-KwaMhlanga [S 25° 46′ 6.3″ E 29° 28′ 42″], refer to Figure 2.4. for the Map of South Africa.

6.3.2. DNA extraction from ticks

The ticks were surface sterilized twice with 75% ethanol then washed once in phosphate buffered saline (PBS) solution before they were dissected and gutted (the engorged) or crushed whole (the males) in individual sterile Eppendorf tubes (Hamburg, Gemany) then later preserved in PBS and then stored at -34°C until further use. Ticks of the same species collected from one animal were grouped as one sample for DNA extraction. Some ticks laid eggs within the collection vials, these were also washed in PBS, spun down at full speed (16 000 xg) in the microcentrifuge then crushed and stored as described above. DNA was extracted from tick extracts and eggs using the salting out method as described by Miller *et al*., (1988). DNA was dissolved in 50 - 200 µl (depending on the size of the pellet) of double distilled water and extracts were stored at -34°C.

6.3.3. Polymerase chain reaction

The experimental procedures were carried out at laboratory of the Parasitology Research Program of the University of the Free State (UFS) - Qwaqwa Campus, South Africa. The extracted DNA (n = 721) from whole ticks together with an additional 55 DNA samples (obtained from dog ticks in the Free State, Phuthaditjhaba area) from a previous study (Leodi, 2010) were subjected to PCR amplification using oligonucleotide sequences FL6 - TTC AGG GTC TCA AGC TTG CAC T and FL7 - GCA TTT TCA ATT TTA GCA AGT GAT G, amplifying the flagellin gene of *B. burgdorferi sensu lato*

(Picken *et al*., 1996; Welc Faleciak *et al*., 2009) as well as B1 - ATGCACACTTGGTGTTAACTA and B2 - GACTTATCACCGGCAGTCTTA which amplifies the 16S rRNA gene of *B. burgdorferi* (Morozova *et al*., 2002). The PCR products were electrophoresed on 1.5% agarose gel stained with GelRed and/or ethidium bromide and size fractionated using a 100 bp DNA ladder (Figure 6.1 - 6.2). PCR was performed using DreamTaq PCR Master Mix (Inqaba Biotechnological Industries (Pty) Ltd, R.S.A.) with the following cycling conditions: Initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55 - 63°C for 30 s and extension at 72°C for 60 s/kb followed by final extension at 72°C for 7 min and held at 4°C ∞ using PRP's Multigene OptiMAx Thermal Cycler (Life Technologies Corporation, U.S.A.).

6.3.4. Purification and sequencing

PCR positive samples were sent to Inqaba Biotechnological Industries (Pty) Ltd (R.S.A.) for sequencing. The sequence data of the PCR products were analyzed using the BLAST 2.0 program (National Center for Biotechnology Information, Bethesda, Maryland, U.S.A.; http://www.ncbi.nlm.nih.gov/blast/) for homology searching. Sequences were analyzed and aligned using BioEdit Sequence Alignment Editor © 1997-2004 (Tom Hall Isis Pharmaceuticals, Inc).

6.4. Results

Using primer set FL6 and FL7 we were able to detect *B. burgdorferi s.l.* from pooled samples of ruminant ticks, however the expected fragment size of the PCR product of 276 bp was not obtained, only short fragments between 80 - 120 bp that seemed like false positives were observed. Repeating PCR and sequencing of the suspected samples revealed positive infection with this pathogen in only two of these samples and for the rest, no sequences were generated at all. The short sequences generated matched 100% to partial sequences of *B. burgdorferi* (Accession no.: Y15099.1;

Y15100.1). A new set of primers, B1 and B2 was purchased and using these to amplify the 16S rRNA gene, the presence of *B. burgdorferi* was confirmed.

The prevalence of infection of ticks with *B. burgdorferi* was 8% in FS (n = 161); 24% in EC (n = 79) and 0% in MP (n = 17), NW (n = 16) as well as in KZN (n = 115) samples. All (n = 115) the dog-, cat-, and vegetation-tick DNA samples were negative for *B. burgdorferi* using both sets of primers.Cattle ticks (n = 133) carried 19% of the pathogen whilst goat (n = 64) and sheep (n = 77) ticks carried 9% and 1.3% respectively. The overall prevalence of infection (depicted on Table 6.1.) in this study for the five sampled Provinces was 8.2% (32/389). Of the positive samples 31% was carried by *Rhipicephalus* spp. and 31% again by *R. decoloratus* whilst *A. hebraeum* and *R. evertsi eversti* each carried 19% of the pathogen.

6.5. Discussion and conclusions

No information regarding the tick vector of *B. burgdorferi* in South Africa is currently available, nevertheless it has been suggested that the abundance of host and tick vectors in the country would favor the establishment of the disease (Fivaz and Petney, 1989). Besides the fact that there are no *Ixodes* tick species known to transmit *B. burgdorferi* in R.S.A., in 1992 a study to investigate the capacity of local ticks to transmit the pathogen was conducted and all but one of the four genera tested failed to transmit it (Strijdom and Berk, 1996). Although the vector of *B. burgdorferi* is specific in most areas, in the United States there is evidence of its occurrence among *Amblyomma*, *Haemaphysalis* and *Dermacentor* (Fivaz and Petney, 1989). In China Niu *et al*., (2011) suggested that *Rhipicephalus* species might play a role in transmitting *Borrelia* spirochetes; this postulated from their study where the data obtained showed that the infection rate of *Rhipicephalus* with *Borrelia* was relatively high. They reported 31% and 32% infection in *Rhipicephalus* spp. and *R. decoloratus* and this is consistent with the findings in the current study where the same infection rate was obtained in *Rhipicephalus* spp. as well as in *R. decoloratus* ticks. The infection rate of the latter two was considerably much higher than that of other species such *R. evertsi evertsi* and *A.*

hebraeum. As *Rhipicephalus* does not readily attach to humans though, it was further suggested that it could transmit the pathogen between cattle and perhaps sheep, and that these infected animals would become reservoirs for other tick species which will attach to humans. Conversely from this study I would like to bring *Rhipicephalus* spp. and *A. hebraeum* forth as possible vectors and reservoirs of the pathogen more especially among livestock, however if ticks like *Amblyomma* were to display vectorial capacity of this pathogen there would be disastrous consequences judging by the 19% infection rate obtained and because they readily feed on humans.

The incidence of LD in South Africa is unknown as laboratory methods used in identification of the organism have only just become popular in the early nineties. Other than that, there are a couple of anecdotal cases reported regarding this pathogen. Serological data has also been used to speculate that cases of outbreaks of an unknown disease with associated symptoms similar to those of LD in Natal in 1982/3 may have been due to *B. burgdorferi* (Fivaz *et al*., 1990; Strijdom and Berk, 1996). In 1988 domestic animals from a riding school were tested using IFAT after an increasing number of reports on the disease, 61% of the horses and 55% of the dogs tested positive for the pathogen. In the same school, the owner fell ill after a tick bite and presented with symptoms of LD and thereafter workers at the farm who had also been bitten by ticks showed the same symptoms. They were all treated for the disease after confirmation with IFAT and ELISA (Fivaz *et al*., 1990). From these cases it could not be confirmed for sure that the causative agent of the disease in dogs and horses was *B. burgorferi* as sera were not screened against other *Borrelia* that occur in Africa such as *B. duttoni* and *B. theileri*. From this tentative data obtained from serological studies on domestic animals that LD does indeed occur in South Africa, subsequent research (both serological and molecular) in the country has not been successful in revealing its presence (Frean and Isaacson, 1995; Strijdom and Berk, 1996). Use of non-specific primer sets could be responsible for not obtaining the desired result in previous studies. In the current study it is suspected that the short fragment and sequence sizes of the product generated using primer set FL6 and FL7 may have been due to poor quality of DNA or poor sequencing reactions. However it has also been published that these

primers are short and have a much lower G+C content (Picken *et al*., 1996), this may have also played a major role in the initial results obtained.

In this study it can be confirmed that the infection detected from the ticks was indeed *B. burgdorferi* since two pairs of specific primers were used to screen for the pathogen and confirmed with sequencing. However the pathogen was only detected amongst ticks collected from sheep, cattle, and goats and not from the dogs, cats, horses and the vegetation. Since the pathogen was only detected in *Rhipicephalus* spp., *R. decoloratus, R. evertsi evertsi* and *A. hebraeum* ticks this could explain its absence amongst dog-, cat- and vegetation-ticks in the current study as they were represented by *R. appendiculatus*, *R. sanguineus* and *Haemaphysalis elliptica* tick species only.

Previous studies focused on restricted areas such as the Witwatersrand and Natal (Fivaz *et al*., 1990; Strijdom and Berk, 1996) and there are no data available for much of the areas outside these, this is possibly the reason why there is a lack of reports on the incidence of the disease in South Africa. This study therefore reports on positive detection of *B. burgdorferi* with PCR, detected from ticks collected from livestock in Eastern Cape and Free State Provinces. Moreover it can be suggested that the aforementioned ticks are possible vectors of the pathogen and that the ruminants act as hosts for adult stages of the tick vectors as well as reservoirs of the pathogen. It is yet to be determined by genetical characterization whether or not the strain obtained is pathogenic to man. Furthermore, clinicians/physicians should consider LD as part of routine screening and in differential diagnosis of patient samples.

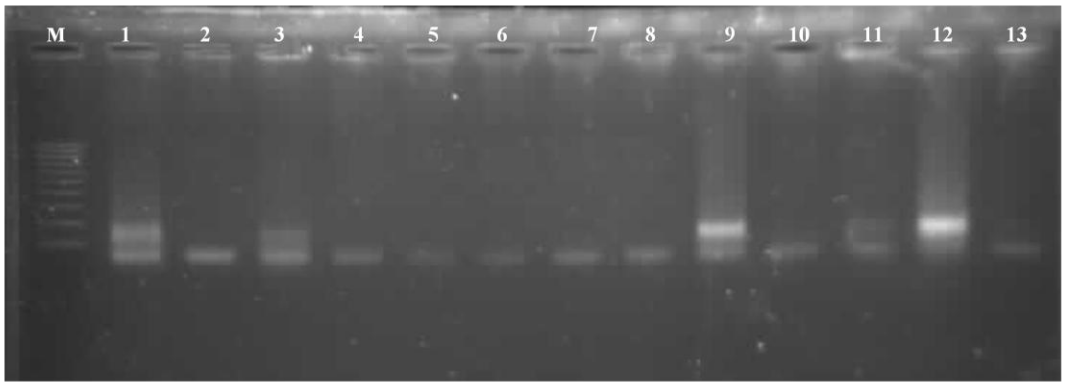

Figure 6.1. Gel electrophesis of PCR products obatined using primer set FL6 and FL7, amplifying the flagellin gene of *B. burgdorferi* . Lane M = Molecular marker (100 bp), 1;3;9&12 = suspected positive samples, 2; 4-8;10-11 = negative samples, 13 = negative control (DDH_2O). Expected size 250 bp, Product size obtained between 100-150 bp.

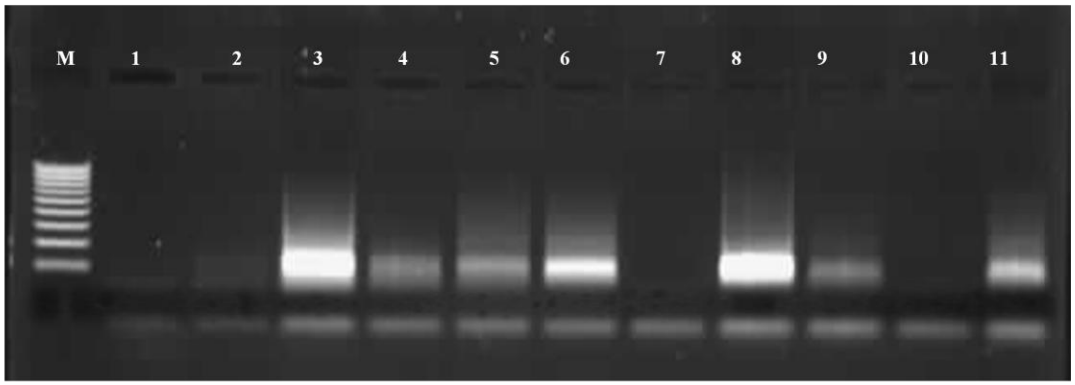

Figure 6.2. Gel electrophesis of PCR products using primer set B1 and B2 amplifying the 16 S rRNA gene of *B. burgdorferi* . Lane M= Molecular marker (100 bp), 3-6;8-9 = positive samples, 1;2;7 = negative samples, 10 = negative control (DDH_2O); 11 = sample that was positive using FL6 and FL7 primers (positive control). Expected size 250 bp, Product size obtained between 100-150 bp.

Table 6.1. Prevalence of *B. burgdorferi* in tick across five sampled Provinces

Animal population	KwaZulu-Natal	Free State	Eastern Cape	North West	Mpumalanga	Overall % (+) per animal population
Dogs						
Tot tested	63	19	-	16	5*	**103**
No. Pos	0	0	-	0	0	**0**
%	**0**	**0**	-	**0**	**0**	**0**
Sheep						
Tot tested	-	77	-	-	-	**77**
No. Pos	-	1	-	-	-	**1**
%	-	**1.3**	-	-	-	**1.3**
Goats						
Tot tested	-	22	29	-	13	**64**
No. Pos	-	0	6	-	0	**6**
%	-	**0**	**21**	-	**0**	**9**
Cattle						
Tot tested	52	31	50	-	-	**133**
No. Pos	0	12	13	-	-	**25**
%	**0**	**39**	**26**	-	-	**19**
Vegetation						
Tot tested	-	12	-	-	-	**12**
No. Pos	-	0	-	-	-	**0**
%	-	**0**	-	-	-	**0**
Total Screened	115	161	79	16	18	**389**
Overall (+) samples	0	13	19	0	0	**32**
Overall % (+) per Province	**0**	**8**	**24**	**0**	**0**	**8.2**

* 1 dog and four cats DNA samples

Chapter 7: General discussion, conclusions and recommendations

7.1. Discussion

In the current study the aim was to detect with PCR the presence of selected zoonotic tick-borne pathogens previously unknown to occur in South Africa and co-incidentally detect and determine the prevalence of other pathogens that they may co-exist with in their tick vectors. It was hypothesized that since there is an abundance of tick vectors in the country surely the establishment of zoonotic pathogens like *A. phagocytophilum*, *B. burgdorferi*, *C. burnetii* and *Rickettsia* species (*R. conorii* and *R. africae*) would prove not to be a difficult task. In addition to this the presence of well known pathogens of livestock such as *E. ruminantium* and *E. canis* was predicted and therefore their detection was included as part of the aims of the study as well.

As a first step, tick samples were collected from livestock (cattle, sheep, goats, dogs, cats and horses) in different study areas across five Provinces of South Africa i.e. KwaZulu-Natal, Free State, Mpumalanga, North West and Eastern Cape. Tick samples were processed, identified and quantified. A total count of 1947 ticks was obtained and tick species included common genera found in South Africa i.e. *Rhipicephalus*, *Amblyomma*, *Hyalomma* and *Haemaphysalis* (Norval and Horak, 1994; Strijdom and Berk, 1996; Mbati *et al*., 2002; Hlatshwayo *et al*., 2002; Marufu, 2008, Marufu *et al*. 2011; Horak *et al*., 2010; Walker *et al*., 2007). All the tick species obtained are known to carry pathogens that are problematic in the livestock industry, causing notorious diseases such as heartwater, redwater and other fevers leading to significant economic losses (Matjila *et al*., 2008; Berrada and Telford, 2009; Crowder *et al*., 2010). I was however unsuccessful in finding ticks of the genus *Ixodes* from which I had expected to find the zoonotic pathogens as previous studies have reported (Jongejan and Uilenberg, 2004; Dumler *et al*., 2005; de La Fuente *et al*., 2005, Butler *et al*., 2008).

Without dismay, DNA was extracted from the tick samples and a total of 776 DNA samples were obtained when combined with 55 other DNA samples obtained from a

previous study (Leodi, 2010), thereafter PCR was employed to reveal the prevalence of infection with the target pathogens. The number of samples screened per Province and the percentage prevalence of pathogen infections are summarized in Table A-1. The prevalence of infection of ticks with *C. burnetii*, *Rickettsia* spp., *A. phagocytophilum*, *B. burgdorferi*, *E. canis* and *E. ruminantium* varied significantly from Province to Province (X^2 = 618.7, df = 24, P > 0.01) and infection rates varied quite significantly regardless of the group of animals from which they were collected (X^2 = 437.7, df = 24, P > 0.01) when Chi-squared test was used.

Although previously, literature indicates that *C. burnetii* is quite prevalent amongst domestic animals (sheep, cattle and goats), wild dogs as well as in humans in South Africa with serology (Schutte *et al*., 1976; Gummow *et al*., 1987; Kelly *et al*., 1993; van Heerden *et al*., 1995; Matthewman *et al*., 1997; Fournier *et al*., 1998; Frean and Blumberg, 2007), there were no apparent infections reported in domestic dogs in the country. Serological methods do however have both their pros and cons. In this study molecular methods were used to evaluate the infection of ticks with *C. burnetii*, the study is the first of its kind in the country and it therefore reports the presence of this pathogen in ticks (possible vectors and reservoirs) collected from sheep, cattle, dogs and goats, with the exception of those collected from the vegetation, horses and cats. The pathogen was successfully isolated and characterized with a reported overall prevalence of 16±1.6%.

Some of the sequences obtained indicated that *C. burnetii* might have been an endosymbiont when analyzed for homology with published sequences on the database. Andreotti *et al*., (2011) reported that most pathogens develop endosymbiotic relationships with their tick hosts and have also reported that there is relatedness between *R. microplus* and *C. burnetii*, however that still remains to be determined here. It is just as well that the prevalence of this pathogen remains very low as it has the potential of becoming an agent of bioterrosism and higher levels could spell trouble on a national scale (Moodie *et al*., 2008; Massung *et al*., 2012). Because Q-fever is rarely a notifiable disease, its incidence in humans cannot be assessed in most countries, however recent studies indicate that it should be considered a public health concern in

countries like France, United Kingdom, Italy, Spain, Germany, Israel, Greece, Canada, Australia, Japan and Austria (Fournier *et al*., 1998; Heinzen *et al*., 1999; Skerget *et al*., 2003; Capuano, 2004; Psaurolaki *et al*.,2006; Cooper *et al*. 2011; Havas and Burkman, 2011; Massung *et al*., 2012).

Rickettsia species that were isolated and characterized in the current study were *R. africae* and *R. conorii* as had been expected and the combined prevalence was 26±1.7%. Serological data however states otherwise with 75% up to 100% infection rates reported amongst indigenous people (Ndip *et al*., 2004; Kelly, 2006). Ticks may act as amplifiers for most of *Rickettsia* species, while vertebrates are bacteremic for short periods of time and whether or not they can serve as reservoirs is another subject (Vorou *et al*., 2008). It is not often that *Rickettsia* species infections are reported in the country except through incidence from visiting tourists, since animal hosts/reservoirs of the infection remain asymptomatic (meaning that *Rickesttsia* spp. have no apparent effects on the livestock industry) and as a consequence rickettsias are neglected (Raoult *et al*., 2001; Parola and Raoult, 2001; Delfos *et al*., 2004; Rutherford *et al*., 2004; Parola *et al*., 2005; Parola, 2006; Frean *et al*. 2008; Roch *et al*., 2008; Althaus *et al*., 2010). However potential damage to the tourism industry could be witnessed if tourists are not made aware of the situation in South Africa and if physicians do not consider them as part of their routine screening, death may result. For endemics there doesn't seem to be any reports of the infection with rickettsias as they might have developed some form of immunity against the infection (Kelly, 2006), nonetheless this does not mean they should not be considered as a cause of illness amongst these people.

Circumstantial evidence of occurrence of *B. burgdorferi* can now be verified regardless of the absence of specific vectors of LD. The overall prevalence of infection with *B. burgdorferi,* 8.2±1.4%, was an unexpected finding since it has, to the best my knowledge, not been detected and characterized by molecular methods in South Africa. The detected organism may or may not be pathogenic as *B. burgdorferi* is a complex of about 13 species with only three being pathogenic to man (Gern and Falco, 2000; Niu *et al*., 2011), but, since there exists evidence of infection from previous case studies,

infection with this pathogen is inevitable and should therefore be considered in patient routine screenings in the country. The vectorial capacity of ticks from which it was detected has either not been determined or the ticks have failed to transmit the pathogen in reported experiments (Fivaz and Petney 1989; Strijdom and Berk, 1996). Some like *Rhipicephalus*, have been reported to harbor *B. burgdorferi* but their preferred hosts don't seem to include human beings (Niu *et al*; 2011). *Amblyomma*, *Haemaphysalis* and *Dermacentor* have also been reported to carry the pathogen (Fivaz and Petney 1989). This presents a challenge though, because despite the 8.2% infection rate obtained in this study there is no possible way of determining whether or not South Africans become infected with *B. burgdorferi* unless blood samples are obtained and from there isolate the organism. There is an interesting finding though of a woman in Johannesburg who has been living with the disease for over 25 years and has now become a chronic LD patient due to misdiagnosis and ineffective administration of treatment (http://lymediseaseinsouthafrica.blogspot.com/2011/04/borrelia-burgdorferi-can-be-passed-from.html). A riding school owner and a couple of workers in the same school were reported to have been infected by *B. burgdorferi* and results thereof confirmed with serology and subsequently treated for LD (Fivaz *et al*., 1990; Strijdom and Berk, 1996). Therefore based on this and the fact that they had all remembered being bitten by a tick very well and also looking at the findings of the current study the possibility of LD should not be ruled out, regardless of the absence of known vectors.

Only one positive PCR confirmation of *A. phagocytophilum* has been reported in the country (Inokuma *et al*., 2005; Matjila *et al*., 2008, Allsopp and McBride, 2009), since then no other studies have been successful in detecting this pathogen. The bacterium was detected from whole blood samples of four dogs from the FS Province. In this study 9±1.2% prevalence of infection can be reported from ticks collected from cats, cattle, dogs, goats and sheep. The tick vectors for *A. phagocytophilum* and *B. burgdorferi* (*I. ricinus*, *I. pacificus*, *I. scapularis*, *I. persulcatus*) are not known to occur in the country and both pathogens have not been isolated from ticks in South Africa previously, however in the absence of established vectors I managed to isolate *A. phagocytophilum*

from *R. sanguineus, R. appendiculatus, R. e. evertsi* and *H. elliptica* and isolated *B. burgdorferi* from *Rhipicephalus* spp., *R. e. evertsi*, *R. decoloratus* and *A. hebraeum*.

R. sanguineus has been implicated as a vector of *A. phagocytophilum* in Egypt where it was found harboring the pathogen and probably transmitting it to Egyptian goats, sheep and dogs (known for their global competence as reservoirs of *A. phagocytophilum*) which it usually parasitizes (Ghafer and Amer, 2012). Migratory birds are said to play a role in the transmission of *A. phagocytophilum* as gene sequences of the bacterium were detected in infected ticks of these birds as well as from humans and domestic animals in Sweden (Olsen *et al.,* 1995; Vorou *et al*., 2007). Therefore the possibility of alternate vectors for both *A. phagocytophilum* and *B. burgdorferi* cannot be eliminated. The ticks may act as main vectors and reservoirs for livestock infections but as for their transmissibility to human hosts I remain uncertain of their vectorial capacity, nevertheless tick species of *Amblyomma* readily bite humans and may be able to transmit both pathogens and could therefore pose a serious threat to the public. Although being the second detection of *A. phagocytophilum* in the country this is the first report of the pathogen from ticks. This however, does not confirm the vectorial competency of the tick species, and so this work is a crucial initial step in vectorial competence studies. Identifying the competent vectors utilized by *A. phagocytophilum* in South Africa will help in understanding the global epidemiology of HGA as well as in designing and implementation of efficient prevention and control measures.

Prevalence of *E. ruminantium*, *E. canis* and *Ehrlichia*/*Anaplasma* was determined to be 28±2.1%, 20±3.6%, and 18±3.8% respectively. Nothing out of the norm was observed with their prevelance but, it was noted that in the EC Province ticks collected from cattle were all negative for *E. ruminantium* yet about 76% of the goat ticks from the same area were positive for the pathogen. Since previous studies in that area and surrounding ones have reported absence of the pathogen even by serological methods (Marufu, 2008; Bryson *et al*., 2002), it was concluded that there might be a situation of endemic instability or it could be that the goats together with their ticks may have recently come from an area in which *E. ruminantium* is highly endemic. It cannot however be ignored

that *E. ruminantium* has been implicated in four deaths in South Africa and should therefore be considered as an ermerging zoonotic agent in the country (Louw *et al*., 2005; Allsopp and McBride, 2009).

No significant *Ehrlichia*/*Anaplasma* species were characterized except for *A. phagocytophilum* as reported above, however it was found that using the set of primers which detects *Ehrlichia*/*Anaplasma* simultaneously, there seemed to have been a bias towards *E. canis* detection and I might therefore have missed significant infections by pathogens like *E. chaffeensis* which has been recently reported serologically in a patient and a dog in the region (Pretorius and Kelly, 1998). This revealed the essence of using species specific primers in the detection of genetically related organisms.

The overall prevalence of infection of ticks collected from four major groups of animals in this study, as well as from the vegetation with the target pathogens, across the five sampled Provinces of South Africa is shown in Figure B-1 and B-2. *E. ruminantium* infection was very high amongst goat ticks (69%) and very low amongst cattle ticks (18%). *C. burnetii* was observed to be much higher amongst dog (41%) and sheep(32%) ticks when compared to cattle (5%) and goat (6.5%) ticks and was absent in ticks collected from the vegetation. A more or less similar infection rate was observed for *Rickettsia* species across ticks from all groups of sampled animals as well as in the ticks collected from the vegetation ranging from 16 - 39%. *A. phagocytophilum* infections were almost the same even though not very high in goat (18%) and dog (19%) ticks, but very low in cattle (6%) and sheep (1.25%) ticks and it was not detected from the ticks collected from the vegetation. Worth noting is the detection of this pathogen from the four cat-tick DNAs posing a potential danger of infection as most people are highly fond of cats. The observed infection rates with this pathogen were however tangible as it was not at all expected. Infection rates with *B. burgdorferi* were observed mainly in cattle (19%) and goat (9%) ticks whilst they remained very low amongst sheep (1.3%) and the bacterium was absent from dog and cat ticks as well as from those collected from the vegetation.

Qualitative and quantitative information on the composition of bacterial communities (especially zoonotic ones) in ticks are scarce in South Africa, despite the fact that determination of relationships between ticks and non-pathogenic bacteria was initially documented at the beginning of the 20th century in other parts of the world. In addition to carrying multiple bacterial communities such as *Borrelia*, *Bartonella*, *Anaplasma*, *Ehrlichia* and *Coxiella*, ticks also harbor bacterial endosymbionts which can have commensal, mutualistic or parasitic relationships with their host ticks (Andreotii *et al*., 2011).

The ticks collected here had varying rates of co-infection with the screened pathogens, both low and very high in other instances; this is consistent with previous findings (Ginsberg, 2008). At least 39±1.8% (305/776) of the tick DNAs screened were positive for one pathogen. A significant number were however uninfected and it was observed that in three separate farms in the FS a total of 118 tick DNA samples were negative for all target and co-incidentally screened pathogens. Double infection rates in the current study were 13±1.2% (103/776), triple infection rates were 5±0.8% (37/776) and less than 1% (5/776) of quadruple infections were recorded. The trend of co-infection of ticks with different pathogens is depicted on Table C-1. The most common co-infections in ticks being between *E. ruminantium* - *Rickettsia*, *C. burnetii* - *Rickettsia, Rickettsia* - *A. phagocytophilum*, *E. canis* - *Rickettsia*, *E. canis* - *C. burnetii*, *A. phagocytophilum* - *C*. *burnetii, B. burgdorferi* - *A. phagocytophilum*, and lastly *B. burgdorferi* - *E. ruminantium*.

For the most frequent co-infections, Ginsberg's co-infection index (I_c) was calculated to determine departure of the mixed infections from independence. This is calculated as the difference of the number of co-infections from the number of expected due to chance alone, as a percentage of the total number of infected ticks in the sample. If *a* = number infected with both pathogens, *b* = number infected only with pathogen 1, *c* = number infected only with pathogen 2, *d* = number not infected with either pathogen, then the number of observed co-infections (*O*) equals *a*, the expected number of co-infected ticks due to chance alone (*E*) is given by: $E= ((a + b)(a + c)/a + b + c + d)$, and the total number of ticks infected by either or both pathogens *(N)* is: $N= a +b + c$. The

index of co-infection is therefore given as (I_c) = ((*O*-*E*)/*N*) x 100. With this index it was found that the number of co-infections with the above pathogens were greater than had been expected due to chance alone (Table C-2).

The crushed egg-DNA samples (n = 17) were mostly negative for the target pathogens except for three samples positive for *C. burnetii*, two for *E. canis* , four for *Rickettsia* spp. and three for *E. ruminantium*. This is consistent with the fact that *B. burgdorferi* and *A. phagocytophilum* are transtadially and not transovarially transmitted amongst ticks (Dumler *et al*., 2005, Ghafar and Amer, 2012). In MP and NW mixed infections were not frequent. While KZN and EC had moderate infections rates, the FS depicted high mixed infection rates.

Co-infection patterns in field collected samples can be distributed by chance or as a vast majority of cases have revealed it can be explained on the basis of vertebrate host associations of the pathogens, without invoking interactions between pathogens within ticks. Nevertheless, some studies have demonstrated antagonistic interactions, and some have suggested potential mutualisms, between pathogens in ticks. Negative or positive interactions between pathogens within ticks can affect pathogen prevalence, and thus transmission patterns. Probabilistic projections suggest that the effect on transmission depends on initial conditions. When the number of tick bites is relatively low (e.g., for ticks biting humans) changes in prevalence in ticks are predicted to have a proportionate effects on pathogen transmission. In contrast, it has been stated that when the number of tick bites is high (e.g., for wild animal hosts) changes in pathogen prevalence in ticks have relatively little effect on levels of transmission to reservoir hosts, and thus on natural transmission cycles (Sparagano *et al*., 1999; Ginsberg, 2008).

There are several published reports on the effects of pathogen co-infections be they negative, positive or null in ticks to which reference can be made e.g. *B. burgdorferi* has been reported to suppress replication of tick borne encephalitis virus (TBEV) while *C. burnetii* has been reported to spread increasingly into tissues of *Dermacentor* ticks in the presence of *Rickettsia phytoseiuli* (Sutakova and Rehacek, 1990). *E. chafeensis* and *E. ewingii* as well as *R. emblyommii* and *Borrelia lonestari* have been observed to

have higher than random levels of association in *A. americanum* ticks. These however could have resulted from ecological factors relating to pathogen infections in reservoir hosts and tick feeding preferences, rather than mutualistic interactions of pathogens within ticks (Mixon *et al*, 2006; Ginsberg, 2008). No evidence of interference in transmission of *B. burgdorferi* and *A. phagocytophilum* by singly and co-infected *I. persulcatus* to white-footed mice in the lab has been reported (Levin and Fish, 2000). Hodzic *et al*., (1998) however report that *B. burgdorferi* and *A. phagocytophilum*'s vector tick sharing does have an effect on transmission of *A. phagoyctophilum*. It is said that upon feeding, *B. burgdorferi* gets activated and enhanced replication is observed while *A. phagocytophilum* transmission is dose dependant hence despite their co-transmission by *I. scapularis* in endemic areas, prevalence of *A. phagocytophilum* is usually much less than that of *B. burgdorferi*. Swanson and Norris (2001) found that *B. burgdorferi s.s.* and *Rickettsia* spp. were independently distributed in *I. scapularis* ticks, additionally they reported that other pathogens co-existed with *B. burgdorferi* and more frequently than expected due to chance alone and these findings were also attributed to shared enzootic cycles rather than to interaction with ticks. Proportions of mixed infections in nymphs would naturally be expected to be lower than in adult ticks, because the nymphs have only fed once while adults have fed twice, and the adults might have picked up infections from different host species (Ginsberg, 2008), however I was unable to prove this factor in the current study as there were not that many nymphal stages in my collection.

Valuable insights into processes occurring within the tick vector, such as stages of a pathogen's life cycle, can be obtained by the application of molecular biology techniques. Such techniques have been used to demonstrate molecular polymorphism of pathogens and also for the detection of mixed pathogen populations. A number of tick-borne organisms, such as some *Theileria* or *Rickettsia* species, may not be pathogenic to livestock animals. Therefore, discriminating techniques are necessary at species or strain levels to differentiate between those which are pathogenic, and those which are not (Sparagano *et al*., 1999). Co-infection with *Ehrlichia* and *Borrelia* for instance have been so frequently reported in Europe that a PCR-hybridization assay

that enables the simultaneous detection and species identification of a variety of *Ehrlichia*, *B. burgdorferi*, and *Bartonella* species in a single sample has been designed (Schouls *et al*.,1999). A multiplex PCR system for detection of *A. phagocytophilum* and *B. burgdorferi* was developed for the same reasons (Courtney *et al*., 2004). These have become efficient tools in endemic areas where more than one pathogen are transmitted by the same species of tick.

Impacts of vaccination trials or tick eradication programs rely on pathogen discrimination, which may not be possible by blood smear examination or cell culture isolation (Sparagano *et al*., 1999). Functional and genomic characterization of endosymbionts for example could provide opportunities for genetic engineering whereby transformants could be developed and used as microbial acaricides (Andreotti *et al*., 2011). Molecular tools can therefore provide a better understanding of the epidemiology of such targeted pathogens (Sparagano *et al*., 1999). The number of described tick-borne pathogens affecting humans in South Africa could increase as research on tick biology and ecology increases and the use of efficient and expedient molecular methods continue to be employed where necessary.

7.2. Conclusions

All in all, *Rhipicephalus* spp., *R. appendiculatus*, *R. decoloratus*, *R. simus*, *R. e. evertsi*, *R. sanguineus*, *H. m. rufipes*, *A. hebraeum* and *H. elliptica* ticks were found to be infesting cattle, dogs, goats and sheep in this study. The target pathogens were successfully isolated by PCR, characterized and validated through sequencing reactions and the study provides compelling evidence of occurrence of *A. phagocytophilum*, *B. burgdorferi*, *C. burnetii* and *Rickettsia* species in the country. Furthermore there seems to be transovarial transmission of *C. burnetii*, *E. canis*, *E. ruminantium* and *Rickettsia* spp., as they were positively detected by PCR from DNA extratced from eggs, this collates previous findings. Presence of these pathogens from ticks does not necessarily mean that they are able to transmit them to mammalian host. However, their positive detection also indicates the possibility of their presence in the mammalian hosts, as it can be assumed that ticks acquired the infections from their

hosts for example *A. phagocytophilum* which is transstadially transmitted. Possibility of tick transmission or mechanical transmission of these pathogens to humans cannot be ruled out. This study has provided current information on the presence of these pathogens in the studied South African Provinces i.e. Eastern Cape, Free State, KwaZulu-Natal, Mpumalanga and North West.

7.3. Recommentations

- There is a need to conduct research in order to determine the virulence of the different zoonotic pathogen strains in both animals and humans.

- Vectorial capacity of the ticks obtained in this study for pathogens detected needs to be studied.

- Detailed epidemiological studies of zoonotic diseases including Erhlichiosis, Anaplasmosis, Rickettsiosis and Borreliosis using serology and DNA diagnosis needs to be conducted countrywide.

- Molecular detection of zoonotic pathogens observed in this study should be conducted in the other four remaining Provinces of South Africa.

- Tourists visiting South African national parks with presence of ticks need to be adviced on preventive measures such as the use of anti-tick lotions, wearing clothing that covers entire body and brightly coloured clothing where ticks can be easily visible etc.

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APPENDIX A

Table A-1. Collective prevalence of infection of ticks with the different pathogens per sampled Province

Prevalence of infection per Province	***C. burnetii***	***Rickettsia* spp.(CS)**	***Rickettsia* spp. (RpCS)**	***E. ruminantium***	***E canis***	***Ehrlichia/ Anaplasma***	***A. phagocytophilum***	***B. burgdorferi***
KZN (+)	21	34	23	21	10	12	4	0
Tot screened	164	75	82	82	55	55	143	115
% (+)	13	45	28	26	19	22	3	0
Prevalence (%±SD)[a]	**13±2.6**	**45±5.7**	**28±5.0**	**26±4.8**	**19±5.3**	**22±5.6**	**3±1.4**	**0**
FS (+)	56	39	42	87	13	09	16	13
Tot screened	203	229	119	237	50	50	263	161
% (+)	28	17	35	37	26	18	6	8
Prevalence (%±SD)[a]	**28±3.2**	**17±2.5**	**35±4.3**	**37±3.1**	**26±6.2**	**18±5.4**	**6±1.5**	**8±2.1**
NW (+)	5	-	6	-	0	0	10	0
Tot screened	16	-	16	-	16	16	16	16
% (+)	31	-	38	-	0	0	63	0
Prevalence (%±SD)[a]	**31±11.6**		**38±12.1**	-	**0**	**0**	**63±12.1**	**0**
MP (+)	1	0	0	0	1	1	6	0
Tot screened	17	13	5	13	5	4	17	18
% (+)	6	0	0	0	20	25	35	0
Prevalence (%±SD)[a]	**6±5.8**	**0**	**0**	**0**	**20±17.9**	**25±21.7**	**35±11.6**	**0**
EC (+)	0	29	-	22	-	-	14	19
Tot screened	123	122	-	124	-	-	123	79
% (+)	0	24	-		-	-	11	24
Prevalence (%±SD)[a]	**0**	**24±3.9**	**-**	**18±3.5**	**-**	**-**	**11±2.8**	**24±4.8**
Overall prevalence(%±SD) [a]	**16±1.6**	**23±2.0**	**32±3.1**	**28±2.1**	**20±3.6**	**18±3.5**	**9±1.2**	**8.2±1.4**

CS- PCR results of primer set CS-78 and CS-323; RpCS- PCR results of primer set RpCS-877 and RpCS-1273r

[a] Calculated as $\sqrt{\frac{p \times (100-p)}{n}}$ **, where *p* is the percentage of positive samples, *n* the sample size**

APPENDIX B

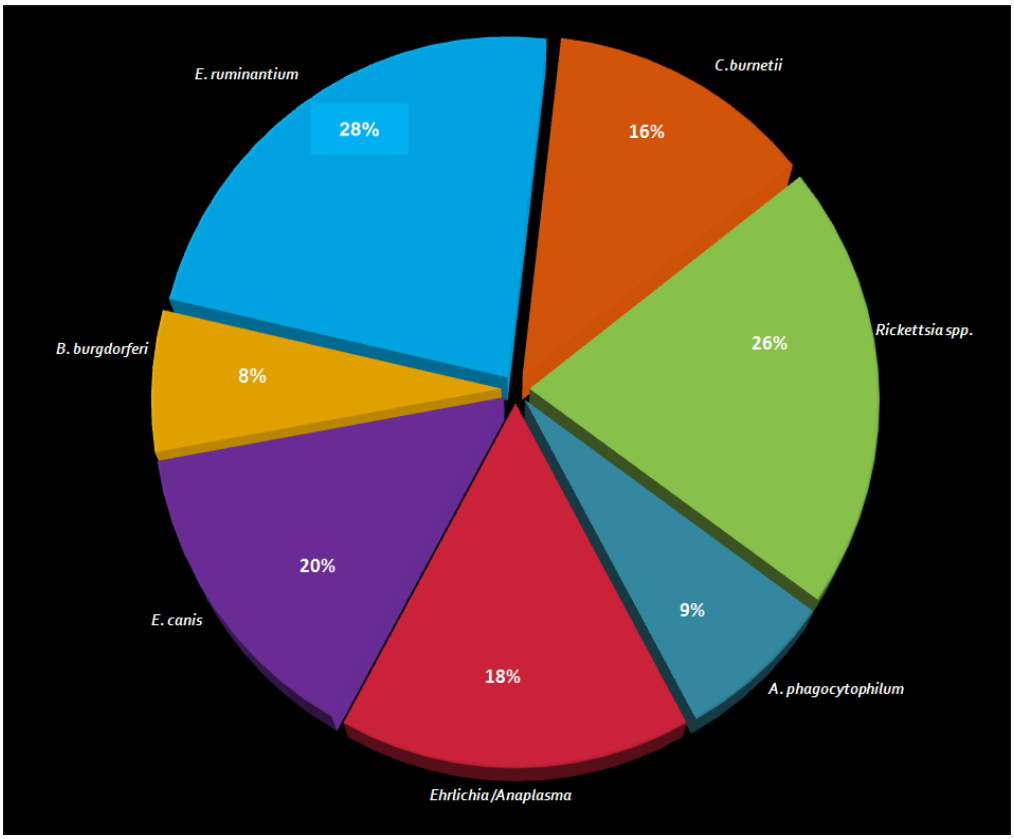

Figure B-1: Overall prevalence of infection of ticks across five sampled Provinces with the different pathogens

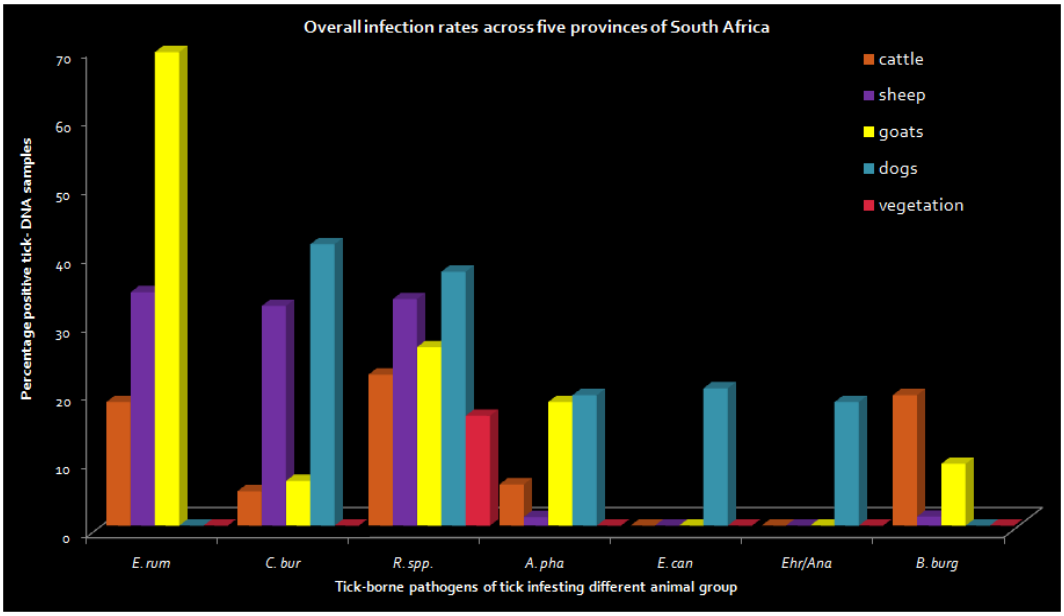

Figure B-2: Overall prevalence of infection of ticks collected from livestock and from the vegetation with six pathogens across five Provinces of South Africa. *E. rum*: *Ehrlichia ruminantium*, *C. bur*: *Coxiella burnetii*, *R. spp.* : *Rickettsia species*, *A. pha*: *Anaplasma phagocytophilum, E. can*: *E. canis*, *Ehr/Ana: Ehrlichia/ Anaplasma*, *B. burg*: *Borrelia burgdorferi*.

APPENDIX C

Table C-1. Co-infection trend between pathogens in ticks

E. ruminantium	*E. canis*	*Rickettsia* spp. (CS)	*Rickettsia* spp.(RpCS)	*B. burgdorferi*	*A. phagocytophilum*	*C. burnetii*
Single infection tallies						
X	x	x	x	x	X	x
X	x	x	x	x	X	x
X	x	x	x	x	X	x
X	x	x	x	x	x	x
X	x	x	x	x	x	x
X	x	x	x	x	x	x
X	x	x	x	x	x	x
X	x	x	x	x	x	x
X	x	x	x	x	x	x
X		x	x	x	x	x
X		x	x			x
X		x	x			x
X		x	x			x
X		x	x			x
X		x	x			x
X		x	x			x
X		x	x			x
X		x	x			x
X		x				x
X		x				x
X		x				x
X		x				x
X		x				x
X		x				x
X		x				x
X		x				x
X		x				
X		x				
X		x				
X		x				
X		x				
X		x				
X		x				
X		x				
X		x				
X						
X						
X						

E. ruminantium	***E. canis***	***Rickettsia* spp. (CS)**	***Rickettsia* spp.(RpCS)**	***B. burgdorferi***	***A. phagocytophilum***	***C. burnetii***
Double infection trends						
X					x	
X					x	
X		x				
X		x				
X		x				
X		x				
X		x				
X		x				
X		x				
X		x				
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X		x				
X		x				
X		x				
X			x			
X			x			
X			x			
X			x			
X			x			

E. ruminantium	*E. canis*	*Rickettsia* spp. (CS)	*Rickettsia* spp.(RpCS)	*B. burgdorferi*	*A. phagocytophilum*	*C. burnetii*
X			x			
X			x			
X			x			
			x		x	
			x		x	
			x		x	
			x		x	
			x		x	
			x		x	
			x		x	
X					x	
X					x	
X					x	
X					x	
X					x	
		x		x		
		x		x		
		x			x	
		x			x	
				x	x	
				x	x	
				x	x	
	x		x			
	x		x			
	x		x			
					x	x
					x	x
					x	x
					x	x
					x	x
			x			x
			x			x
			x			x
			x			x
			x			x
			x			x
			x			x
		x				x
		x				x
		x				x
		x				x

E. ruminantium	*E. canis*	*Rickettsia* spp. (CS)	*Rickettsia* spp.(RpCS)	*B. burgdorferi*	*A. phagocytophilum*	*C. burnetii*
		x				x
		x				x
		x				x
	x					x
	x					x
	x					x
X				x		
X				x		
X				x		
X			x			
X			x			
	x		x			
	x		x			
			x		x	
	x		x			
		x	x			
X			x			
X			x			
		x			x	
X						x
X			x			
				x		x
Triple infection trend						
X		x				x
X		x				x
X		x				x
X		x				x
X		x				x
X		x				x
X		x				x
X		x				x
X		x				x
X		x				x
X		x	x			
X		x	x			
X		x	x			
X		x	x			
X		x	x			
X		x	x			
X		x			x	
X		x			x	
	x		x			x

E. ruminantium	*E. canis*	*Rickettsia* spp. (CS)	*Rickettsia* spp.(RpCS)	*B. burgdorferi*	*A. phagocytophilum*	*C. burnetii*
	x		x			x
	x		x			x
	x		x			x
	x		x			x
	x		x			x
	x		x			x
	x				x	x
	x				x	x
			x		x	x
			x		x	x
X				x	x	
X		x			x	
	x		x		x	
			x	x		x
				x	x	x
X					x	x
X			x			x
		x		x	x	
Quadruple infection trend						
X		x		x	x	
X		x		x	x	
	x	x			x	x
X		x	x			x
X			x	x		x

Table C-2. Ginsberg's co-infection index

Pathogen 1 (infected with pathogen 1 only)	Pathogen 2 (infected with pathogen 2 only)	No. sampled	No. co-infected	Total no. infected	I_c
E. ruminantium (61)	*Rickettsia* spp. (104)	1099	69	303	21.7
Rickettsia spp. (135)	*C. burnetii* (44)	1183	39	256	12.5
A. phagocytophilum (63)	*Rickettsia* spp. (155)	1222	18	223	2.83
E. canis (11)	*C. burnetii* (70)	694	13	107	10.6
C. burnetii (71)	*A. phagocytophilum* (31)	1085	12	133	10.5